Activation and silencing of secondary metabolites in *Streptomyces albus* after transformation with cosmids containing the thienamycin gene cluster from *Streptomyces cattleya*

Alfredo F. Braña[1], Miriam Rodríguez[1], Pallab Pahari[3], Jurgen Rohr[3], Luis A. García[2] and Gloria Blanco[1]*

1. Departamento de Biología Funcional e Instituto Universitario de Oncología del Principado de Asturias, Universidad de Oviedo, 33006 Oviedo, Spain
2. Departamento de Ingeniería Química y Medio ambiente. Universidad de Oviedo, Spain.
3. Department of Pharmaceutical Sciences. College of Pharmacy. University of Kentucky, USA

Running title: Activation and silencing of secondary metabolites in *Streptomyces*

Key words: Antibiotic, biosynthesis, paulomycin, paulomenol, pyrazines, tetramethyl-pyrazine,

* To whom correspondence should be addressed. Tel/Fax +34-985-103205.
e-mail gbb@uniovi.es

Abstract

Activation and silencing of antibiotic production was achieved in *S. albus* J1074 and *S. lividans* Tk21 after introduction of genes within the thienamycin cluster from *S. cattleya.* Dramatic phenotypic and metabolic changes, involving activation of multiple silent secondary metabolites and silencing of others normally produced, were found in recombinant strains harbouring the *thn* cluster in comparison to the parental strains. In *S. albus*, UPLC purification and NMR structural elucidation revealed the identity of four structurally related activated compounds: the antibiotics paulomycins A, B and the paulomenols A and B. Four volatile compounds whose biosynthesis was switched off were identified by GC-MS analyses and databases comparison as pyrazines; including tetramethylpyrazine, a compound with important clinical applications to our knowledge never reported to be produced by *Streptomyces*. In addition, this work revealed the potential of *S. albus* to produce many others secondary metabolites, including compounds of medical and industrial interest normally obtained from plants, suggesting that it might be an alternative model for their industrial production. In *S. lividans*, actinorhodins production was strongly activated in the recombinant strains whereas undecylprodigiosins were significantly reduced. Activation of cryptic metabolites in *Streptomyces* species might represent an alternative approach for pharmaceutical drug discovery.

Introduction

Species of the *Streptomyces* genus are the main producers in nature of antibiotics and other bioactive secondary metabolites of great medical and industrial significance. Genomics has recently revealed that the biosynthetic potential of these bacteria has been largely underestimated. *Streptomyces* genomes contain a great reservoir of secondary metabolite biosynthetic gene clusters, most of which are silent under standard laboratory conditions (Baltz, 2008). Cryptic gene clusters encode putative natural products whose production has not been detected. There is a large difference between the number of biosynthetic gene clusters present in sequenced genomes and the number of known produced molecules, since cryptic metabolites outnumber the known produced metabolites by an order of magnitude. Activation of silent gene clusters for antibiotics and other bioactive secondary metabolites is therefore one of the most important areas of research for novel drug discovery in *Streptomyces*.

Different approaches have been reported to successfully activate silent cryptic biosynthetic gene clusters in microorganisms. Most strategies have been recently reviewed (Zhu et al. 2013; Chiang et al, 2011; van Wezel and McDowall, 2011). These include changes in environmental factors, like empirical variation of growth conditions, and co-cultivation with microorganisms from the same ecosystem in an attempt to simulate their natural habitat (Brakhage and Schroeckh, 2011, Pérez et al., 2011). An alternative approach involves overexpression of regulatory genes, either pleiotropic or pathway-specific activator genes, as reported for the awakening of the expression of a

silent biosynthetic gene cluster in *Aspergillus nidulans* (Zerikly and Challis, 2009).

Among antibiotics, the beta-lactam carbapenem thienamycin (Kahan et al. 1979) is the most potent, most broad-spectrum of all natural antibiotics known so far (Demain, 2009). Cloning of the thienamycin biosynthetic gene cluster (*thn*) from the producer *Streptomyces cattleya* (Núñez et al. 2003) has contributed to the knowledge of thienamycin biosynthesis (Freeman et al. 2008; Hamed et al. 2009; Bodner et al. 2010; Rodríguez et al. 2011), and its regulation (Rodríguez et al. 2008; Rodríguez et al. 2010). In the course of previous attempts to produce thienamycin in two genetically well characterized *Streptomyces* strains, *S. albus* J1074 and *S. lividans* TK21, by transformation with cosmids containing the *thn* gene cluster, strong pigmentation was induced in both recombinant strains. Comparative metabolite profiling of the recombinant strains with the parental strains performed by UPLC and GC-MS analysis revealed that, surprisingly instead of the expected thienamycin product, dramatic changes in the metabolome of the recombinant strain were observed. Here we report the successful activation of silent antibiotic biosynthetic pathways and silencing of others normally produced in both *Streptomyces* hosts after transformation with the *thn* gene cluster from *S. cattleya*. Although the mechanism underlying this event has not been identified, our study might represent a promising approach for the discovery of many secondary metabolites, whose gene clusters remain otherwise cryptic in *Streptomyces* genomes.

Materials and Methods

Bacterial strains, culture conditions and cloning vectors

Streptomyces albus J1074 and *Streptomyces lividans* TK21 ATCC 55251 were used in this study. *Escherichia coli* DH10B (Invitrogen) was used as a cloning host. *E. coli* ET12567 (*dam*$^{-}$ *dcm*$^{-}$ *hsdS*), harbouring pUB307 (Flett et al. 1997) was used as the donor for intergeneric conjugation as a source of non-methylated DNA (MacNeil et al. 1992) to prevent plasmid degradation by the methylation restriction system of *S. cattleya* NRRL 8057. For antibiotic production cultures were grown on R5A medium (Fernández et al., 1998). Cosmids cosCAT25, cosCAT14 and cosCAT22 are pKC505 derived low copy number vectors (Nuñez et al., 2003) containing different regions of the *thn* and *cph-II* gene clusters from *S.cattleya*. Cosmid cosCAT32 is a pHZ1358 derived high copy number vector, obtained (L.E. Núñez, unpublished data) during the generation of a Δ*thnD-cphU* deletion mutant (Rodríguez et al. 2011); it harbours the complete nucleotide sequence under Accession number AJ421798. pUC18 (Pharmacia) was used as *E.coli* cloning vector. The pEM4T expression vector, which contains the constitutive P*ermE** promoter (Menéndez et al. 2006), was used for gene expression experiments. When needed, antibiotics were added to a final concentration of 25 μg ml^{-1} for thiostrepton, apramycin, kanamycin, chloramphenicol and nalidixic acid and 100μg ml^{-1} for ampicillin.

DNA manipulations, sequencing and subcloning experiments

Plasmid DNA preparations, restriction endonuclease digestions, ligations, protoplasts transformation and other DNA manipulations were performed according to standard procedures for *E. coli* (Sambrook and Russell, 2001) and for *Streptomyces* (Kieser et al. 2000). PCR amplifications were performed with

the high fidelity *Pfx* DNA polymerase (Invitrogen) in a total volume of 50μl. The reaction mixture contained 0.1 μg of DNA, 30 pmol of each oligonucleotide primer, dNTPs (final concentration of 0.3 mM), 1x amplification buffer and 2.5U of DNA polymerase. Reactions were performed on a MJ Research Peltier Effect Cycling PTC-100TM with the following program: 1 cycle of denaturation at 94ºC (2 min), 30 cycles of denaturation/ annealing/ synthesis at 94ºC (30 s) / 62ºC (1min) / 68ºC (30 s) and 1 cycle of final extension at 68ºC (5 min). DNA fragments obtained after PCR amplification were purified with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). For *thnI* amplification the following primers were used: F*thnI* (5'-ATCGTCTAGAAGCGCACAGGAGCAGCGAATG-3', XbaI underlined) and R*thnI* (5'-ATCGGAATTCTCAGCACACCTCGGTGGAGGA-3', EcoRI underlined); for *cphU*: (5'- ATCGGCATGCGGTTTGGGGGGATCCACTGATG-3', SphI underlined) and (5'-ATCGTCTAGAGTCAGGGCACCGCCGCGCC-3', XbaI underlined). DNA sequencing was performed on double-stranded DNA templates by using the dideoxynucleotide chain termination method and the Thermo Sequenase Labelled Primer Cycle Sequencing Kit with 7-deaza-deoxyguanosine triphosphate (Amersham Biosciences) and an ALF-express automatic DNA sequencer (Pharmacia). pEM4T was used as expression vector for subcloning BamHI fragments containing regulatory genes (Fig. 1). The plasmids pMRB1 and pMRB7 were generated after subcloning the 4.8 Kb *Bam*HI fragment containing *thnB* in two different orientations into pEM4T, previously digested with the same restriction enzyme. pMRB4 and pMRB6 were obtained by subloning the 7.6 Kb *Bam*HI fragment containing *thnI* in both orientations in the same vector. pEM4AT was generated by insertion of

aac(3)IV gene (encoding the apramycin resistance cassette) in the *Eco*RV restriction site of pEM4T, which lies in the coding region of the thiostrepton resistance gene (*tsr*). pEM4AT, digested with *Eco*RI and blunt ended with Klenow, was used as expression vector for subcloning independently the PCR amplified *thnI* and *cphU* regulatory genes. Each amplified gene, previously subcloned in pUC18 and sequenced was then rescued after *Hin*dIII-*Eco*RI digestion, blunt ended and then subcloned in pEM4AT after the constitutive P*ermE** promoter, generating pMR*thnI* and pMR*cphU*, respectively. Introduction of these constructions, with the corresponding control vectors, in *S. albus* J1074 was achieved through intergeneric conjugation from *E. coli* ET12567/pUB307 as described (Mazodier et al., 1989).

Chromatographic analysis

Routinely, compounds produced by *Streptomyces* strains were assessed in cultures on R5A solid medium. Agar plugs taken from the plates were extracted with ethyl acetate and 1% formic acid. The organic fraction was evaporated and the residue redissolved in 100 μl of a mixture of DMSO and methanol (50:50). These samples were analyzed by reversed phase chromatography in an Acquity UPLC equipment with a BEH C18 column (1.7μm, 2.1 x 100 mm, Waters), with acetonitrile and 0.1% trifluoroacetic acid as solvents. Samples were eluted with 10% acetonitrile during 1 min, followed by a linear gradient from 10% to 100% in 7 min and an additional isocratic hold with 100% acetonitrile during 2 min, at a flow rate of 0.5 ml/min and a column temperature of 35ºC. For HPLC-MS analysis, an Alliance chromatographic system coupled to a ZQ4000 mass spectrometer and a Symmetry C18 (2.1 x

150 mm, Waters) was used. Solvents were the same as above and elution was performed with an initial isocratic hold with 10% acetonitrile during 4 min followed by a linear gradient from 10% to 88% acetonitrile over 26 min, at 0.25 ml/min. MS analysis were done by electrospray ionization in the positive mode, with a capillary voltage of 3 kV and a cone voltage of 50 V. Detection and spectral characterization of peaks was performed in both cases by photodiode array detection and Empower software (Waters).

Isolation of paulomycins

S. albus/cosCAT25 was cultivated in 3 l of solid R5A medium (approximately 100 plates) at 28ºC during 10 days. Agar cultures were removed from the plates, placed in three 2 liter Erlenmeyer flasks, covered with ethyl acetate and 1% formic acid, and extracted in an orbital shaker for 3h at 30ºC and 150 rpm. The organic extracts were evaporated *in vacuo* and the extraction was repeated. The resulting dry extract was redissolved in a mixture of DMSO and methanol and the compounds of interest were purified by preparative HPLC using a SunFire C18 column (10 μm, 10 x 250 mm, Waters). Compounds were chromatographed with mixtures of acetonitrile or methanol and 0.05% TFA in water in isocratic conditions optimized for each peak, at 7 ml/min. After every purification step, the collected compounds were diluted fourfold with water and were desalted and concentrated by solid-phase extraction, being finally lyophilized.

NMR spectroscopic and mass spectrometric analysis

NMR spectra were measured on a Varian Vnmr 500 (1H, 500 MHz; 13C, 125 MHz) spectrometer. ESIMS was recorded on a Finnigan LCQ ion trap mass spectrometer. HRMS was recorded by ESIMS on an Agilent LC/MSD TOF (resolution: 10 000; 3 ppm mass accuracy; inlet systems: Agilent Technologies 1200 Series LC pumps) mass spectrometer (manufacturer: Agilent, Palo Alto, CA, USA).

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Qualitative analysis was performed by coupled GC-MS (Chromatograph Agilent 6890N coupled with a 5975B mass spectometer) mainly as described for geosmin detection (Gust 2003). Volatiles released from *Streptomyces* strains during 2 weeks' growth on R5A plates at 28ºC were absorbed onto 100 mg of activated charcoal (Norit GAC 1240) placed in the lid of each petri dish. The charcoal was extracted with 0.5ml of chloroform (Merck) for an hour, and then filtered through cotton wool. One microliter of each extract was analyzed by GC-MS as follows: capillary column, fused silica (30 m; 0.25-mm inside diameter; 0.25-μm film thickness); carrier gas, He (0.82 kPa on column injection); temperature program: isothermal for 1 min at 40ºC, change from 40 to 210ºC at a rate of 10ºC per min, and isothermal for 25 min at 210ºC; energy of ionization, 70 eV. The identity of these volatile compounds was determined by comparing their mass spectra with the Whiley and NIST (National Institute of Standards and Technology) libraries.

RESULTS

Phenotypic changes detected in *S. albus and S. lividans* harbouring cosmids containing genes of the *thn* cluster

Genes involved in thienamycin biosynthesis reside in a single cluster, *thn* gene cluster (Nuñez et al 2003), (Fig.1), located in the central region of the 1,8 Mb pSCAT megaplasmid (spanning from nucleotides 900,546 to 932,876) as has been reported in the recently sequenced *S. cattleya* genome (Barbe, et al. 2011). After protoplast transformation of two genetically well characterized host strains, *S. albus* J1074 and *S. lividans* TK21, with cosmids (Nuñez et al 2003), containing different combinations of *thn* biosynthetic and regulatory genes (Fig.1) striking phenotypic changes involving strong pigmentation were noticed in some of the recombinant strains (Fig.2).

When the *S. albus* strains harbouring the pKC505 derived cosmids cosCAT25 or cosCAT14 were grown on R5A solid medium, changes in the mycelium pigmentation colour involving the appearance of a redish brown pigment were observed. In addition, differences in the odour of sporulated cultures were also detected after olfactory analysis, carried out by smelling solid cultures of these strains. In contrast, no changes were detected in relation to the parental strain in *S. albus* harbouring cosCAT22, which displayed the same phenotype as the negative controls (*S. albus* parental strain and *S. albus* harbouring the pKC505 empty vector). Similar phenotypic and metabolic changes were also detected when we used cosCAT32, derived from the multicopy cosmid vector pHZ1358, containing the entire 32 kb region (Accession number AJ421798) represented in Fig.1. In *S. lividans* no phenotypic changes were detected with the above mentioned cosmids based

on the pKC505 monocopy cosmid. In contrast, when we used the multicopy cosCAT32, the appearance of an intense blue pigment was clearly detected.

Three regulatory genes present in the *thn* cluster: *thnI, cphU* and *thnB*, are the main candidates for the phenomenon observed, (Fig.1). The LysR-type transcriptional activator ThnI, a key factor in activating thienamycin biosynthesis, involved in the regulation of 10 genes within the *thn* cluster including the regulator itself in a complex regulatory network (Rodríguez et al. 2008; Rodríguez et al. 2010). ThnI was the most obvious candidate to be involved in heterologous activation since it was the only one present in all cosmids displaying a positive phenotype (Fig.1). The SARP-transcriptional activator CphU (formerly ThnU), also linked to this cluster, regulates *in trans* the expression of cephamycin biosynthetic genes located in the main chromosome (Rodríguez et al. 2008); CphU does not seem to be involved in the process, since *cphU* is present in cosCAT22, which displays a negative phenotype, The TetR family member ThnB is a lactone-dependent transcriptional regulator putatively involved in *quorum sensing* regulation (Nuñez, et al. 2003) whose role remains still unknown. No homologues have been found to date in other *Streptomyces* species, and the *thn*-like gene cluster recently identified in *S. flavogriseus* by genome mining does not contain this regulatory gene, although this gene cluster appears to be silent (Blanco, 2012). Although *thnB* is truncated (it lacks the last 25 codons) in cosCAT25, which displays a positive phenotype, the encoded protein might still be active.

To determine whether changes in phenotype and secondary metabolite profiles were due to expression of any of these regulatory genes, relevant regions were subcloned (Fig. 1) and the recombinant plasmids introduced in

S.albus by intergeneric conjugation. We focused on *S. albus,* given that *S. lividans* is not a suitable host since it has been shown to produce ACT in response to very different genetic and environmental stresses. As shown in Fig. 1, different constructions were obtained by cloning relevant BamHI fragments (pMRB1, pMRB7, pMRB4 and pMRB6) or PCR amplifications of individual regulatory genes (pMR*thnI* and pMR*cphU*), in the multicopy pEM4T expression vector; either under control of their own promoter or the constitutive P*ermE** promoter. However, no phenotype or metabolic change was observed with any of these constructions. Information obtained with the different cosmids, allowed narrowing the putative region involved in the process to de DNA included between the *thnB* and *thnM* genes.

Activation and silencing of secondary metabolite biosynthetic pathways in *S. albus*

The striking changes in the phenotypes observed in both heterologous hosts prompted further analysis involving comparative metabolite profiling of the recombinant strains with the parental strains.

Ethyl acetate extracts of R5A solid cultures of the above mentioned *S. albus* strains after 7 days of growth were analysed by ultra performance liquid chromatography (UPLC). Although the reddish brown pigment was not extracted with ethyl acetate (or other organic solvents as methanol or acetone), metabolomic profiling of organic extracts from *S. albus* harbouring cosCAT25 revealed important changes in the peak profile when compared to the control strains, *S. albus* parental strain and *S. albus* carrying the pKC505 vector (Fig. 3). Differences in multiple metabolites were observed when absorbance was

measured at different wave lengths (Fig.3). These involve activation of at least six silent metabolites and silencing of at least two active metabolites, being the corresponding peaks indicated by arrows in the maxplot. Figure 4 shows changes detected at 323 nm, which involve activation of several silent or poorly expressed secondary metabolites (peaks **1** to **4**) and silencing of another (2.4 min retention time). *S. albus* harbouring cosCAT14 (Fig.1) displayed a metabolic profile pattern similar to *S. albus*/cosCAT25, whereas the *S. albus*/cosCAT22 pattern was similar to the control cultures, *S. albus* carrying the pKC505 vector and *S. albus* parental strain (data not shown).

Purification and structural elucidation of compounds 1-4

Four compounds whose production was activated in *S. albus*/cosCAT25 were purified by preparative HPLC and structurally characterized by nuclear magnetic resonance spectroscopy. The NMR spectroscopic and mass spectrometric analysis (Supporting information) proved identity with the respective four structurally related known compounds: paulomycins A, B and paulomenols A and B (Fig. 5). Paulomycins A and B are antibiotics with very potent activity against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus* and other *Streptomyces*) initially described in *S. paulus* (Argoudelis *et al.* 1982) and later in *S. albus* J1074 (Majer and Chater, 1987). Paulomycins A and B display diverse biological activities and are of therapeutical use in the treatment of gonococcal and *Chlamydia* infections (Novak, 1988). Paulomenols A and B have only been reported to date to be produced by *S. paulus* (Argoudelis *et al.*1988), not in *S. albus*. Since they do not contain the paulic acid moiety needed for the bioactivity of the molecule, paulomenols do not show

antibiotic activity; however, they have industrial use as UV light filters (Wiley, 1983).

Comparative volatile metabolite profiling analyses reveals silencing of metabolites normally produced in the parental strain

S. albus parental cultures produce a very characteristic odour unlike that of any other *Streptomyces*, (which almost allows identification simply by smelling). Since changes in odour of some recombinant strains were also detected in R5A solid cultures, a comparative Gas Chromatography-Mass Spectrometry (GC-MS) analysis to detect volatile metabolites by sporulated surface cultures was carried out. As shown in Fig. 6A, a comparison of volatile profiling between the parental *S. albus* strain, *S. albus*/pKC505 and the recombinant *S. albus*/cosCAT25 shows remarkable differences involving silencing of metabolites normaly produced by the *S. albus* parental strain. In agreement with the above mentioned UPLC analysis, *S. albus*/cosCAT14 displayed a similar metabolic profile pattern than *S. albus*/cosCAT25, whereas the *S. albus*/cosCAT22 pattern was equivalent to the controls: *S. albus* parental strain and *S. albus* /pKC505 (data not shown). Among the metabolites differentially produced in the comparative analysis, four volatile compounds whose biosynthesis was switched off in the recombinant strain (peaks **5** to **8**) were identified using the Whiley database (Table 1). All four metabolites are structurally related and belong to a family of flavour compounds known as pyrazines, which have intensive smells and very low odour threshold values (Muller and Rappert, 2010). Pyrazines are a group of 1,4 dinitrogen substituted benzenes whose increased demand in recent years owes to their vast

applications in the fields of food, agriculture and medicine. They are widely distributed in plants and animals, where they are considered to be alerting signals (Muller and Rappert, 2010). Among microorganisms, however, pyrazines production is infrequent and only a few bacteria and fungi have been reported to be able to synthesize them (Rajini et al. 2011). The predominant pyrazines produced by the parental strain (Fig. 6B) and not by the recombinant strain are: 2,3,5,6-tetramethyl-pyrazine (**7**); 2,3,5-trimethyl-6-ethyl-pyrazine (**8**); whereas 2,5-dimethyl-pyrazine (**5**) and 2,3,5-trimethyl-pyrazine (**6**) are present at lower concentrations. Tetramethyl-pyrazine displays important biological activities as therapeutical agent and to our knowledge, its production is unprecedented in *Streptomyces.*

Some remaining metabolites present in both, the recombinant and the parental strains, were also identified in *S. albus* (Table 1). Most of them are aromatic compounds, mainly sesquiterpene hydrocarbons, being the two major products geosmin (**9**); and beta-patchoulene (**10**). Geosmin, the compound responsible for the “smell of the earth” is widespread among *Streptomyces* species (Jachymova *et al.*, 2002; Cane and Ikeda, 2011). In contrast, beta-pachoulene, an aromatic compound usually produced by plants and used as fragrance agents in perfume industry, as far as we know has never reported to be produced by bacteria. Another interesting metabolite produced in lower concentrations by *S. albus* is dihydro-beta-agarofurane (**11**). This compound, previously detected in *S. citreus* (Pollak and Berger, 1996), displays multiple biological activities such as antitumor, anti-VIH, immunossuppresant, multidrug resistance (MDR) reversal and insecticidal activity (Gao et al., 2007).

Activation of actinorhodin production and silencing of undecylprodigiosins in *S. lividans*

Ethyl acetate extracts of R5A solid cultures of the recombinant strain harboring cosCAT32 and the wild type strain were analysed by UPLC after 7 days of growth. As shown in Figure 7, comparative metabolite profiling by UPLC mass analysis revealed changes in *S. lividans* harbouring cosCAT32 in relation to the parental strain and the parental strain harbouring the pHZ1358 control vector (Fig. 7). Differences were observed when absorbance was measured at 499 nm involving activation of several silent or poorly expressed secondary metabolites and down regulation of others normally expressed in the wild type strain. These extracts were also analysed by HPLC-MS and on the basis of their absorption spectra and their mass spectra were identified as actinorhodins (ACT) (m/z[H^+] values ranging from 631.5 to 635.5) and undecylprodigiosins (RED) (m/z[H^+] values of 392.5 and 394.5). Production of ACT compounds was activated in the recombinant strain, whereas that of RED compounds was strongly reduced. The presence of a silent gene cluster for actinorhodin production in *S. lividans* has been previously reported (Horinouchi and Beppu, 1984), and also different ways of activating this antibiotic (Romero et al.1992; Penn et al. 2006).

In contrast to *S. albus*, no differences were found when analyzing the volatile metabolite profiling by GC-MS analysis of the *S. lividans* recombinant strain compared to the control strains (data not shown). The *S. lividans* wild type volatile spectrum has been previously determined (Jachynova et al, 2002). The activation/silencing trigger seems to be different in *S. lividans* from that in

S. albus as only CAT32 transformants show induction of Act and reduction of Red production.

Discussion

Annotations of *Streptomyces* genomes revealed that they contain a much greater reservoir of secondary metabolite gene clusters than expected. Genome mining is a novel approach for the identification of gene clusters for natural products in sequenced genomes, and the prediction of the coded product by connecting genomes and bioinformatics (Zerikly and Challis 2009; Walsh and Fischbach, 2010). Most of these bioinformatically newly discovered cryptic gene clusters are silent or poorly expressed under standard culture laboratory conditions. Interesting speculations for the biological meaning of silencing of antibiotic gene clusters have been proposed in the beta-lactam carbapenem producer *Erwinia carotovora* (Holden *et al*., 1998). Thus, an important challenge is to find out alternative strategies to wake up these otherwise dormant secondary metabolic pathways.

We report here the successful activation of silent antibiotic biosynthetic pathways in two well known *Streptomyces* strains, *S. lividans* and *S. albus*. The *thn* cluster in multicopy activates actinorhodin production in *S. lividans* whereas undecylprodigiosin, normally produced by the wild type strain, seems to be down-regulated. It has been previously shown that in *S. lividans* strains silent clusters, such as the actinorhodin one, can be activated following introduction of heterologous antibiotic biosynthesis clusters (Penn *et al.*, 2006). The mechanism underlying the activation/silencing phenotype showed here has not been identified so far by subcloning individual regulatory genes. Transcriptional

organization in this region is very complex (Rodríguez et al. 2008; Rodríguez et al. 2010), in addition we cannot rule out that expression of multiple genes is needed. An alternative hypothesis to explain the phenomenon observed in heterologous hosts is competition for the pool of precursor metabolites among the different pathways. Although, as mentioned, the final product thienamycin was not produced in any of these heterologous hosts, expression of some biosynthetic and regulatory genes of the cluster is likely to occur. In this way, thienamycin precursors (or intermediates) might affect the imbalance of metabolism, thus changing the overall secondary metabolite profiles in the heterologous hosts. Advances in the knowledge of the thienamycin biosynthetic pathway have been recently reviewed (Hamed et al. 2013) and the precursors for the thienamycin skeleton proposed as L-glutamic acid, coenzyme A, malonyl-CoA, S-adenosyl methionine (SAM) and pantetheine. Precedents of such a regulatory cross-talk have been reported in other *Streptomyces* species. In *S. coelicolor*, cross-regulation of RED and ACT production has been proposed to occur through a mechanism involving competition for the common precursors malonyl-CoA and acetyl-CoA, in a way that a decrease in RED production results in an increase in ACT production (Ou et al. 2009). Furthermore, the methyl donor SAM, reported as a regulator for secondary metabolism in *Streptomyces*, has been shown to enhance streptomycin production in *S. griseus* by activating *adpA* transcription (Shin et al. 2006). Interestingly, all identified compounds involved in the activation/silencing process reported here are methylated products and the *thn* cluster contains three SAM-methyltranspherases (Nuñez et al 2003).

Although *S. albus* J1074 is widely used in many laboratories as heterologous host for producing secondary metabolites from other actinomycetes, our results reveal that it has also the potential to produce multiple secondary metabolites. Dramatic changes in the metabolome of the recombinant strains involving activation of silent secondary metabolite gene clusters, and silencing of others normally active, were revealed by comparative metabolic profiling using UPLC analysis; although many of these metabolites have not been identified. Purification and structure elucidation of four structurally related compounds, actively produced by the recombinant strain, revealed that they correspond to the paulomycins/paulomenols family; only paulomycins show antibiotic activity against Gram-positive bacteria, and as mentioned, is used in gonococcal and *Chlamydia* infections (Novak, 1988). Although the biosynthetic pathway is unknown, our results suggest that paulomycins and paulomenols are synthesized from the same gene cluster. During time courses of *S. albus*/cosCAT25 cultures, we have observed that paulomycins are first produced, whereas paulomenols are detected in the cultures later on (data not shown). Paulomycins yields decrease at the same time that paulomenols yields increase, thus indicating that paulomycins biosynthesis occurs prior to paulomenols biosynthesis, and that paulomycins might be intermediates of the paulomenols pathway.

On the other hand, as a result of the GC-MS analysis of volatile substances released by solid cultures, it was observed that the biosynthesis of several flavour metabolites of the pyrazine family was silenced. Among them, one of the major volatile compounds produced by *S. albus* has been identified as tetramethylpyrazine, also known as ligustrazine since it was originally

isolated from the plant *Ligusticum wallichi*, widely used in traditional chinese medicine. This compound is also the subject of current active research, since it displays multiple biological activities with important medical applications. It is used as a therapeutical agent against cardio-and cerebrovascular diseases (Zhang *et al*. 2003) in lung protection and cystic fibrosis (Rajini *et al.,* 2011). Only two bacterial genera are reported to produce tetramethylpirazine: a mutant of *Corynebacterium glutamicum* obtained with NTG (Demain et al. 1967) and *Bacillus* species (Cane and Ikeda, 2011). To the best of our knowledge this is the first report of tetramethylpyrazine production by *Streptomyces*. Another volatile compound of medical interest produced by *S. albus* parental strain is dihydro-beta-agarofurane, which displays multiple and diverse biological activities such as antitumor, anti-VIH, immunossuppresant, multidrug resistance (MDR) reversal and insecticidal activity (Gao et al., 2007). In addition, *S. albus* also produces great amounts of beta-patchoulene, aromatic compound of interest as fragrance in perfume industry, traditionally obtained from the plant *Pogostemon cablin* (patchouli), which as far as we know has never been reported to be produced by prokaryotes.

These findings might open the field to use *Streptomyces* as an alternative model for industrial production of compounds of pharmacological and medical interests, and also for the perfume industry, usually produced by plants. All together, the present study revealed that the biosynthetic potential of *S. albus* has been greatly underestimated. *S. albus* has the ability to produce multiple secondary metabolites, including compounds of pharmaceutical and industrial interest. Discovery of some metabolic products, together with bioinformatic mining on its recently sequenced genome, might help in linking

gene clusters to produced metabolites, specially when the structure of the products can not be easily predicted from *in silico* analysis.

An interesting point is that molecules, whose production was activated in *S. albus* and *S. lividans* hosts, are structurally unrelated and so are they to the β-lactam carbapenem thienamycin, of the native producer *S. cattleya*. It was previously reported that in streptomycetes, there is an extensive 'cross-talk' between pathway-specific regulators in different biosynthetic pathways (Huang *et al*., 2005). The possibility of a mechanism which functions across different producers represents a promising approach to the discovery of cryptic secondary metabolites in *Streptomyces*. Anyway, further investigations need to be done to identify the mechanism underlying this inter-species activation process. Understanding the nature of this phenomenon might facilitate the discovery of cryptic natural products, by awakening some of the great number of dormant gene clusters of the *Streptomyces* genomes, bringing to light these "hidden treasures".

Acknowledgements

This study was financial supported by the University of Oviedo (Ayuda Puente UNOV-10-MA-4). Thanks to Miguel Campoamor for his excellent GC-MS technical assistance. The authors are grateful to José L. Caso and José A. Guijarro for their continuous support We also want to thank Jose A. Salas, Carmen Méndez and Luz E. Núñez for previous work on thienamycin.

REFERENCES

Argoudelis AD, Brinkley TA, Brodasky TF, Buege JA, Meyer HF, Mizsak SA (1982) Paulomycins A and B. Isolation and characterization. J Antibiot 35: 285-294.

Argoudelis AD, Baczynskyj L, Mizsak SA, Shilliday FB (1988). *0*-Demethylpaulomycins A and B, U-77,802 and U-77,803, Paulomenols A and B, new metabolites produced by *Streptomyces paulus*. J Antibiot 41: 1316-1330.

Baltz RH (2008) Renaissance in antibacterial discovery from actinomycetes. Curr Op Pharmacol 8:557-563.

Barbe V, Bouzon M, Mangenot S, Badet B, Poulain J, Segurens B, Vallenet D, Marlière P, Weissenbach J (2011) Complete genome sequence of *Streptomyces cattleya* NRRL 8057, a producer of antibiotics and fluorometabolites. J Bacteriol 193: 5055-5056.

Blanco G (2012). Comparative analysis of a cryptic thienamycin-like gene cluster identified in *Streptomyces flavogriseus* by genome mining. Arch Microbiol. 194: 549-555.

Bodner JJ, Phelan RM, Freeman MF, Li R, Townsend CA (2010) Non-heme iron oxygenases generate natural structural diversity in carbapenem antibiotics. J Am Chem Soc 132:12-13

Brakhage AA, Schroeckh V (2011) Fungal secondary metabolites - strategies to activate silent gene clusters. Fungal Genet Bio 48:15-22.

Cane D E, Ikeda H (2011) Exploration and mining of the bacterial terpenome. Acc Chem Res 45: 463-72.

Chiang Y-M, Chang S-L, Oakley BR, Wang CC (2011) Recent advances in awakening silent biosynthetic gene clusters and linking orphan clusters to

natural products in microorganisms. Current Opinion in Chemical Biology 15:137-143.

Demain AL, Jackson M, Trenner NR, (1967) Thiamine-dependent accumulation of tetramethylpyrazine accompanying a mutation in the isoleucine-valine pathway. J Bacteriol 94: 323-326.

Demain AL (2009) Antibiotics: natural products essential to human health. Medicinal Research Reviews 29: 821-842

Fernández E, Weissbach U, Sánchez Reillo C., Braña AF, Méndez C, Rohr J Salas JA (1998) Identification of two genes from *Streptomyces argillaceus* encoding two glycosyltransferases involved in the transfer of a disaccharide during the biosynthesis of the antitrumor drug mithramycin J Bacteriol 180: 4929-4937.

Flett F, Mersinias V, Smith CP (1997) High efficiency intergeneric conjugal transfer of plasmid DNA from Escherichia coli to methyl DNA-restricting streptomycetes. FEMS Microbiol Lett 155: 223-229.

Freeman MF, Moshos KA, Bodner MJ, Li R, Townsend CA (2008) Four enzymes define the incorporation of coenzyme A in thienamycin biosynthesis. Proc Natl Acad Sci USA 105: 11128-11133

Gao JM, Wu WJ, Zhang JW, Konishi Y (2007) The dihydro-beta-agarofuran sesquiterpenoids. Nat Prod Rep 24: 1153-1189.

Gust B, Challis GL, Fowler K, Kieser T, Chater K (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci USA 100: 1541-1546.

Hamed RB, Batchelar ET, Mecinovic J, Claridge TD, Schofield CJ (2009) Evidence that thienamycin biosynthesis proceeds via C-5 epimerization: ThnE catalyzes the formation of (2S, 5S)-trans-carboxymethylproline. ChemBioChem 10: 246-250

Hamed RB, Gomez-Castellanos JR, Henry L, Ducho C, McDonough MA, Schofield C J (2013) The enzymes of β-lactam biosynthesis. Nat Prod Rep 30: 21-107.

Horinouchi S, Beppu T (1984) Production in large quantities of actinorhodin and undecylprodigiosin induced by *afsR* in *Streptomyces lividans*. Agric Biol Chem 48: 2131-2133.

Holden MTG, McGowan SJ, Bycroft BW, Stewart GSAB, Williams P, Salmond GPC (1998) Cryptic carbapenem antibiotic production genes are widespread in *Erwinia carotovora*: facile *trans* activation by *carR* transcriptional regulator. Microbiol 144: 1495-1508.

Huang J, Shi J, Molle V, Sohlberg B, Weaver D., Bibb MJ, Karoonuthaisiri N, Lih CJ, Kao CM, Buttner MJ, Cohen SN (2005). Cross-regulationamong disparate antibiotic biosynthetic pathways of *Streptomyces coelicolor*. Mol Microbiol 58: 1276-1287.

Jachymova J, Votruba J, Viden I, Rezanka T (2002) Identification of *Streptomyces* odor spectrum. Folia Microbiol 47: 37-41.

Kahan JS, Kahan FM, Goegelman R, Currie SA, Jackson M, Stapley EO, Miller TW, Miller AK, Hendlin D, Mochales S, Hernandez S, Woodruff HB, Birnbaum J (1979) Thienamycin, a new β-lactam antibiotic.I. Discovery, taxonomy, isolation and physical properties. J Antibiot 23: 1255-1265.

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Practical *Streptomyces* genetics. John Innes Foundation, Norwich, United Kingdom.

Majer J, Chater K, (1987) *Streptomyces albus* G produces an antibiotic complex identical to paulomycins A and B. *J Gen Microbiol* 133: 2503-2507.

MacNeil DJ, Gewain KM, Rub CL, Dezeny G, Gibbons PH, MacNeil T (1992) Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. Gene 111:61-68.

Mazodier P, Petter R, Thompson CJ (1989) Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. J Bacteriol 171: 3583-3585.

Menéndez N, Nur-e-Alam M, Fischer C, Braña AF, Salas JA, Rohr J, Méndez C (2006) Deoxysugar transfer during chromomycin A3 biosynthesis in Streptomyces griseus subsp. griseus: new derivatives with antitumor activity. Appl Environ Microbiol 72:167-77.

Müller R, Rappert S (2010) Pyrazines: occurrence, formation and biodegradation. Appl Microbiol Biotechnol 85: 1315-1320.

Novak E (1988). Treating Chlamydia infections with paulomycin. The Upjohn company. Patent PCT/US1987/002420.

Núñez LE, Méndez C, Braña AF, Blanco G, Salas JA (2003)The biosynthetic gene cluster for the beta-lactam carbapenem thienamycin in *Streptomyces cattleya*. Chem Biol 10:1-20

Ou X, Zhan B, Zhan L, Zhao G, Xiaoming D (2009) Characterization of *rrdA*, a TetR family protein gene involved in the regulation of secondary metabolism in *Streptomyces coelicolor.* App Env Microbiol, 75: 2158-2165.

Penn J, Li X, Whiting A, Latif M, Gibson T, Silva CJ, Brian P, Davies J, Miao V, Wrigley SK, Baltz RH (2006) Heterologous production of daptomycin in *Streptomyces lividans.* J Ind Microbiol Biotechnol 33:121-128.

Perez J, Muñoz-Dorado J, Braña AF, Shimkets LJ, Sevillano L, Santamaría RI (2011) *Myxococcus xanthus* induces actinorhodin overproduction and aerial mycelium formation by *Streptomyces coelicolor*. Microbial biotechnology 4:175-183.

Pollak FC, Berger RG (1996) Geosmin and related volatiles in bioreactor-cultured *Streptomyces citreus* CBS 109.60. Appl. Environ. Microbiol, 62: 1295-1299.

Rajini KS, Aparna P, Salikala Ch, Ramana ChV (2011) Microbial metabolism of pyrazynes. Critical Reviews in Microbiology 37: 99-112.

Rodríguez M, Núñez LE, Braña AF, Méndez C, Salas JA, Blanco G (2011) Mutational analysis of the thienamycin biosynthetic gene cluster from *Streptomyces cattleya*. Antimicrob Agents Chemother 55: 1638-1649.

Rodríguez M, Núñez LE, Braña AF, Méndez C, Salas JA, Blanco G (2008) Identification of transcriptional activators for thienamycin and cephamycin C biosynthetic genes within the thienamycin gene cluster from *Streptomyces cattleya*. Mol Microbiol 69: 633-645.

Rodríguez M, Méndez, Salas JA, Blanco G (2010) Transcriptional organization of ThnI-regulated thienamycin biosynthetic genes in *Streptomyces cattleya*. J Antibiotics 63:135-138.

Romero NM, Parro V, Malpartida F, Mellado RP (1992) Heterologous activation of the actinorhodin biosynthetic pathway in *Streptomyces lividans*. Nucleic Acids Research, 20: 2767-2772.

Sambrook J, Russell D W (2001) Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Shin S-K, Xu D, Kwo, H-J, Syh J-W (2006) *S*-adenosylmethionine activates *adpA* transcription and promotes streptomycin biosynthesis in *Streptomyces griseus.* FEMS Microbiol Letters 259: 53-59.

van Wezel GP, McDowall KJ (2011) The regulation of secondary metabolism of *Streptomyces*: new links and experimental advances. Nat Prod Rep, 28: 1311-1333.

Walsh CT, Fischbach MA (2010) Natural products version 2.0: connecting genes to molecules. J Am Chem Soc 132: 2469-2493.

Wiley FP (1983) Paulomycin derivatives. The Upjohn Company. Patent PCT/US1983/4520191.

Zerikly M, Challis GL (2009) Strategies for the discovery of new natural products by genome mining. ChemBioChem 10: 625-633.

Zhang Z, Wei T, Hou J, Li G, Yu S, Xin W (2003) Tetramethylpyrazine scavenges superoxide anion and decreases nitric oxide production in human polymorphonuclear leukocytes. Life Sciences 72: 2465-2472.

Zhu H, Sandiford SK , vanWezel GP (2013) Triggers and cues that activate antibiotic production by actinomycetes. J Ind Microbiol Biotechnol. DOI 10.1007/s 10295-013-1309-z.

TABLE 1 Predominant volatile metabolites produced by *S. albus* parental and recombinant strains

Compound*	**CAS**	**Probability** %	**Producer strains** *S. albus***	*S.albus/* cosCAT25
2,5-dimethyl-pyrazine (**5**)	000123-32-0	90	+	-
2,3,5-trimethyl-pyrazine (**6**)	014667-55-1	80	+	-
2,3,5,6-tetramethyl-pyrazine (**7**)	001124-11-4	95	+	-
2,3,5-trimethyl-6-ethyl-pyrazine (**8**)	017398-16-2	76	+	-
geosmin (**9**)	019700-21-1	98	+	+
beta-patchoulene (**10**)	000514-51-2	83	+	+
dihydro-beta-agarofurane (**11**)	005956-09-2	96	+	+

*Whiley library matching results

** *S. albus* and *S. albus/* pKC505 displays similar pattern

FIGURE LEGENDS

Fig. 1. Genetic organization of the thienamycin biosynthetic gene cluster (*thn*) from *S. cattleya* (Accession number AJ421798). Arrows indicate the order and direction of transcription of the genes. Double arrows below the genes represent the putative physical boundaries of the *thn* cluster and the *cph-II* subcluster. Genes shown in colour indicate the regulatory genes *thnB*, *thnI* and *cphU*. Bars coloured in amber at the bottom represent the nucleotide sequence in cosmids with a positive phenotype (cosCAT25, cosCAT14 and cosCAT32)

harboured by *S. albus* J1074; and grey bars and arrows represent the nucleotide sequence of cosCAT22 and plasmids displaying a negative phenotype. The blue coloured bar indicates the positive phenotype of cosCAT32 harboured by *S. lividans*. Dotted lines in cosCAT14 and cosCAT22 indicate the part of the nucleotide sequence were the boundaries are unknown.

Fig. 2. Phenotypic changes of *S. albus* and *S.lividans* recombinant strains carrying respectively cosCAT25 and cosCAT32 in comparison with the respective parental strains. Cultures were grown in R5A agar plates. Pictures were taken after seven days of growth.

Fig. 3. Chromatograms of extracts of the recombinant strain *S. albus*/cosCAT25 compared with the parental strains. The chromatograms shown are maxplots, i.e. chromatograms at absorbance maximum for each analyte, obtained from spectrophotometric detection in the range from 210 to 500 nm. Arrows in the *S. albus*/cosCAT25 chromatogram indicate differential peaks.

Fig. 4. Chromatograms at 323 nm of extracts of the recombinant strain *S. albus*/cosCAT25 in comparison with the parental strains. Peak numbers indicate the compounds whose structure has been elucidated by NMR analyses and correspond to: 1, paulomenol B, 2, paulomenol A, 3, paulomycin B and 4, paulomycin A. UV spectra of paulomenol B and paulomycin B are also shown.

Fig. 5. Chemical structures of paulomycins and paulomenols.

Fig. 6. **A**. GS-MS comparative analysis among *S. albus* wild type, *S. albus*/pKC505 and the recombinant strain harbouring cosCAT25. Peak numbers indicate the compounds identified by comparison with the Whiley database as: 2,5-dimethyl-pyrazine (**5**); 2,3,5-trimethyl-pyrazine (**6**) 2,3,5,6-tetramethyl-

pyrazine (**7**); 2,3,5-trimethyl-6-ethyl-pyrazine (**8**); geosmin (**9**); beta-patchoulene (**10**); dihydro-beta-agarofurane (**11**). **B**. Chemical structures of predominant pyrazines identified in *S.albus* wild type strain.

Fig. 7. Chromatograms at 499 nm of extracts of *S. lividans, S. lividans*/pHZ1358 and the recombinant strain harbouring cosCAT32. Compounds belonging to the ACT and RED families are indicated.

Figure 1
Click here to download high resolution image
Click here to view linked References

Fig. 1

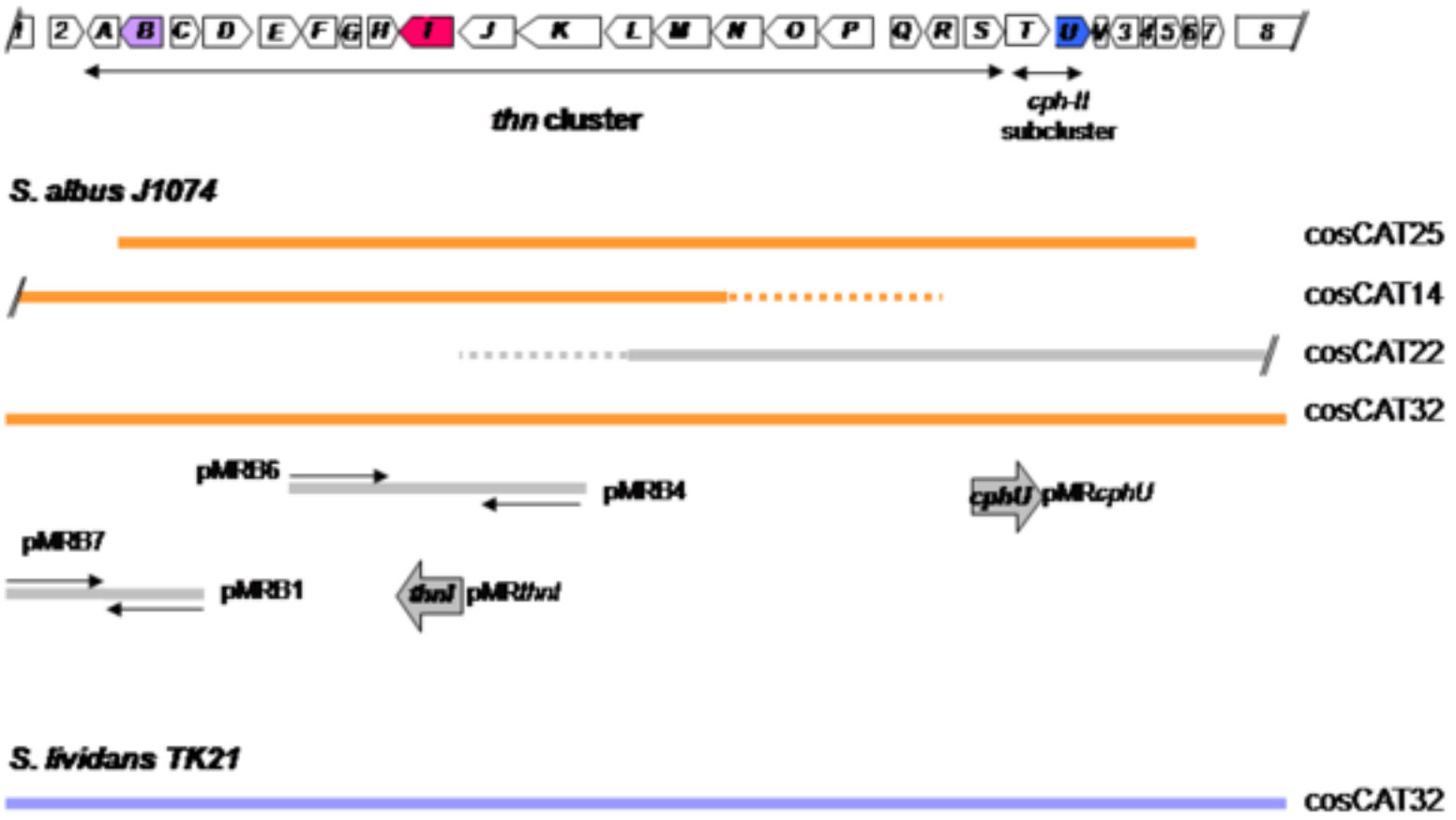


Figure 2

Click here to download high resolution image

Click here to view linked References

Fig. 2

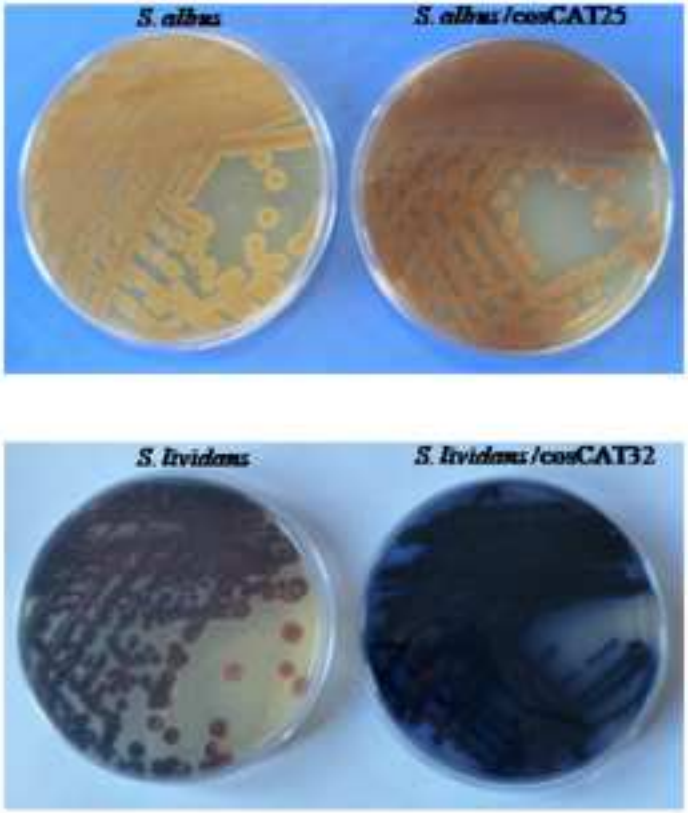


Figure 3

Click here to download high resolution image

Click here to view linked References

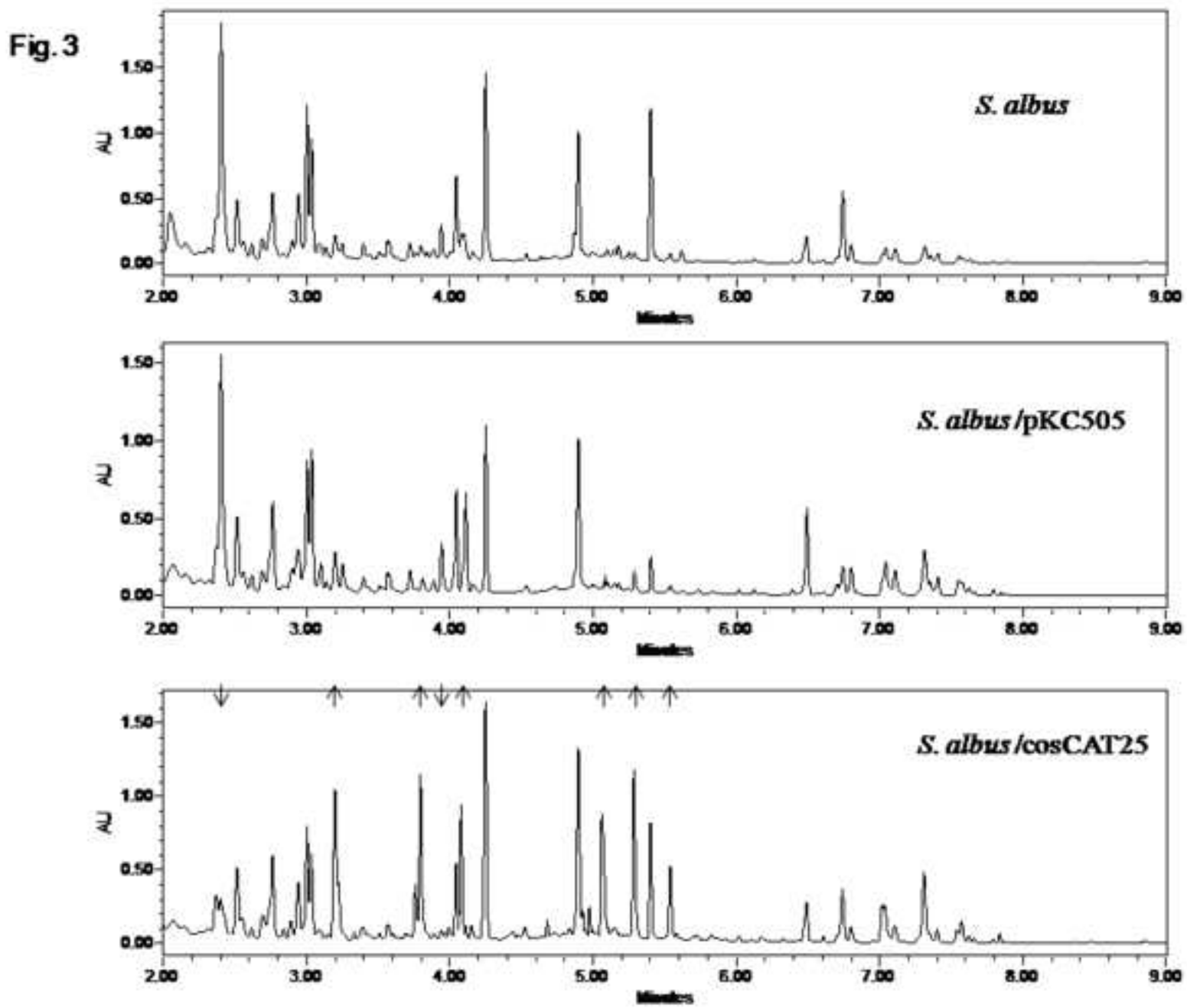


Figure 4

Click here to download high resolution image

Click here to view linked References

Fig. 4

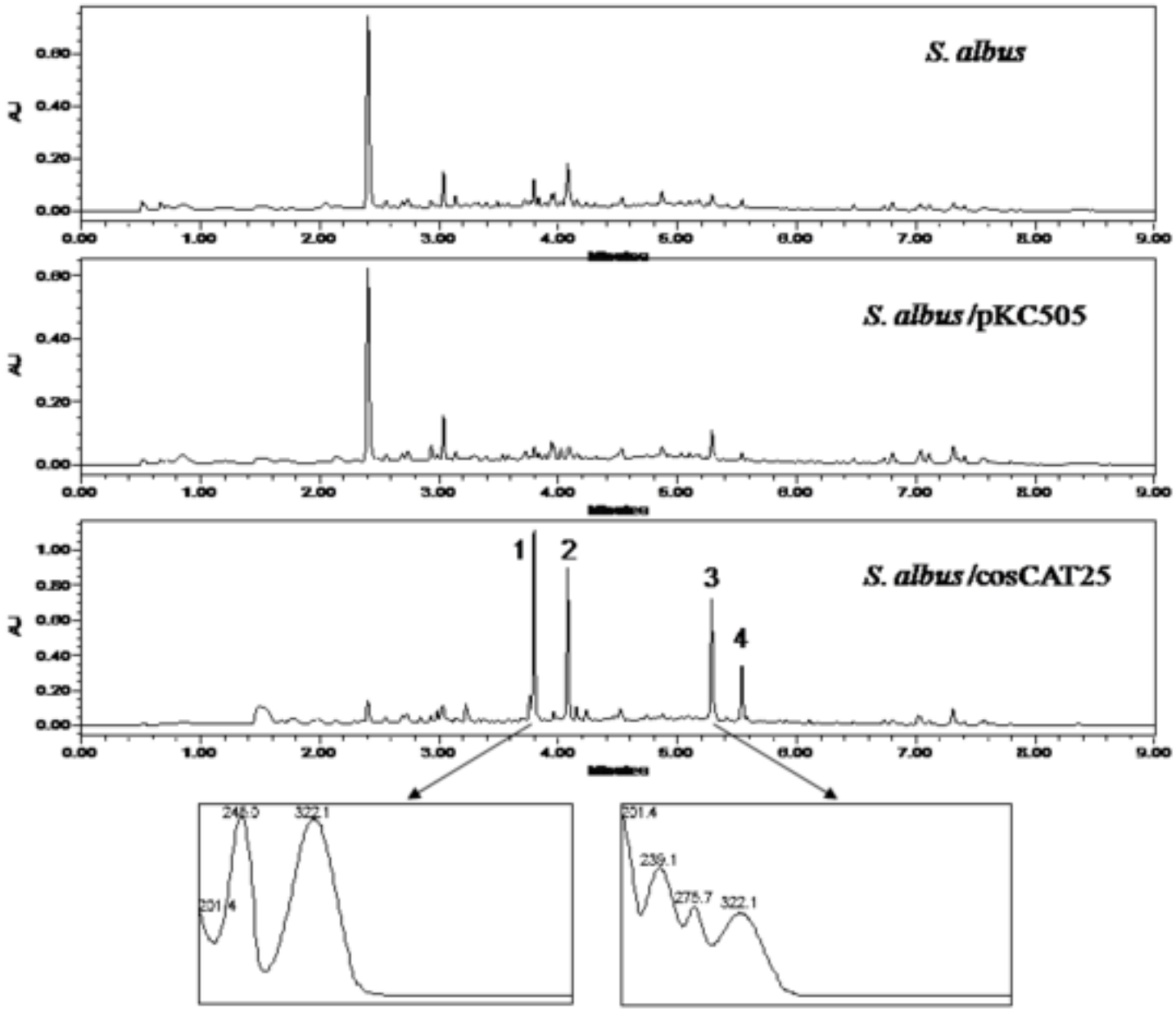
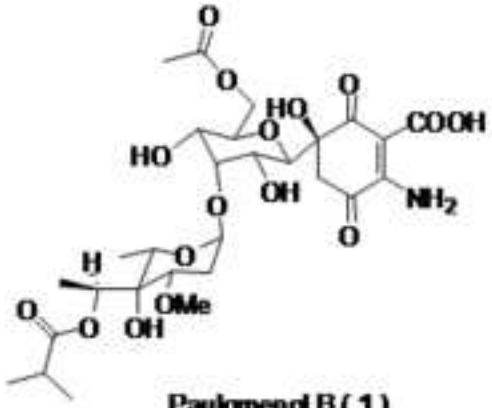


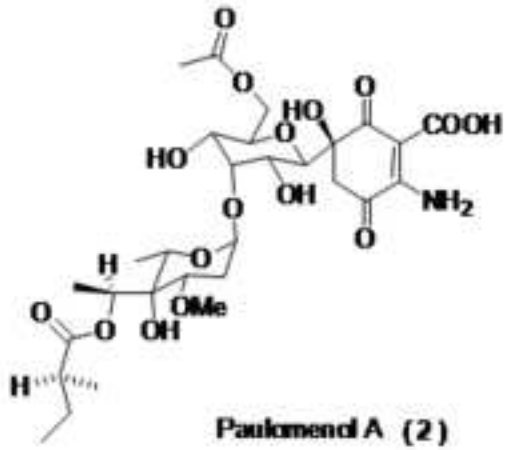
Figure 5

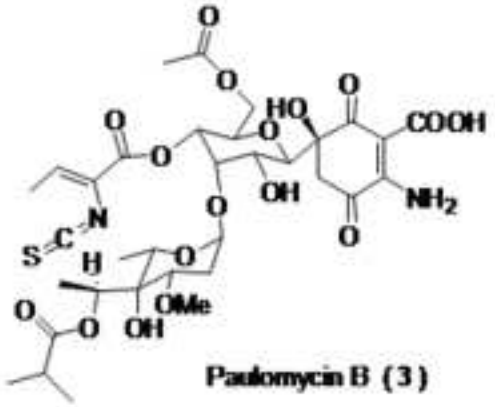
Click here to download high resolution image

Click here to view linked References

Fig. 5







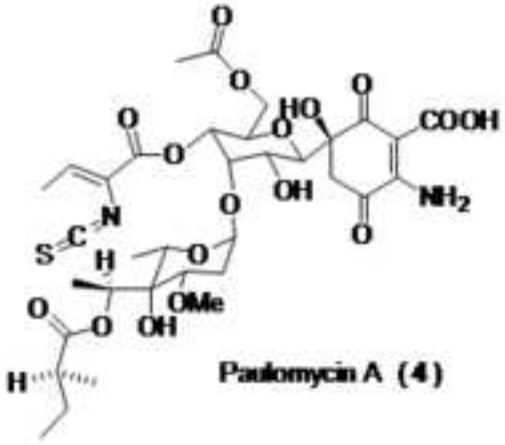


Figure 6

Click here to download high resolution image

Click here to view linked References

Fig. 6

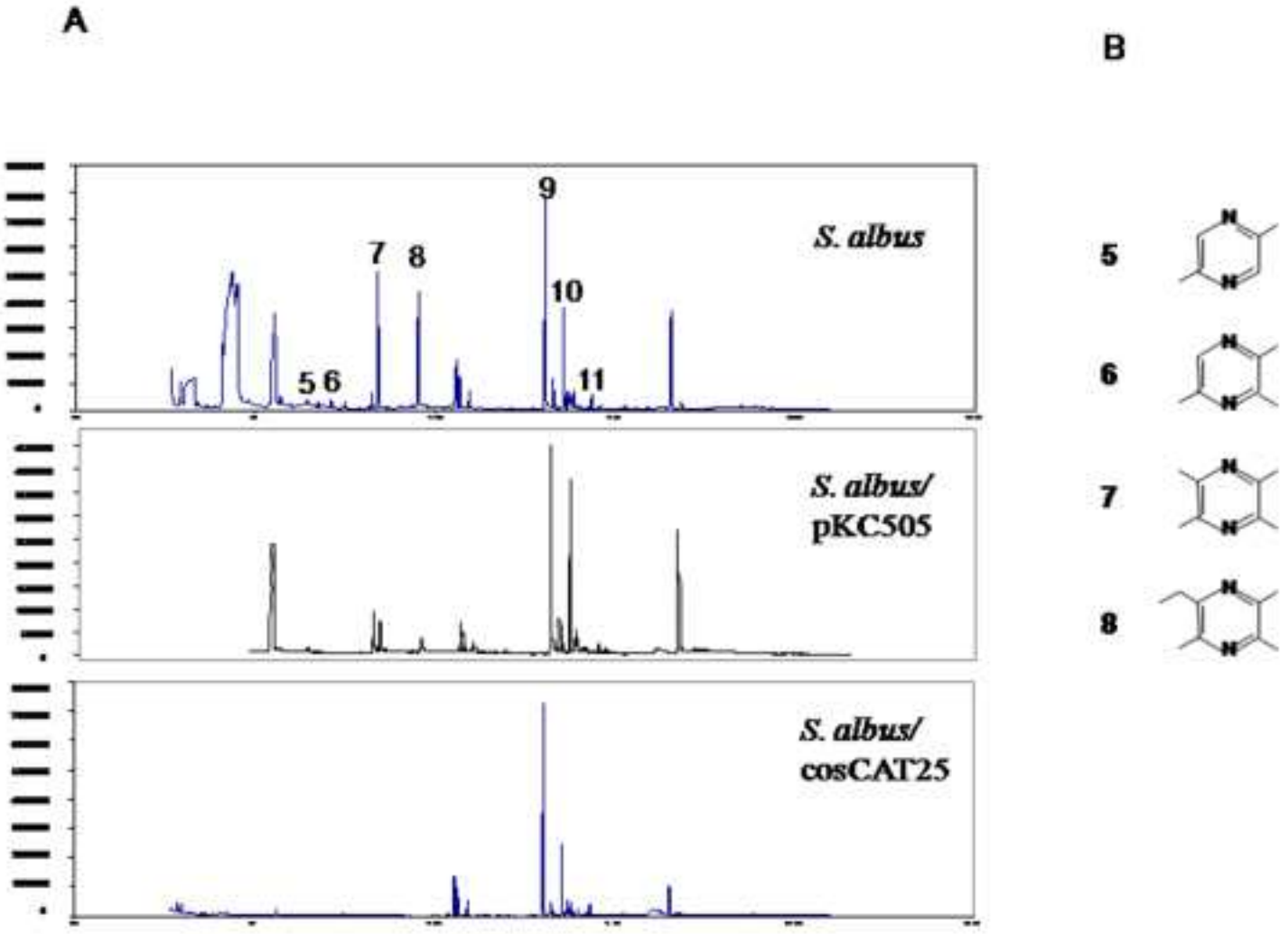
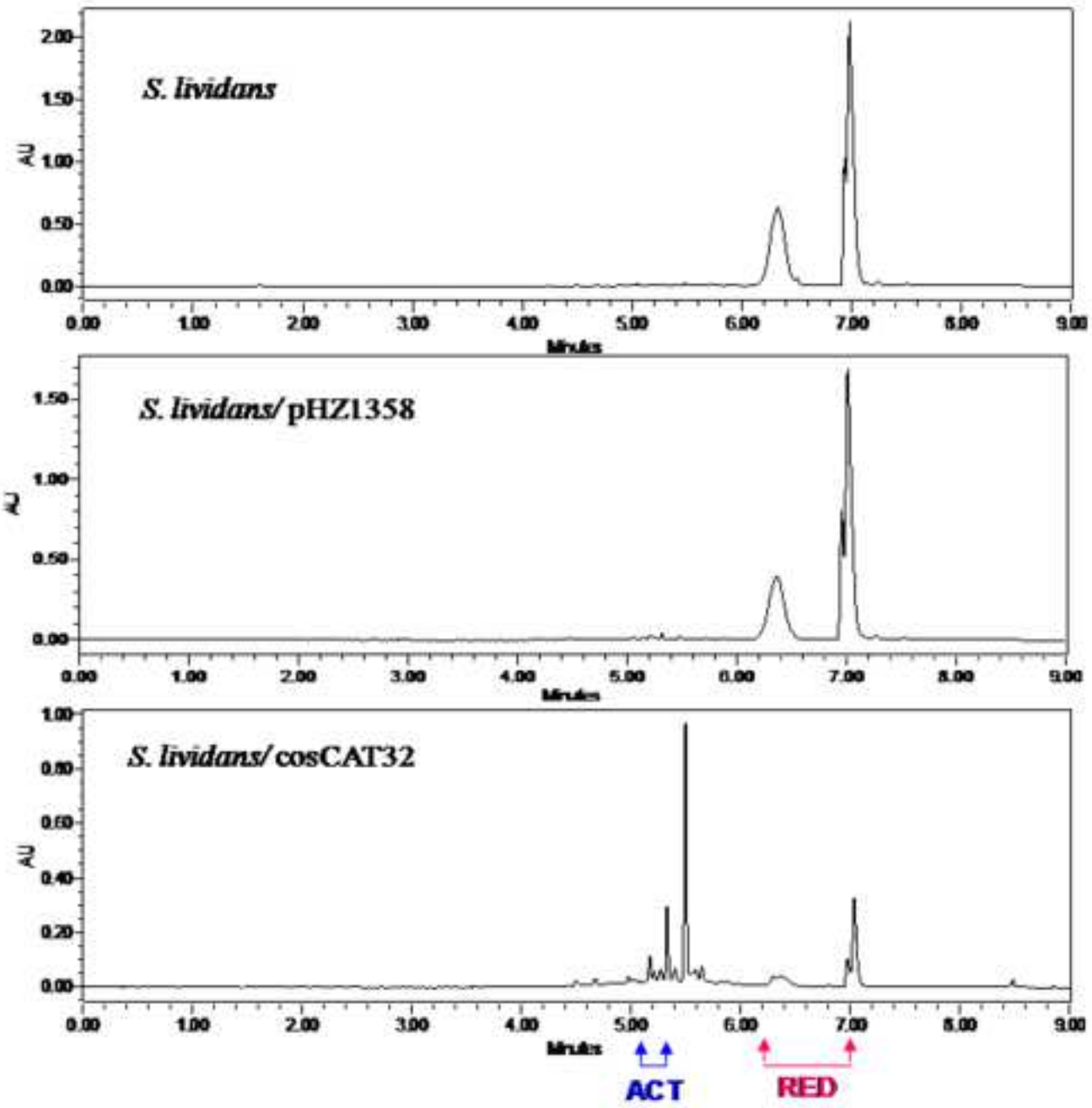


Figure 7

Click here to download high resolution image

Click here to view linked References

Fig. 7

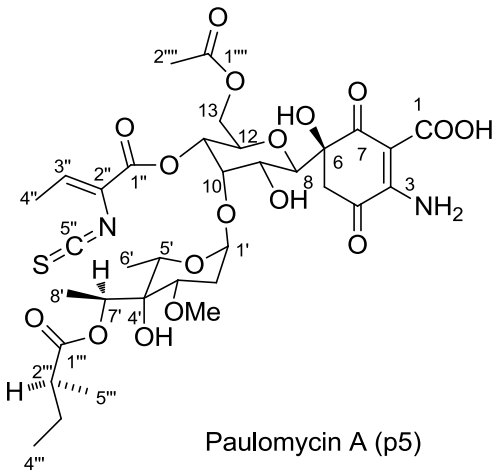


Supporting information

Click here to download Manuscript: Supporting information.doc

Click here to view linked References

Spectral data comparison table of compound p5 with paulomycin A[1]

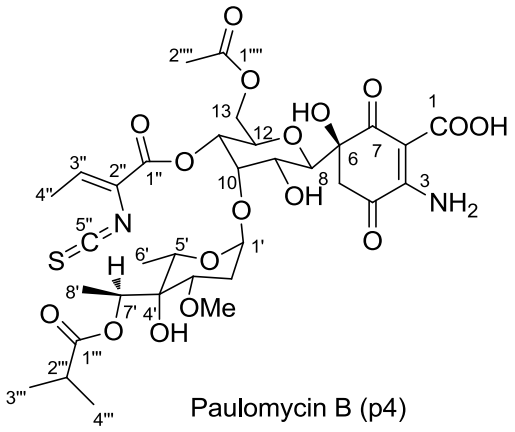


Paulomycin A (p5)

Position	^{1}H NMR of paulomycin A	^{1}H NMR of p5	^{13}C NMR of paulomycin A	^{13}C NMR of p5
1	-----	-----	169.35	170.0
2	-----	-----	100.72	101.1
3	-----	-----	159.37	160.2
4	-----	-----	198.50	199.4
5	3.16, 3.23 (AB)	3.19, 3.32 (ABq, 18.0)	48.01	48.7
6	-----	-----	78.20	78.9
7	-----	-----	188.39	189.1
8	3.83 (d)	3.84 (d, 10)	78.26	79.0
9	3.70 (ddd)	3.72 (ddd, 10, 6, 2.5)	69.20	69.9
10	4.31 (dd)	4.31 (dd, 2, 2)	76.18	76.9
11	4.82 (dd)	4.82 (dd, 10, 2)	70.73	71.5
12	4.18 (ddd)	4.19 (ddd, 10, 4.5, 2.5)	72.29	73.0
13	3.88, 3.99 (ABX)	3.88, 3.99 (ABX, 12.5, 4.5, 2)	62.30	62.9
1’	4.95 (dd)	4.95 (dd, 4, 2)	99.04	99.8
2’	1.90, 2.22 (ABMX)	1.90, 2.22 (ABMX, 13, 6, 2)	30.56	31.3
3’	3.65 (dd)	3.65 (dd, 11, 5)	74.40	75.1
3’-OMe	3.33 (s)	3.34 (s)	56.62	57.3
4’	-----	-----	73.62	74.3
5’	4.52 (q)	4.52 (q, 6.5)	67.18	68.5
6’	1.27 (d)	1.29 (d, 6.5)	15.28	16.0
7’	5.36 (q)	5.36 (q, 6.5)	69.93	70.6
8’	1.22 (d)	1.24 (d, 6.5)	15.39	16.1
1’’	-----	-----	160.25	160.9

2''	-----	-----	123.36	124.1
3''	6.83 (q)	6.83 (q, 6.5)	136.64	137.4
4''	1.97 (d)	1.95 (d, 6.5)	14.11	14.8
5''	-----	-----	142.64	143.3
1'''	-----	-----	175.15	175.9
2'''	2.45 (ddq)	2.45 (ddq, 7, 6, 1.5)	41.51	42.2
3'''	1.50, 1.70 ($ABMX_3$)	1.53, 1.72 ($ABMX_3$, 13, 7, 7)	26.65	27.4
4'''	0.94 (t)	0.97 (t, 7)	11.39	12.1
5'''	1.17 (d)	1.18 (d, 7)	16.73	17.5
1''''	-----	-----	170.18	170.9
2''''	1.98 (s)	1.99 (s)	19.99	20.7

Spectral data comparison table of compound p4 with paulomycin B[1]

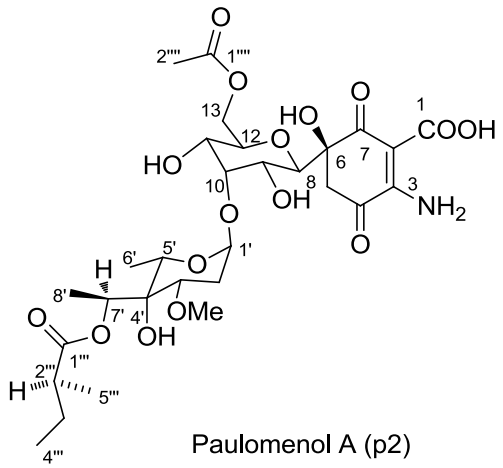


Paulomycin B (p4)

Position	^{1}H NMR of paulomycin B	^{1}H NMR of p4	^{13}C NMR of paulomycin B	^{13}C NMR of p4
1	-----	-----	169.35	170.1
2	-----	-----	100.14	100.9
3	-----	-----	159.36	160.2
4	-----	-----	198.37	199.1
5	3.15, 3.35 (AB)	3.19, 3.29 (ABq, 18.0)	47.95	48.7
6	-----	-----	78.15	79.0
7	-----	-----	188.40	189.1
8	3.86 (d)	3.84 (d, 10)	78.14	78.9
9	3.70 (ddd)	3.72 (ddd, 13, 7, 2.5)	69.28	70.1
10	4.28 (dd)	4.31 (dd, 3.5, 3)	75.91	76.8
11	4.81 (dd)	4.82 (dd, 14, 3)	70.71	71.5

12	4.21 (ddd)	4.20 (ddd, 14, 6.5, 3)	72.21	73.0
13	3.86, 3.99 (ABX)	3.88, 3.99 (ABX, 16, 6.5, 4.0)	62.23	62.9
1’	4.93 (dd)	4.95 (dd, 4, 2)	98.34	99.7
2’	1.90, 2.23 (ABMX)	1.89, 2.22 (ABMX, 16, 5, 2)	30.33	31.3
3’	3.65 (dd)	3.66 (dd, 11.5, 6)	74.38	75.2
3’-OMe	3.33 (s)	3.34 (s)	56.69	57.3
4’	-----	-----	73.66	74.4
5’	4.58 (q)	4.52 (q, 8)	67.65	68.5
6’	1.28 (d)	1.28 (d, 8)	15.23	15.9
7’	5.39 (q)	5.35 (q, 8)	69.96	70.7
8’	1.19 (d)	1.23 (d, 8)	15.44	16.2
1’’	-----	-----	160.25	161.0
2’’	-----	-----	123.32	124.1
3’’	6.88 (q)	6.83 (q, 9.0)	136.66	137.4
4’’	1.97 (d)	1.95 (d, 9.0)	14.13	14.8
5’’	-----	-----	142.54	143.4
1’’’	-----	-----	175.71	176.4
2’’’	2.65 (ddq)	2.63 (ddq, 9, 4)	34.15	34.9
3’’’	1.19 (d)	1.21 (d, 8.5)	18.93	19.6
4’’’	1.17 (d)	1.19 (d, 9.5)	18.77	19.5
5’’’	-----	-----	-----	-----
1’’’’	-----	-----	170.18	170.9
2’’’’	2.02 (s)	1.99 (s)	19.98	20.7

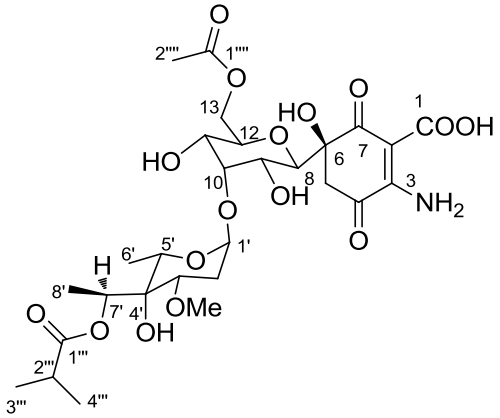
Spectral data comparison table of compound p2 with paulomenol A[2,3]



Paulomenol A (p2)

Position	^{1}H NMR of paulomenol A	^{1}H NMR of p2	^{13}C NMR of paulomenol A	^{13}C NMR of p2
1	-----	-----	170.01	170.1
2	-----	-----	100.53	100.7
3	-----	-----	160.01	160.2
4	-----	-----	197.60	199.4
5	3.16 (dd)	3.10, 3.20 (ABq, 20)	48.76	48.9
6	-----	-----	78.98	79.1
7	-----	-----	188.66	188.8
8	3.92 (d, 8)	3.88 (d, 8)	78.98	79.1
9	3.49 (m)	3.52-3.44 (m)	68.58	68.7
10	4.10 (m)	4.11-4.07 (m)	81.37	81.5
11	3.49 (m)	3.52-3.44 (m)	70.34	70.5
12	4.08 (m)	4.07-4.04 (m)	75.72	75.9
13	3.73 (m)	3.75, 3.79 (ABX, 13, 7, 2)	64.04	64.2
1’	5.17 (q)	5.15 (dd, 5, 2)	100.3	100.5
2’	1.92, 2.27 (m)	1.92, 2.27 (ABMX, 16, 6, 3)	30.91	31.0
3’	3.65 (m)	3.66 (dd, 13, 7)	75.02	75.1
3’-OMe	3.34 (s)	3.32 (s)	57.26	57.3
4’	-----	-----	74.23	74.3
5’	4.49 (q, 6)	4.47 (q, 8)	68.24	68.4
6’	1.29 (d, 6)	1.27 (d, 7)	15.99	16.1
7’	5.39 (q, 6.5)	5.37 (q, 8.5)	70.59	70.7
8’	1.31 (d, 6.5)	1.29 (d, 7)	15.98	17.4
1’’	-----	-----	-----	-----
2’’	-----	-----	-----	-----
3’’	-----	-----	-----	-----
4’’	-----	-----	-----	-----
5’’	-----	-----	-----	-----
1’’’	-----	-----	175.07	175.8
2’’’	2.46 (m)	2.45 (ddq, 12, 8, 2)	42.15	42.3
3’’’	1.64 (m)	1.53, 1.73 (ABMX_3, 16, 9, 4.5)	27.32	27.5
4’’’	0.98 (t, 6))	0.96 (t, 9)	11.87	12.1
5’’’	1.21 (d, 8)	1.19 (d, 9)	15.99	16.1
1’’’’	-----	-----	171.5	171.2
2’’’’	2.04 (s)	2.02 (s)	20.73	20.8

Spectral data comparison table of compound p1 with paulomenol B[2,3]

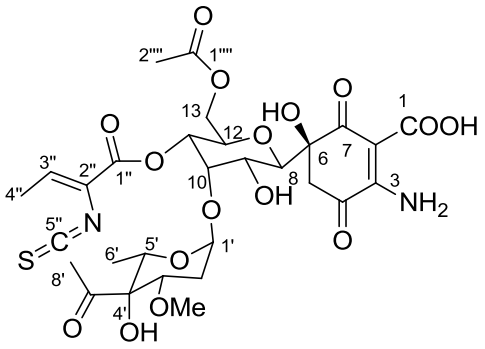


Paulomenol B (p1)

Position	^{1}H NMR of p1	^{13}C NMR of paulomenol B	^{13}C NMR of p1
1	-----	170.06	170.1
2	-----	100.56	100.7
3	-----	160.05	160.2
4	-----	197.45	199.4
5	3.10, 3.20 (ABq, 20)	48.76	48.9
6	-----	78.94	79.1
7	-----	188.72	188.8
8	3.88 (d, 7.5)	78.94	79.1
9	3.52-3.44 (m)	68.35	68.7
10	4.11-4.07 (m)	81.07	81.5
11	3.52-3.44 (m)	70.37	70.5
12	4.07-4.04 (m)	75.68	75.9
13	3.76, 3.80 (ABX, 12, 7.5, 2)	64.07	64.2
1’	5.16 (dd, 4.5, 2)	100.19	100.5
2’	1.92, 2.27 (ABMX, 13.5, 5.5, 3)	30.92	31.0
3’	3.66 (dd, 13, 6)	75.08	75.1
3’-OMe	3.32 (s)	57.24	57.3
4’	-----	74.31	74.3
5’	4.48 (q, 8)	68.45	68.4
6’	1.29 (d, 9)	15.92	16.1
7’	5.35 (q, 8.5)	70.71	70.7
8’	1.28 (d, 9)	15.93	17.4
1’’	-----	-----	-----
2’’	-----	-----	-----
3’’	-----	-----	-----

4''	-----	-----	-----
5''	-----	-----	-----
1'''	-----	175.70	175.8
2'''	2.68-2.58 (m)	34.85	42.3
3'''	1.22 (d, 9)	19.39	27.5
4'''	1.19 (d, 9)	19.59	12.1
5'''	-----	-----	-----
1''''	-----	171.19	171.2
2''''	2.02 (s)	20.75	20.8

Spectral data comparison table of compound p3 with paulomycin E[4]



Paulomycin E (p3)

Position	^{1}H NMR of paulomycin E	^{1}H NMR of p3
1	-----	-----
2	-----	-----
3	-----	-----
4	-----	-----
5	3.15, 3.35 (AB)	3.32-3.16 (m)
6	-----	-----
7	-----	-----
8	3.96 (d)	3.96 (d, 10)
9	3.72 (ddd)	3.74 (ddd, 13, 10, 2.5)
10	4.31 (dd)	4.32 (dd, 2.5, 2)
11	4.82 (dd)	4.83 (dd, 11, 2)
12	4.26 (ddd)	4.27 (ddd, 11, 5, 2)
13	3.88, 3.99 (ABX)	3.89, 4.01 (ABX, 12, 8.5, 5.0)
1'	5.05 (dd)	5.05 (dd, 4, 2)
2'	1.88, 2.32 (ABMX)	1.84, 2.31 (ABMX, 16, 5, 2)

3’	4.11 (dd)	4.18 (dd, 11.5, 4)
3’-OMe	3.23 (s)	3.25 (s)
4’	-----	-----
5’	4.56 (q)	4.55 (q, 6.5)
6’	0.94 (d)	0.95 (d, 6.5)
7’	-----	-----
8’	2.24 (s)	2.26 (s)
1’’	-----	-----
2’’	-----	-----
3’’	6.84 (q)	6.83 (q, 7.0)
4’’	1.94 (d)	1.96 (d, 7.0)
5’’	-----	-----
1’’’	-----	-----
2’’’	-----	-----
3’’’	-----	-----
4’’’	-----	-----
5’’’	-----	-----
1’’’’	-----	-----
2’’’’	1.98 (s)	1.99 (s)

References

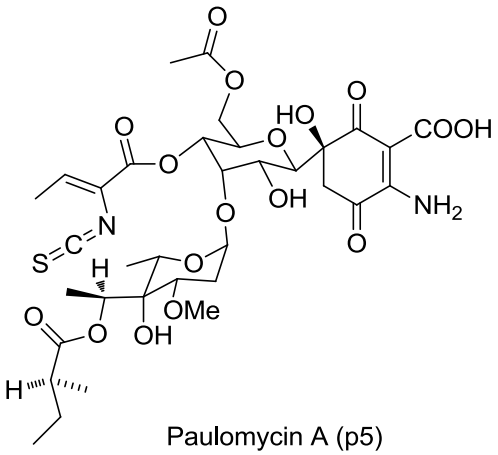
1. Argoudelis, A. D.; Brinkley, T. A.; Brodasky, T. F.; Buege, J. A.; Meyer, H. F.; Mizsak, S. A. *J. Antibiot.* **1982**, *25*, 285-294.

2. Argoudelis, A. D.; Baczynskyj, L.; S. A. Mizsak, S. A.; Shilliday, F. B. *J. Antibiot*. **1988**, *31*, 1316-1330.

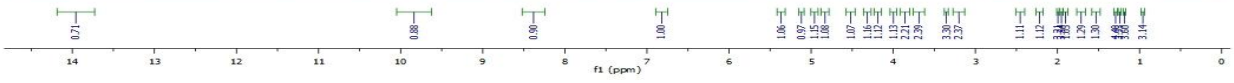
3. Wiley, P. F.; Mizsak, S. A.; Baczynskyj, L.; Argoudelis, A. D.; Duchamp, D. J.; Watt, W. *J. Org. Chem.* **1986**, *51*, 2493-2499.

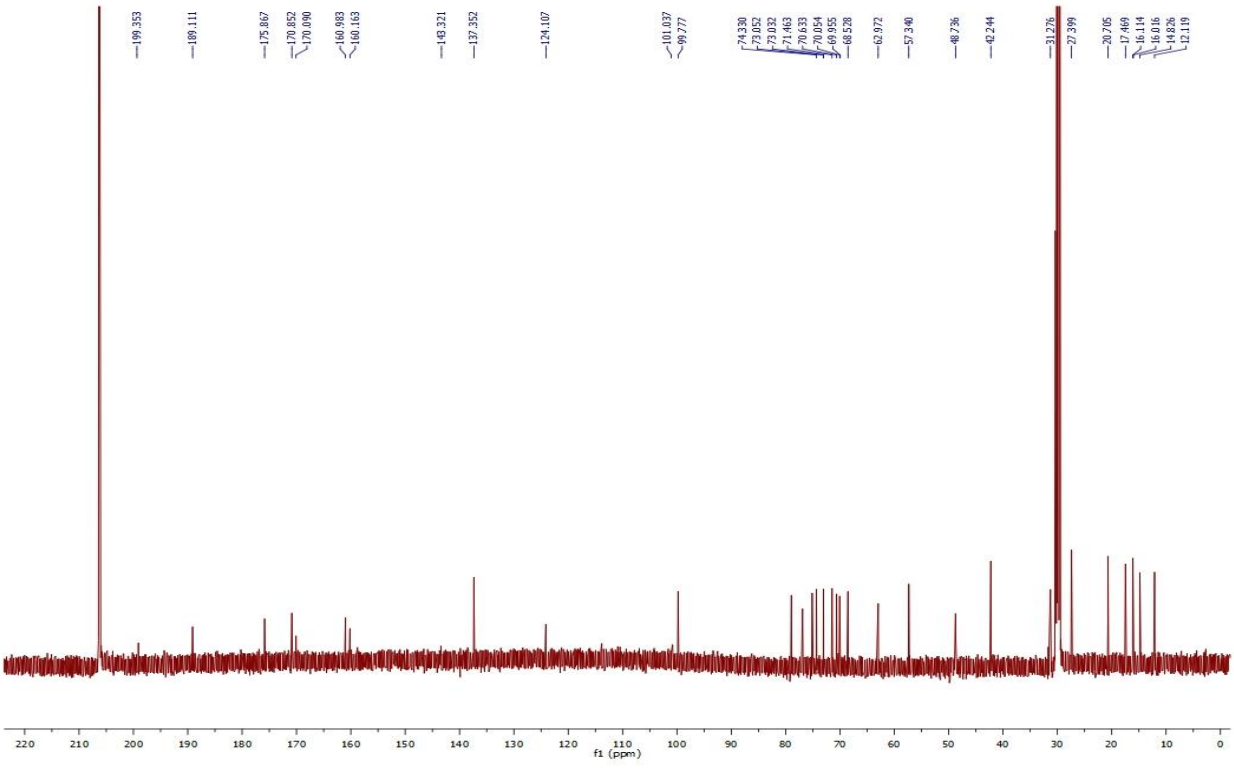
4. Argoudelis, A. D.; Baczynskyj, L.; Haak, W. J.; Knoll, W. M.; Mizsak, S. A.; Shilliday, F. B. *J. Antibiot.* **1988**, *31*, 157-169.

1H and 13C spectra of compound p5 (paulomycin A)

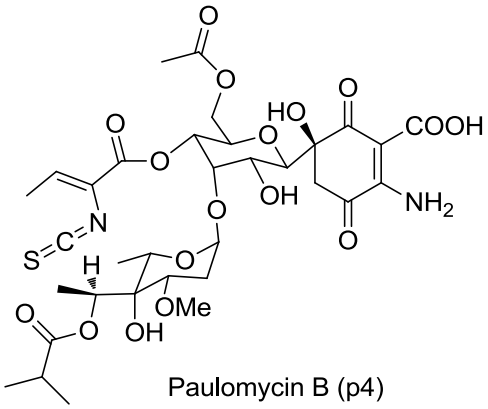


Paulomycin A (p5)

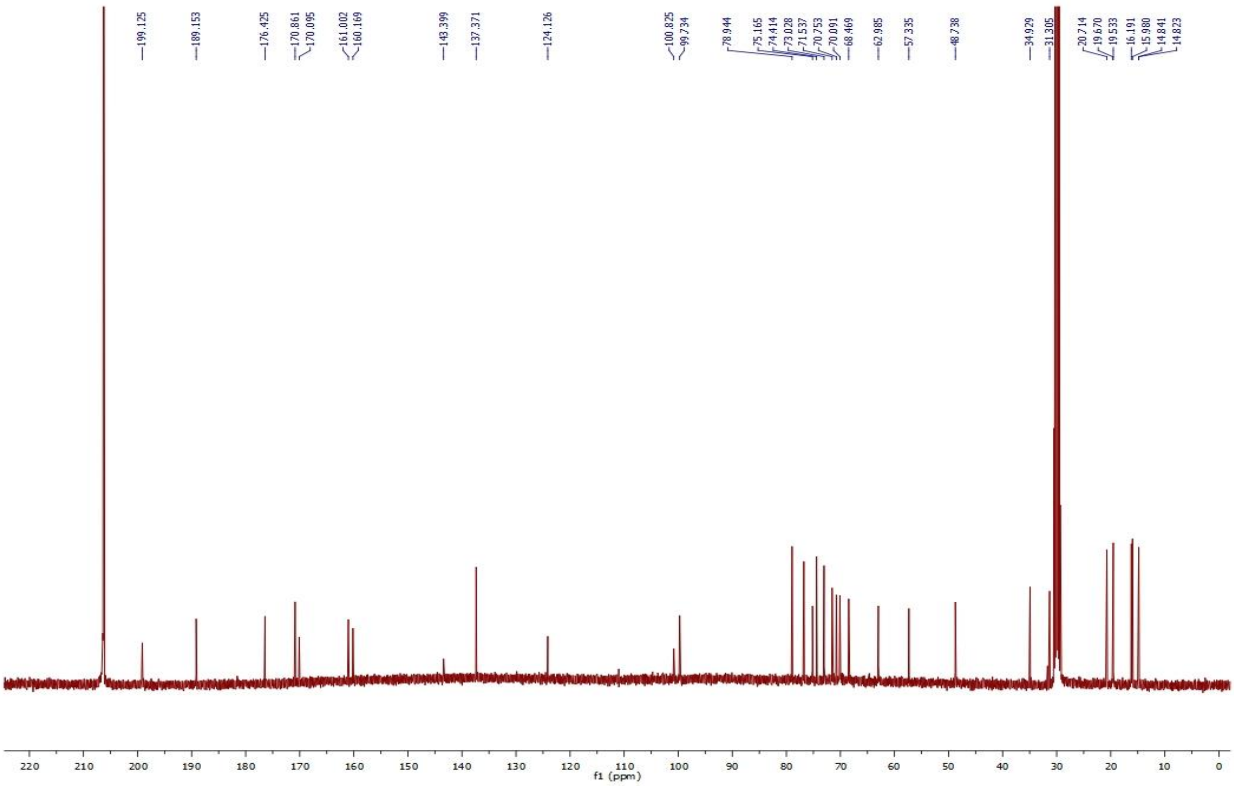




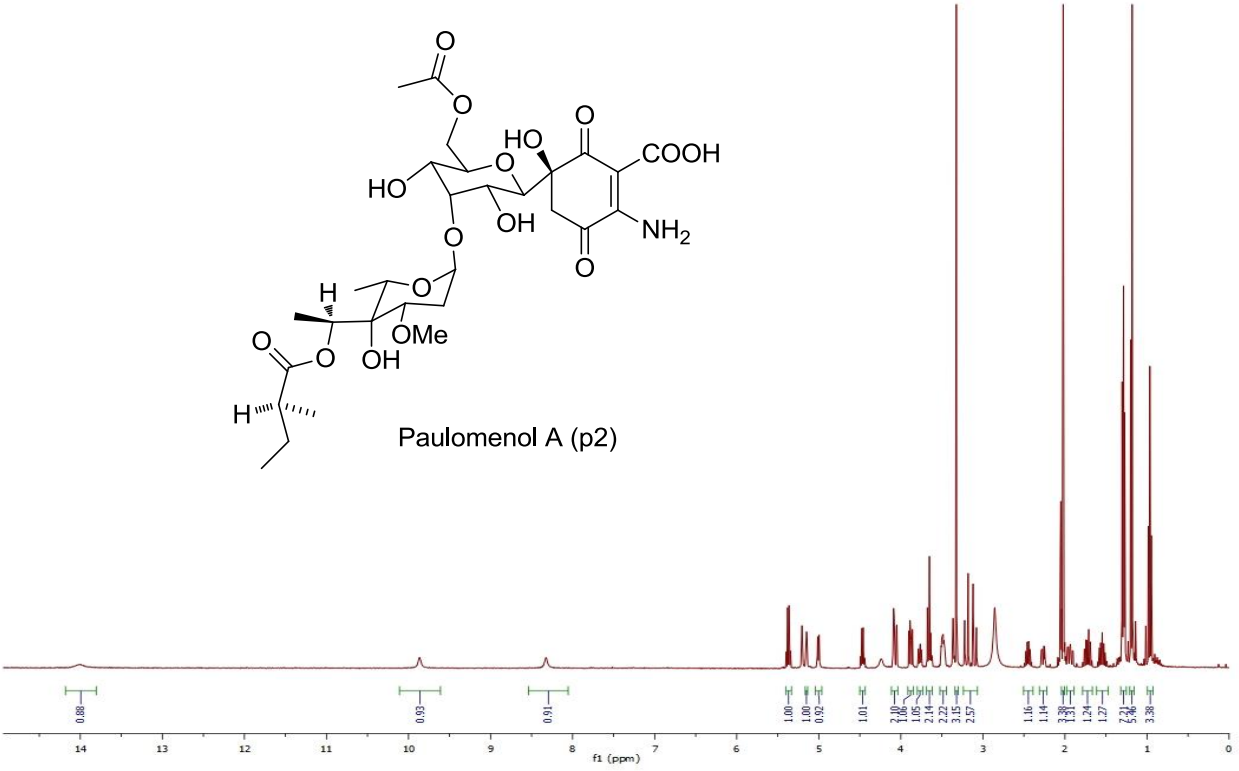
1H and 13C spectra of compound p4 (paulomycin B)

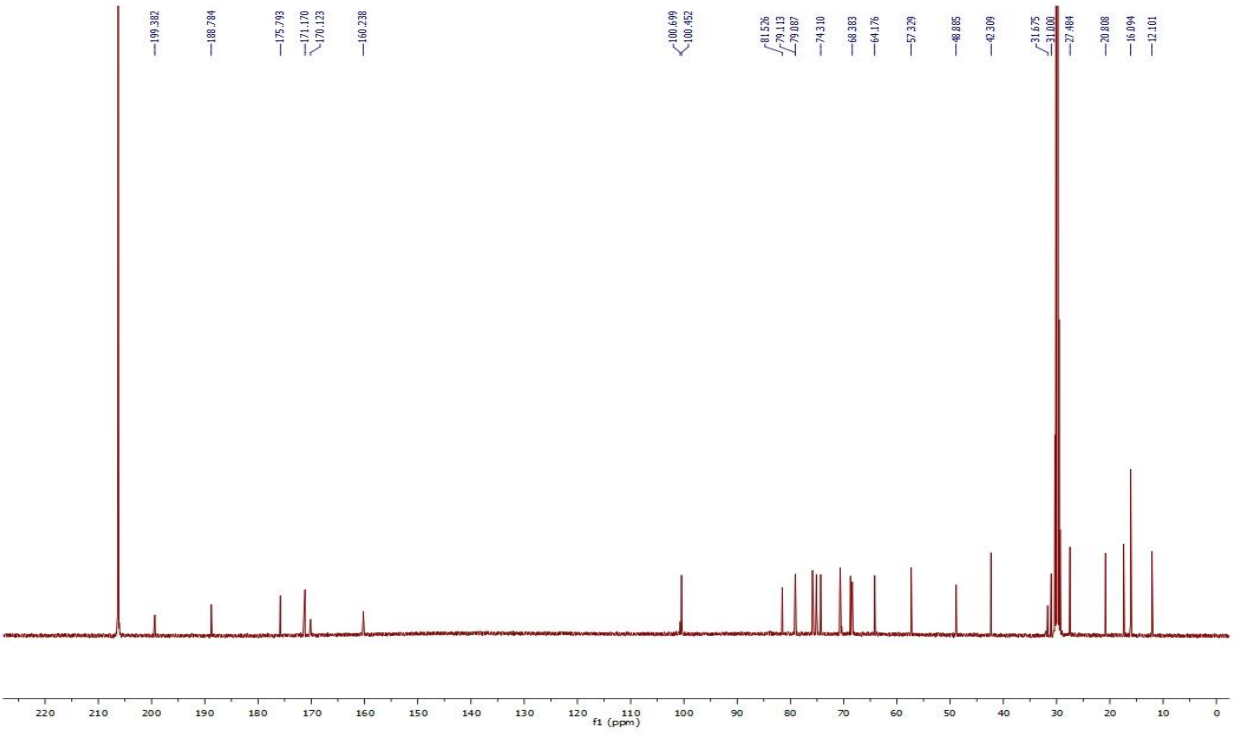


Paulomycin B (p4)

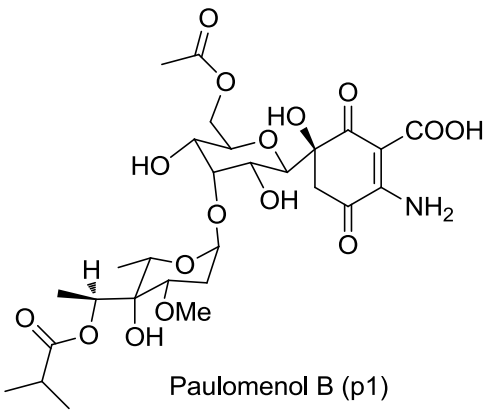


1H and 13C spectra of compound p2 (paulomenol A)

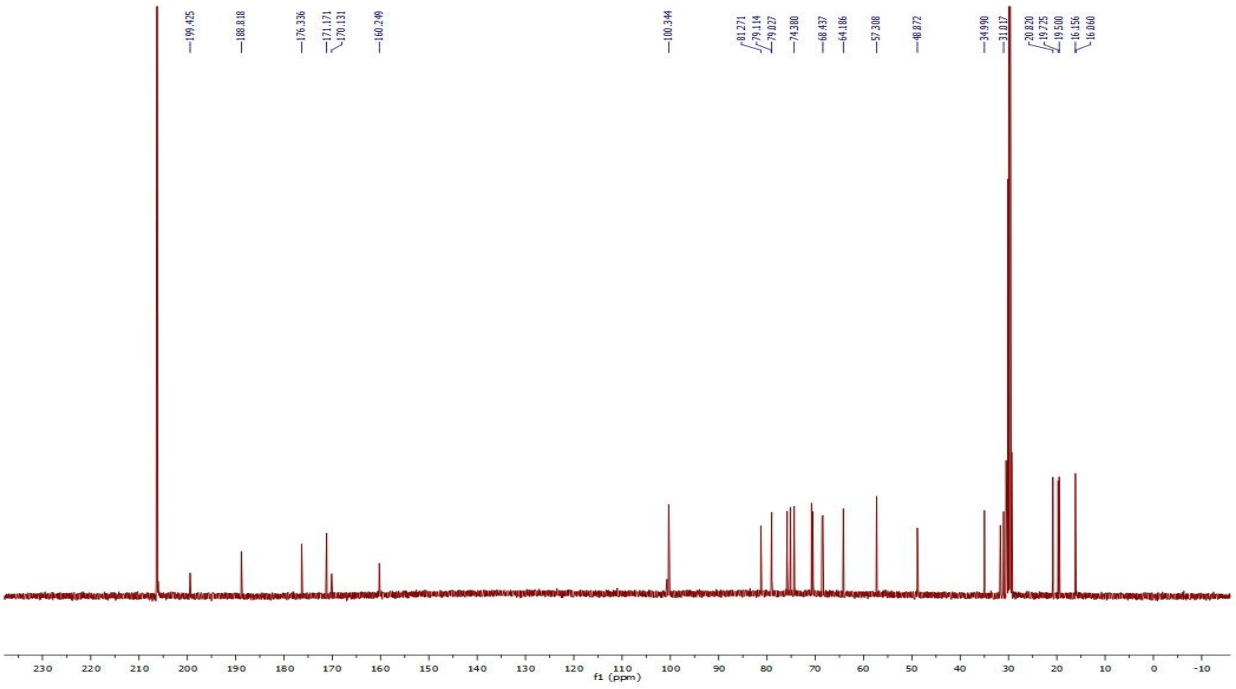




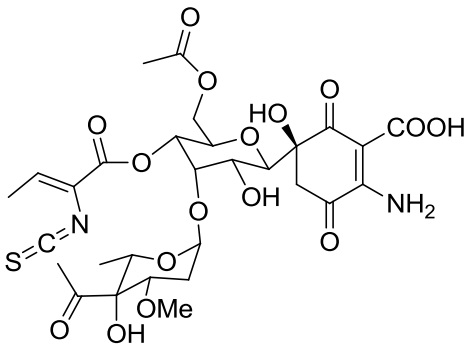
1H and 13C spectra of compound p1 (paulomenol B)



Paulomenol B (p1)



1H spectra of compound p3 (paulomycin E)



Paulomycin E (p3)