VOL. 54 NO. 3, MAR. 2001 THE JOURNAL OF ANTIBIOTICS pp. 239 — 249

Collinone, a New Recombinant Angular Polyketide Antibiotic Made by an Engineered *Streptomyces* Strain

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(Received for publication August 17, 2000)

Large chromosomal DNA fragments containing different parts of the putative rubromycin polyketide synthase gene cluster were cloned and functionally expressed in *S. coelicolor* CH999. Expression of these clones yielded 5~10 metabolites that were not detected in *S. collinus* culture extracts. This paper focusses on one of the new metabolites, termed collinone, that was isolated in large quantities and purified for spectroscopic structure determination and biological screening assays. Collinone is a heavily oxidized angular hexacyclic compound containing an unusual 1,4,5,8(2*H*,3*H*)-anthracenetetrone moiety previously only reported to be present in antibiotics SF2446A1, A2, A3, B1 and B2 isolated from *Streptomyces* sp. SF2446. Structure analysis of collinone indicates a tridecaketide with a 26 carbon backbone. The basic benz[a]naphthacene ring system of collinone is angular, similar to the aglycones of the well-known angucycline and angucyclinone antibiotics. While collinone showed antibacterial activity against vancomycin-resistant enterococci, no antifungal or significant antiviral activities were detected. Collinone could be a good starting point to obtain new bioactive angucyclin(on)e-like compounds by further genetic engineering of its pathway.

Aromatic polyketides belong to a large group of structurally diverse secondary metabolites that exhibit a broad range of biological activities. More than half of the clinically important polyketides are produced in *Streptomycetes via* initially a common biosynthetic pathway which then diverges on the specific enzymes individual species possess. Polyketide synthase (PKS) complexes catalyze the reactions which are analogous to the fatty acid synthesis, in which CoA-activated short carboxylic acids are used in repetitive decarboxylative condensations to build a polyketide chain. Structural diversity arises from a PKS encoded variability in the chain length and from the subsequent reactions such as ketoreductions, ring closures, aromatizations, hydroxylations, methylations, dimerizations, glycosylations, *etc.*

KHOSLA and coworkers established a genetic engineering approach to generate novel, non-natural polyketides by combining the biosynthetic pathways from several organisms[1,2]. However, this approach has several limitations: i) owing to the substrate specificities of the enzymes involved, not all recombinant constructs will result in new metabolites; ii) the number of genes that can be recombined in a cloning system is limited to approximately 10 at the most; and iii) the existing pool of characterized pathways and sequenced genes as well as their availability are limited. To address these limitations, we isolated and partially sequenced a new polyketide biosynthetic gene cluster from *Streptomyces collinus* DSM 2012, a strain that makes rubromycins[3,4]. α-Rubromycin, also termed collinomycin, possesses considerable *in vitro* activity

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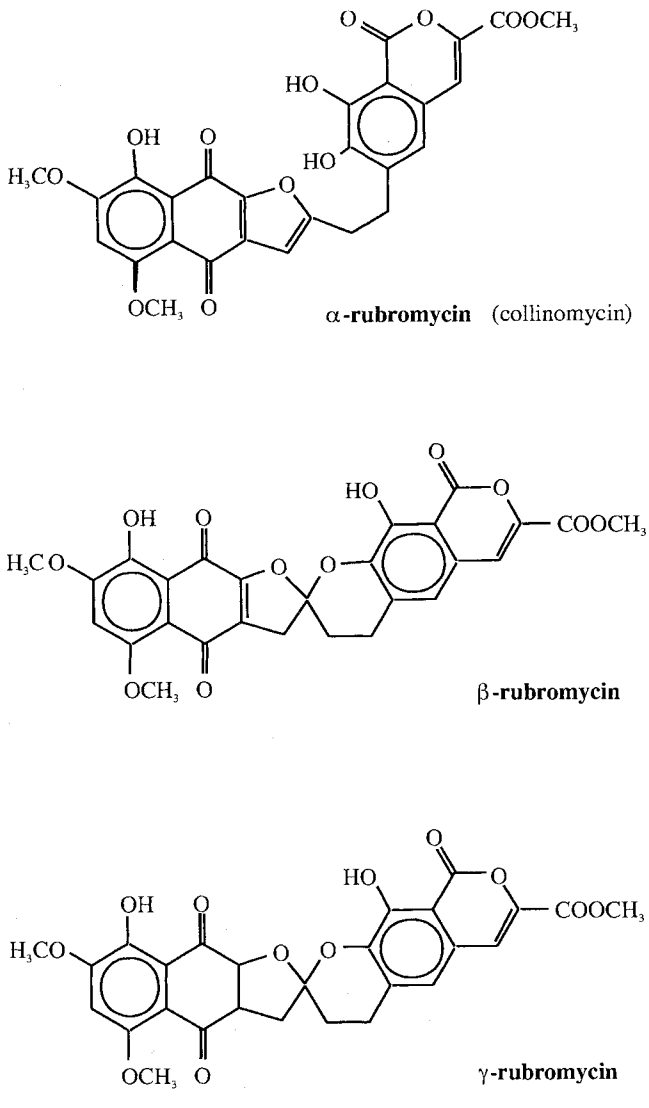
against *Staphylococcus aureus*, while β- and γ-rubromycin were shown to selectively inhibit HIV-1 reverse transcriptase[3~6]. The rubromycins possess numerous oxidized functionalities, some of which are methylated while others are cyclized forming a furan ring or a bicyclic acetal through the reaction of hydroxyl groups with enol functionalities in the vicinity (see Fig. 1).

The genes of the enzymes catalyzing these chemical changes in the rubromycins were at the center of our interest in the *S. collinus* polyketide biosynthesis gene cluster with the aim to generate new recombinant pathways.

Here we report the isolation of cosmid clones that contain some 35 kilobases (kb) of the putative rubromycin gene cluster and their expression in a heterologous host. Several metabolites that were not detected in *S. collinus* were produced by the recombinat strains. This paper focuses on the chemical structure and biological activity of one of the new metabolites, collinone.

Fig. 1. Chemical structures of the rubromycins.



Materials and Methods

Bacterial Strains and Plasmids

S. coelicolor CH999[1] and *S. collinus* DSM2012[7], *Escherichia coli* XL-1 Blue (Stratagene), *E. coli* ET12567 (*dam*$^-$, dcm$^-$, hsdM)[8], used to isolate unmethylated DNA for transformation into *S. coelicolor* CH999, *E. coli* K803 (*supE*, *hsdR*, *gal*, *metB*)[9], needed for transfection of packaged cosmids, and the *E. coli*-*Streptomyces* shuttle cosmid pKC505 (ampR, apR, ColE1, SCP2*)[10] are described in the indicated reviews. pRM5 and pSEK4[1] were obtained from C. KHOSLA, Standford University, Ca. *E. coli* vectors, pBluescript IISK+ (Stratagene) and pGEM3 Zf (+) (Promega), were obtained from commercial sources.

Standard medium for *Streptomyces* plate cultivations was R2YE; YEME and TSB were used for liquid cultures[11]. All cultivations were carried out at 30°C. Liquid cultures were incubated in a gyratory shaker (model G25, New Brunswick Scientific) at 300 rpm. Unless stated otherwise, all strains of *E. coli* were grown in LB medium (Bacto-tryptone 10 g/liter, Bacto-yeast extract 5 g/liter, NaCl 5 g/liter). For solid LB, 20 g/liter Bacto agar were added. *E. coli* strains were incubated at 37°C. Liquid cultures were incubated at 225 rpm in a gyratory shaker. Antibiotics, ampicillin (100 μg/ml), apramycin (20 μg/ml in liquid and 200 μg/ml in solid medium), and tobramycin (10 μl/ml), were added as required.

Protocols for genetic manipulation of *Streptomyces*, isolation of DNA, and protoplast transformation were previously described[11]. Standard techniques and protocols for molecular genetic techniques in *E. coli*, including plasmid isolation, DNA transformation, Southern hybridization, and gel electrophoresis have been described elsewhere[9].

Enzymes and Chemicals

All restriction endonucleases, T4 DNA ligase, and alkaline phosphatase were obtained from commercial sources and used according to the supplier's instructions. Chemicals were obtained from commercial sources and were at least of analytic grade quality. Organic solvents for polyketide extraction and HPLC were HPLC grade.

Preparation of the *S. collinus* Genomic DNA Library

About 100 μg of *S. collinus* DSM 2012 genomic DNA was partially digested with 4 U *Sau*3AI in a total volume of 1 ml. Aliquots of 120 μl were removed after 15 and 30 seconds, and after 2, 4, 6, 8 and 10 minutes at 37°C. The digestion was stopped by phenol extraction. The aliquots were pooled, isopropanol-precipitated, redissolved in 70 μl water, and treated with alkaline phosphatase. The dephosphorylated DNA was precipitated, washed with 70% ethanol, air-dried, and resuspended in 25 μl TE buffer, pH 8.

About 25 μg of the cosmid pKC505 was digested with *Hpa*I, dephosphorylated and subsequently digested with *Bam*HI. Approximately 1 μg of *Hpa*I and *Bam*HI digested pKC505 was ligated with 1.2 μg of *Sau*3AI partially digested *S. collinus* chromosomal DNA. The ligation mixture was packaged into phage particles using the Giga-Pack Gold packaging kit (Stratagene) according to the Manufacturer's protocol. A 50 ml, overnight grown culture of *E. coli* K803 was infected with 100 μl of the packaged phage solution and cultured according to the supplied protocol. Aliquots of 250 μl of the infected cell suspension were plated onto ten TSB plates of 145 mm diameter containing tobramycin (10 μl/ml) and incubated overnight at 30°C. The following day, the transductants were used to proceed to colony lifting on nylon membrane and the plates were stored at 4°C.

Colony Lifting and Southern Hybridization

The genomic library was screened by colony lifting[9]. The DNA probe for detection of putative PKS genes contained the ketoacyl synthase genes for the α- and β-subunits from the actinorhodin pathway of *S. coelicolor* A3(2)[12]. These genes are highly conserved throughout type-II polyketide synthase genes. This fragment was isolated as a 2.6 kb *Pac*I-*Xba*I fragment from pRM5[1]. The probe was labeled for chemiluminescent detection using the DIG-labelling and DIG-detection kits (Roche Molecular Biochemicals) according to the protocols supplied.

Isolation and Purification of Collinone from Recombinants

The recombinant strains MRB3B, MRB3C, MRB3E and MRB6C produced ample amounts of red-blue pigmented secondary metabolites. For large scale isolation of metabolites, thirty R2YE plates (9 cm diameter, approximately 3 liter medium) containing the appropriate antibiotic were inoculated each with 150 μl of a TSB culture (grown for 48 hours) of the respective strain. After four weeks incubation at 30°C, the agar was cut into small pieces and extracted twice with approximately one volume of ethyl acetate/methanol 4 : 1 containing 1% acetic acid. The extracts were pooled and filtered through a paper filter. Water was eliminated from the organic phase by adding approximately 50 g of anhydrous magnesium sulfate. The organic phase was filtered to remove the magnesium sulfate, and air dried. The dark red dried powder was redissolved in 25 ml ethyl acetate and loaded onto a flash silicagel column (J. T. Baker 7024-01) for further purification. A small fraction of this crude extract was used directly for TLC or HPLC analysis. After elution of the flash column with ethyl acetate, the colored fractions were collected and dried. The dried extract was redissolved in 50 ml acetonitrile, filtered though a 0.2 μm PTFE membrane filter and loaded onto the preparative HPLC column. Preparative HPLC purifications were carried out on a DeltaPrep 4000 system (Waters) equipped with a Waters™ 486, Tunable Absorbance Detector and a preparative C18 reverse phase column (Delta-Pak C18 300 H, 15 μm, cartridge 40×100 mm, Waters). Peak fractions were collected manually. The mobile phase consisted of acetonitrile in distilled water with 0.1% trifluoroacetic acid (TFA), the flow was 50 ml/minute, and the detection wavelength was set to 280 nm. After sample injection (5 ml) a programmed 50 minutes linear gradient was started followed by a column cleaning step with 80% acetonitile. The gradient for collinone purifications started with 25% acetonitrile and ended at 40%. Peak fractions from each run were collected and an aliquot was removed for peak purity assessment on an analytical HPLC (Perkin-Elmer Liquid Chromatograph Model Series 410, Series 200 Diode Array Detector) equipped with a C18 reverse phase column (cc 250/4 Nucleosil 100-5 C18 HD, Macherey-Nagel, Switzerland). Running the appropriate acetonitrile gradient containing 1% acetic acid, peaks were detected at 280 nm. The remainder of the fractions was extracted with approximately 0.2 volume of HPLC-grade ethyl acetate. A high acetonitrile content in some fractions required the addition of distilled water until the separation of the phases occurred. Further washing of the organic phase with water was done until the pH of the aqueous phase was 5.5 to 6. The organic phase was dried, the amount of dried powder determined, and used for spectroscopic analysis, structure elucidation and biological assays. Following this procedure with MRB6C grown on 1.5 liter medium, 250 mg collinone could be purified with a 99.9% purity.

NMR Spectroscopy

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500

spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{XH}=145$ Hz and $^vJ_{XH}=10$ Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). Mass spectra were recorded with a Jeol SX102 spectrometer, while the melting point (uncorrected) was determined with a Reichert microscope.

MS (FAB ionization, with addition of NaOAc), *m/z*: 557.0671 ($M+Na^+$, $C_{27}H_{18}O_{12}Na$ requires 557.0696).

Screening for Biological Activities of Collinone

Antibacterial activities, minimum inhibitory concentrations (MICs), were determined on Müller-Hinton (MH) agar (Difco). Collinone was dissolved in a small volume of acetonitrile, diluted with water and incorporated in two-fold serial dilutions into the agar. It was first verified that acetonitrile alone had no antibacterial effect at the concentrations used. Ampicillin, penicillin G and vancomycin were dissolved in water. Plates and solutions were prepared fresh daily. The inoculum was prepared from overnight cultures of strains, diluted and applied to the agar surface by using a multipoint inoculator. The size of the inoculum was about 10^4 colony-forming units per spot. The plates were inoculated 20 hours at 35°C. The MICs were determined as the lowest concentrations that prevented visible growth, disregarding less than 3 colonies or a faint haze.

For antifungal susceptibility testing, we used the modified microdilution method of the National Committee for Clinical Laboratory Standards (NCCSL) M27-A with the spectrophotometric method. YNBPB (=YNB+1%glucose+0.25%K_2HPO_4, pH 7.0) was used for yeasts and YNBPA (=YNB+1%glucose+0.25%K_2HPO_4+LMPA, pH 7.0) was used for filamentous fungi. A conidial suspension was added 1/100 volume of medium to test medium and the inoculum size for all strains was 1 to 3×10^4 conidia/ml in final concentration. Microtest plates were incubated at 35°C for a suitable period for each species, such as 1 to 2 days for most species except for the slow growing strains such as Dermatophytes species. The MIC end point of fluconazole (FCZ) was determined by the concentration of drug that produced an 80% reduction of turbidity compared to that of the drug free control measured by the microplate reader at 630 nm.

The antiviral assays were based on inhibition of virus-induced cytopathicity in either E_6SM (Herpes simplex virus-1, Herpes simplex virus-2, Vaccinia virus, Vesicular stomatitis virus), Vero (Parainfluenza-3 virus, Reovirus-1, Sindbis virus, Coxsackie-B4 virus, Punta Toro virus) or HeLa (Vesicular stomatitis virus, Coxsackie-B4 virus, Respiratory syncytial virus) cell cultures, following previously established procedures[13,14]. Briefly, confluent cell cultures in microtiter 36-well plates were inoculated with 100 $CCID_{50}$ of virus, 1 $CCID_{50}$ being the virus dose required to infect 50% of the cell cultures. After a 1-hour virus adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying dilutions of the test compound (80~16 μg/ml) collinone. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compound.

The anti-HIV activity assays were run as described previously[15]. Briefly, human MT-4 ($\sim4\times10^5$ cells ml^{-1}) cells were infected with 100 $CCID_{50}$ of HIV-1 (III_B) ml^{-1} and seeded in 200 μl wells of a microtiter plate, containing appropriate dilutions of the test compounds. After 5 days (MT-4) or 4 days (CEM) of incubation at 37°C, the number of viable (MT-4) cells was determined in a blood cell counting chamber by trypan blue dye exclusion or giant (CEM) cell formation was examined microscopically.

Results

Construction of the Recombinant Strains

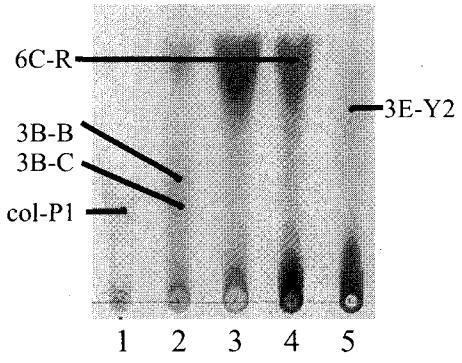
The cosmid pKC505 which replicates in both *Escherichia coli* and *Streptomyces* and is capable of carrying inserts between 30 to 40 kb was used to construct the *S. collinus* DSM 2012 genomic library, resulting in 4000 clones carrying inserts of the expected size. We isolated from this library by Southern hybridization using the polyketide synthase specific *actI* DNA probe, several cosmid clones that contained PKS genes. These clones could be grouped into two classes, representing two putative PKS gene clusters. DNA sequence analysis revealed that one gene cluster has strong homology to PKS genes encoding spore pigments while the other gene cluster has significant homology to the antibiotic PKS genes[16]. Four cosmids, p3B, p3C, p3E, and p6C, of the latter PKS cluster, carried inserts of 30~42 kb covering about 52 kb of the *S. collinus* chromosomal DNA. When these cosmids were transformed into *S. coelicolor* CH999, resulting in

strains MRB3B, MRB3C, MRB3E, and MRB6C, respectively, all transformants were found to produce considerable amounts of pigmented secondary metabolites (Fig. 2). Two strains, MRB3C and MRB6C that released a dark red compound were chosen for more detailed analysis of their secondary metabolites. A 6 kb *Xba*I-*Eco*RI fragment of p6C, containing the PKS genes and 5 additional genes, was subcloned into *Eco*RI-*Pac*I digested pSEK4 and transformed into *S. coelicolor* CH999. The resulting strain MRB1 made a different pigmented metabolite.

Culture extracts from *S. collinus* DSM 2012 and MRB6C were analyzed by analytical HPLC (Fig. 3). The different elution profiles of their metabolites supported the previous findings from TLC clearly showing that MRB6C produces a compound that is not present in wild type *S. collinus*. About 250 mg of this main metabolite made by MRB6C was purified for the structural analysis and the determination of potential biological activity. Between 10~20 μg of the MRB1 metabolite were purified for structure determination.

Fig. 2. TLC analysis of culture extracts from *S. collinus* DSM 2012 (lane 1), and the recombinant strains MRB3B (lane 2), MRB3C (lane 3), MRB3E (lane 4) and MRB6C (lane 5).

Culture extracts dissolved in ethyl acetate were applied onto a silicagel TLC plate and resolved with ethyl acetate/methanol 7:3+1% acetic acid. The main products in the extracts were collinone (6C-R) and 3 so for unidentified compounds (marked 3B-B, 3B-C, 3E-Y2) and putative collinomycin (col-P1) made by *S. collinus*.



Structure Determination

Collinone was obtained as a dark-red solid, m.p. 222~225°C. The structure of collinone (Fig. 4) was determined by NMR spectroscopy and mass spectrometry. The latter technique showed that the molecular weight of collinone is 534, and high resolution measurements suggested that the elemental composition is $C_{27}H_{18}O_{12}$. This was confirmed by the presence of 27 signals in the ^{13}C NMR spectrum and signals integrating for a multiple of 18 protons in the ^{1}H NMR spectrum (1D NMR data are given in Table 1). Consequently, collinone has an unsaturation index of 19, and as the NMR data suggest the presence of 5 carbonyl groups and 8 carbon-carbon double bonds indicates a 6 ring structure. The structure elucidation was complicated by the presence of no less than 18 non-protonated carbons, but careful examination of the long-range ^{1}H-^{13}C correlations (the correlations observed in HMBC experiments are summarized in Fig. 5) established the structure. The 3-methyl substituted isocoumarin moiety was demonstrated by the strong HMBC correlations between 26-H_3 and C-24 as well as C-25, and the weak correlation to C-23, the HMBC correlations between 24-H and C-2, C-22, C-25 and C-26, between 22-H and C-1, C-2, C-4, C20 and C-24, and between the hydrogen bound 3-OH and C-2, C-3 and C-4. The presence of a $-CH_2-CH_2-$ unit connected to C-21 could be established by the COSY correlations between 19-H_2 and 20-H_2, and the HMBC correlations from 19-H_2 to C-21 as well as between 20-H_2 to C-4, C-21 and C-22. The additional HMBC correlations between 20-H_2 and C-18 and between 19-H_2 and C-5, C-17 and C-18 show that C-19 is connected to C-18 which has C-5 and C-17 as neighbours. Both C-5 and C-18 appear from their ^{13}C chemical shifts to be quarternary oxygenated carbons, and a weak HMBC correlation between 22-H and C-5 closes the six-membered ring adjacent to the isocoumarin system. In the other end of the molecule, HMBC correlations from the methoxy protons to C-11 and (weakly) to C-12 indicate the presence of a methyl enol ether, and this is supported by the chemical shifts for the double bond carbons. 19-H gives strong HMBC correlations to C-10, C-11 and C-14 as well as weak correlations to C-13 and C-15. This supports together with the observed HMBC correlations from the two remaining hydrogen bonded hydroxyl protons (from 8-OH to C-7, C-8, C-9 and (weakly) C-10, and from 15-OH to C-14, C-15, C-16 and (weakly) C-13) of the suggested dihydroxy-methoxy-1,4-naphthoquinone substructure. No HMBC correlations could be observed to C-6, but a very weak correlation from 19-H_2 to C-16 indicates that C-16 is connected to either C-5 or C-17. By connecting C-16 to C-17 and incorporating the remaining carbonyl group (C-6) into the sixth and final ring (between C-5 and C-7), the structure shown in Figure 4 was obtained. An alternative

Fig. 3. HPLC analysis of culture extracts from *S. collinus* DSM 2012 (upper panel) and MRB6C (lower panel).

Chromatograms are shown on the left and the spectral properties of the numbered peaks are shown on the right. The major red colored peak in the *S. collinus* extract (3) eluts at ~30 minutes, while the major peak of MRB6C (5, collinone) elutes at 26 minutes. This peak fraction of MRB6C was isolated and purified for structural analysis. Peaks 2, 4 and 6 are polyketide related compounds that have not been isolated so far. As judged from the elution profile, peak 1 may not be a polyketide related substance. Separation conditions are described in detail in Materials and Methods.

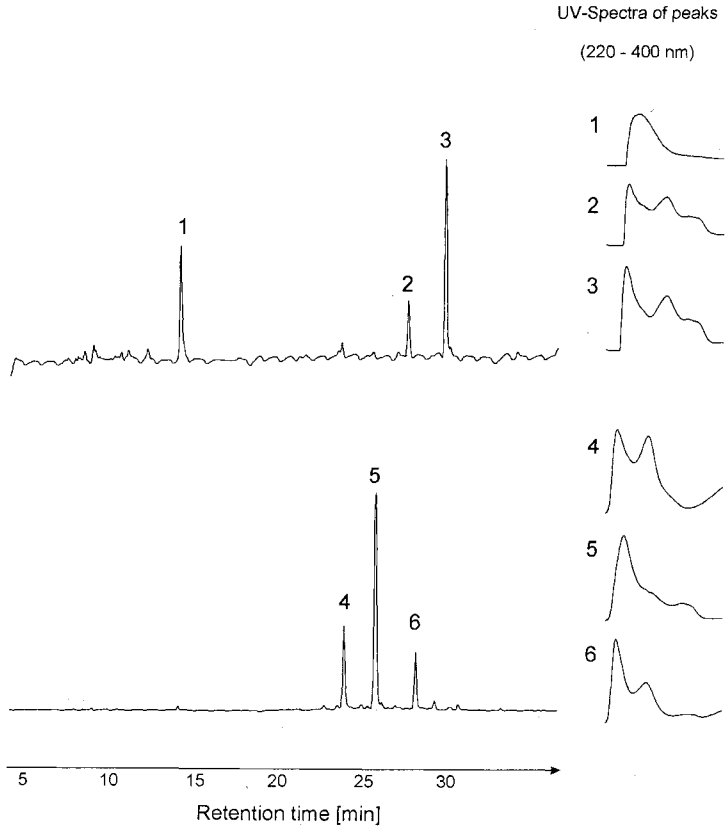
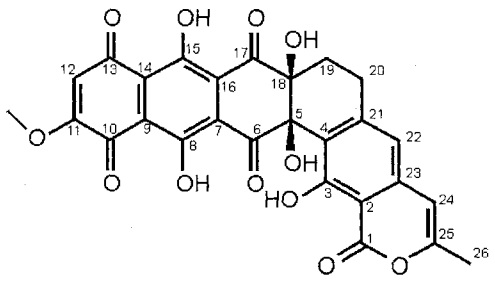


Fig. 4. The structure and atom numbering of collinone.

The stereochemistry is tentatively assigned.



structure, obtained by connecting C-16 to C-5 and placing C-6 between C-7 and C-17 in the last ring, is not plausible as the difference in ^{13}C chemical shift between C-7 and C-16 would be expected to be much larger than that observed (2.3 ppm). The relative stereochemistry of the C-5/C-18 bridge is suggested to be *cis*, based on the analysis of Dreiding models of the possible isomers/comformers and comparing the dihedral angles between 19-H_2 and 20-H_2 and the ^1H-^1H coupling constants. In DMSO-d_6, although this solvent was not suitable for NMR spectroscopy (*vide infra*), the ^1H chemical shifts for 20-Ha and 20-Hb were separated by 0.08 ppm and all coupling constants for this spin system could be extracted. The coupling constant between 19-Ha (3.10 ppm) and 20-Ha (2.32 ppm) is 1 Hz, indicating that the dihedral angle between them is close to

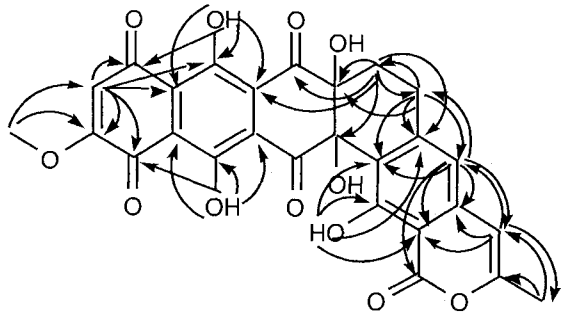
Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for collinone, in $CDCl_3$ with the solvent signals (7.26 and 77.0 ppm, respectively) as reference.

	δ ^{1}H (ppm)	multiplicity	J (Hz)	δ ^{13}C (ppm)	multiplicity
C					
1	-	-	-	166.4	s
2	-	-	-	103.3	s
3	-	-	-	159.5	s
4	-	-	-	119.6	s
5	-	-	-	78.7	s
6	-	-	-	194.3	s
7	-	-	-	130.9	s
8	-	-	-	154.4	s
9	-	-	-	116.7	s
10	-	-	-	182.6	s
11	-	-	-	160.7	s
12	6.17	s	-	110.7	d
13	-	-	-	188.0	s
14	-	-	-	115.9	s
15	-	-	-	155.4	s
16	-	-	-	128.6	s
17	-	-	-	191.0	s
18	-	-	-	80.8	s
19a	2.43	m	-	25.7	t
19b	2.24	ddd	8, 8, 13	-	-
20	3.09	m	-	27.3	t
21	-	-	-	148.0	s
22	6.53	s	-	115.8	d
23	-	-	-	137.8	s
24	6.03	s	-	104.0	d
25	-	-	-	155.0	s
26	2.17	s	-	19.3	q
3-OH	11.52	s	-	-	-
5-OH	5.34	brs	-	-	-
8-OH	12.53	s	-	-	-
11-OMe	3.93	s	-	57.1	q
15-OH	13.48	s	-	-	-
18-OH	3.55	brs	-	-	-

90°. This condition is fulfilled in the conformation of the *cis* isomer having the C-6 and C-17 carbonyl groups in approximately the same plane as the neighboring aromatic ring (as suggested by the ^{13}C chemical shifts for C-6 and C-17), but not with the *trans* isomer. No attempts were made to determine the absolute stereochemistry.

As indicated above, DMSO-d_6, as well as other solvents and combinations of solvents, were not suitable for NMR measurements with collinone, because the ^{13}C NMR signals for the dihydroxy-methoxy-1,4-naphthoquinone system could not be observed even at different temperatures. In addition, the compound turned out to be unstable in many solvents (DMSO, acetone, methanol). This may be caused

Fig. 5. Summary of HMBC correlations observed with collinone in $CDCl_3$.



by a rapid interchange between the 1,4-quinone and 1,4-hydroquinone functionalities, making it susceptible to oxidation by molecular oxygen, for example. The same complications hampered the spectroscopic investigation of MRB1, the structure of which is presently inconclusive: current analysis indicated a dimethylene subunit and structure similarity to collinone is inevitable.

Biological Activities

To evaluate the pharmaceutical potential of collinone, it was tested for antibacterial, antifungal, antiviral, and cytotoxic activity. Collinones activity against Gram (+) and Gram (−) bacteria included representative strains of pathogenic species. The results showed it inhibited consistently Gram (+) cocci such as staphylococci, streptococci and enterococci at concentrations of 16 or 32 μg/ml (Table 2). It was, however, inactive against Gram (−) bacteria up to 64 μg/ml, the highest concentration tested (data not shown). Thus the range of susceptible bacteria is similar to that of vancomycin, but vancomycin is generally 4~16 times more active. Penicillin-susceptible cocci were much more sensitive to ampicillin and penicillin G than to collinone. Noteworty was collinones activity against methicillin-resistant staphylococci (MRS) and vancomycin-resistant enterococci (VRE) (Table 2) which required MICs (minimum inhibitory concentration) of 16 μg/liter and of 16 to 32 μg/ml, respectively. Antifungal tests revealed no biological activity of collinone against *Candida albicans* ATCC 48130 and *Aspergillus fumigatus* 437 as indicator strains and fluconazole as a positive control. Unfortunately, collinone proved cytotoxic at concentrations of ≥16 μg/ml in various

Table 2. Antibacterial activities of collinone compared to ampicillin, penicillin G and vancomycin against Gram(+) bacteria including representative strains of pathogenic species and methicillin-resistant staphylococci (MRS) and vancomycin-resistant enterococci (VRE).

Minimal inhibitory concentration (MIC) in presented as [μg/ml]

Strain	MIC [μg/ml]			
	Collinone	Ampicillin	Penicillin G	Vancomycin
Staphylococcus aureus 6538	16	0.25	<0.1	2
Staph. aureus 887	16	8	16	1
Staph. aureus 743 (MRS)	16	>64	>64	2
Staph. aureus 270A (MRS)	16	32	32	2
Staph. aureus 42080 (MRS)	16	>64	64	4
Staph. aureus H19982 (MRS)	16	64	64	4
Streptococcus pyogenes β15	16	<0.1	<0.1	0.5
Streptococcus pneumoniae 907	32	<0.1	0.1	0.5
Enterococcus faecalis 6	16	2	1	4
Enter. faecalis ATCC 29212	16	2	1	4
Enterococcus faecalis E III haem	16	2	2	2
Enterococcus faecium 73-92	16	64	>64	>64
Enterococcus faecium VanB E45-7	32	64	>64	>64
Enterococcus faecium VanB E11-10	32	64	64	>64
Enterococcus faecium QK 5/90	32	16	64	>64

cell lines (*i.e.* human skin-embryo fibroblasts, Hela cells, Vero cells and human T-lymphocyte MT-4 cells). An even lower IC_{50} value for collinone of about 1.8 μg/ml was determined for HeLa S3 cells in plasma protein free medium. Tests for antiviral activity (Human immunodeficiency virus, Herpes simplex virus, Vaccinia virus, Vesicular stomatitis virus, Respiratory syncytial virus, Parainfluenza-3 virus, Reovirus-1, Sindbis virus, Coxsackie-B virus and Punta Toro virus) did not demonstrate any specific antiviral activity at a concentration of $\leq$16 μg/ml (not shown). In summary, collinone is a biologically active compound that despite its cytotoxicity also has interesting biological antibacterial activity. Current studies focus on genetic modifications of the biosynthetic pathway leading to collinone to further alter the structure in a specific way. This approach combined with a better understanding of the mode of action of collinone could lead to a more potent secondary metabolite synthesized *in vivo*.

Discussion

Angucycline/angucyclinone-derived secondary metabolites possess a broad spectrum of biological activities. According to present knowledge, biosynthesis of the angucycline group of antibiotics seems to be characteristic of actinomycetes. Although the biosynthetic formation of decaketide chain is possible, for example, in the group of *Fungi imperfecti*, not a single 'angu'-metabolite has been found in these organisms[17]. Furthermore, angucycline group metabolites have not been discovered in other natural sources[17]. Introduction of cosmid p6C composed of about 35 kb of the putative rubromycin PKS gene cluster from *S. collinus* into *S. coelicolor* CH999 (MRB6C) resulted in the production of several compounds not detected in *S. collinus*. TLC analysis of culture extracts showed that MRB6C and also MRB3C make a dark red pigmented compound named collinone which is by far the predominant compound produced by these two transformants.

The chemical structure of collinone suggests that it is an angular hexacyclic tridecaketide, with a backbone made of 26 carbons, in which one of the keto groups has been reduced to a methylene group (C-19) and several carbons have been either reduced or oxidized. The term "angucycline" has been ascribed to every natural product consisting of (or derived from) an angular tetracyclic structural moiety which is biosynthetically derived from a

decaketide (20 carbons) chain derived *via* the polyketide biosynthetic pathway[17]. Since collinone is an hexacyclic molecule, it could be argued that collinone might not match the strict interpretation of the above definition. Focussing on the angular structure as the main criterion, we suggest that collinone is regarded as a member of the angucyclinone class of metabolites. To our knowledge, no structure like collinone has previously been described, and the only structures that might resemble some similarity to collinone are those of benanomicin A and B, molecules derived from a dodecaketide, an alanin, and a methionine which also fails to meet the strict criteria for an angucycline classification[18].

A small number of reports demonstrate the feasibility to obtain an angular metabolite upon expression of a PKS in an heterologous host[19~21]. However, collinone is the first example of a recombinant hexacyclic angucylinone most likely derived from a tridecaketide that is synthesized in a heterologous host. In addtion, collinone is a heavily oxidized angular hexacyclic compound containing an unusual 1,4,5,8(2*H*,3*H*)-anthracenetetrone moiety previously only reported to be present in antibiotics SF2446A1, A2, A3, B1 and B2 isolated from *Streptomyces* sp. SF2446[22]. Similar tridecaketides are antibiotic KS-619-1, an inhibitor of Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase isolated from *Streptomyces californicus*[23], and antibiotics WS79089A, B and C, inhibitors of the endothelin converting enzyme isolated from *Streptosporangium roseum*[24]. These molecules, however, contain an anthraquinone moiety in the place of the (2*H*,3*H*)-anthracenetetrone moiety present in collinone.

The biosynthetic pathway of collinone and the rubromycins in *S. collinus* DSM 2012 still needs to be determined, but it can be imagined that collinone is an intermediate (or a derivative of an intermediate) in the biosynthesis of α-, β- and γ-rubromycins. Collinone has two adjacent asymmetric carbons, C-5 and C-18, which both have hydroxyl groups that are *cis* oriented. The same relative stereochemistry has been reported for angucyclines with a similar substructure[22]. Further oxidation at C-5/C-6 could lead to the elimination of both carbons, which would yield the rubromycin skeleton. Similar chemical rearrangements and carbon elimination steps have been reported for the biosynthesis of the kinamycins from their angucyclinone precursor[25]. The finding of the same dimethylene subunit (C-19/C-20 in collinone) in the rubromycins and in collinone indicates that they are formed along a common biosynthetic pathway. Moreover, the dimethylene subunit at position C-19 and C-20 could already be detected in MRB1 extracts[16] expressing a ~6 kb subfragment from p6C that contains 8 open reading frames (ORF) (not shown) of which ORF 1~3 code for the type-II polyketide synthase, ORF 4, 5 and the *N*-terminal half of ORF 6 have similarty to three *Streptomyces*, polyketide-related cyclases[16], and ORF 6 *C*-termial half and ORF 7 have homology to putative oxidoreductase genes found in the *Actinomadura hibisca* pradamycin gene cluster, while ORF 8 has homology to a gene of unknown function of the same gene cluster[26]. Even though the products of the latter three genes remains to be verified, the functional requirements for the reduction of the keto group (C-19 in collinone) to form the dimethylene subunit are a ketoreduction, and a dehydration followed by an carbon-carbon double bond reduction. Since MRB1 already shows this dimethylene subunit, all genetic information for these reactions should be contained within these 8 ORFs. Knock-out experiments of ORFs 6~8, that are currently under way, should permit the functional characterization of these gene products. The enzymes catalyzing the angular cyclization and the further conversions will be very valuable for use in generating novel hybrid polyketides and in designing biosynthetic pathways for altered molecules.

Unfortunately, only scattered information is available on the structure-function-relationship of angucylin(on)es most of which has been reviewed recently[17]. Most of the angucyclines/angucyclinones exhibit weak antibacterial activity, especially against Gram-positive bacteria[27,28] and, in some cases, weak antifungal activity[29]. More interesting are the inhibitory effect on the reverse transcriptases of the Avian myeloblastosis virus[30] and the activity against the DNA viruses Herpes simplex type 1 and 2[29]. The majority of studies on biological activities, however, have revealed cytotoxicity/cytostatic activity of the angucyclines/angucyclinones[31~34], which might become a potential tumor treatment. Remarkably, some angucyclines (*i.e.* aggreticin) have been described to inhibit the growth of cell lines that are resistant to different cytostatic drugs in clinical use, *e.g.* doxorubicin[35].

Relatively small structural differences, *e.g.* between α-β-, and γ-rubromycin, result in different in biological activities including *in vitro* activity against *Staphylococcus aureus* (α-rubromycin) or the selective inhibition of HIV-1 reverse transcriptase (β- and γ-rubromycin)[3~5]. Collinone is structurally different from the rubromycines (Figs. 1 and 4) and consequently has different biological activities. Its antibacterial activity against multiresistant enterococci (vancomycin- and β-lactam-resistant enterococci, Table 2) is interesting but associated with cytotoxicity; neither antifungal or significant antiviral activities were detected so far. It has been suggested that the antitumor activity of the

angucyclines is due to the chromophore moiety, which in most cases, including collinone, is a hydroxyanthraquinone or a hydroxynaphtoquinone. Such activity remains to be evaluated. As expected for antitumor compounds, the activity of the angucyclines is accompanied by cytotoxicity[36,37].

Considering that vancomycin is the current last line of defense for the treatment of methicillin-resistant (MRS) *Staphylococcus aureus* infections, and vancomycin resistance in enterococci has risen dramatically from 0.4% incidence in hospital intensive care units in 1989 to 13% by 1993[38], the activity of collinone against these pathogens makes it an interesting starting point for a potentially useful drug candidate. Our current efforts to genetically modify the pathway leading to collinone by introducing and/or eliminating tailoring enzymes that have been isolated from heterologous polyketide gene clusters, will introduce directed modifications into the structure. Together with a better understanding of the mode of action of collinone our genetic approach might lead to a more potent secondary metabolite produced *in vivo*. In this respect, it will be interesting to analyze the chemical and biological properties of the metabolites produced by the other recombinants, MRB3B, MRB3C, and MRB3E, which express different combinations of genes of the putative *S. collinus* rubromycin PKS. Furthermore, possibilities for chemical derivatisation of the molecules will be considered.

Acknowledgements

We thank CHAITAN KHOSLA for helpful discussions, HEIDI ERNST for excellent technical assistance and Eli Lilly & Co. for providing the cosmid pKC505. We are grateful to Drs. ANGEHRN and TSCHAN, Hoffmann-La Roche, Basle, Switzerland, for performing antibacterial and cytotoxicity assays on collinone and Dr. MASAHIRO AOKI, Nippon Roche Research Center, for evaluation of the antifungal activities. This work was supported by the Swiss National Science Foundation grants to W. M. (5002-57799) and to J. E. B. (5002-046082).

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