Hybrid compounds derived from the combination of anthracycline and actinorhodin biosynthetic pathways

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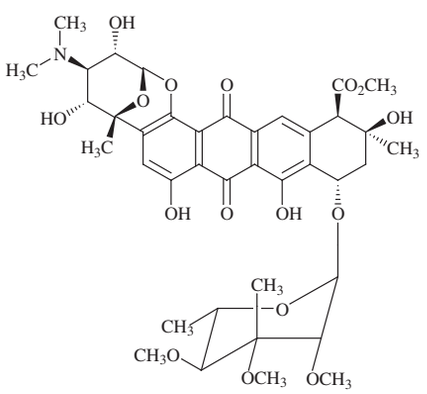
A new approach in the field of polyketide biosynthetic engineering, the combination of the biosynthetic routes of two different sources, is introduced. *Streptomyces nogalater* genes expressed in *S. lividans* TK24 yield the hybrid strain TK24/pSY15. Structural analysis of the products isolated from cultivation of the hybrid strain revealed the ability of the hybrid to produce novel compounds. Instead of accumulating characteristic products (*e.g.* actinorhodin) of the host *S. lividans* TK24, or intermediate compounds expected to be generated by the plasmid pSY15 (*e.g.* nogalamycin precursor), the hybrid strain produces novel compounds reflecting the enzymatic activity of both the host and the expressed plasmid. This implies that genes from two different types of aromatic polyketide biosynthesis are working together. The method described in this work complements earlier targeted biosyntheses.

Introduction

Polyketides are a large group of complex and pharmaceutically important molecules. The aromatic polyketides, and particularly the anthracyclines, play a significant role in combinatorial cancer chemotherapy.[1] Nonetheless the pursuit for ever more effective and selective drugs with minimal side effects continues. The traditional search for novel compounds includes the screening of products from bacteria isolated from soil samples, and this approach has facilitated the discovery of several clinically important anthracyclines including aclacinomycin A and daunorubicin.[2] The mutation of existing strains can lead to compounds with improved curative properties, of which doxorubicin is an example.[3] Furthermore, numerous semisynthetic products have been produced amongst which the most interesting are idarubicin[4] and menogaril.[5] The laborious and random nature of these approaches however demanded the development of a new, more efficient methodology. Knowledge of the biochemical pathways and genetic control of these provide an alternative way to influence the level of production and nature of the products. Early experiments concentrated on replacing or adding a gene for one enzymatic activity at a time and successful examples include the modification of the stereochemistry in actinorhodin to produce granaticin,[6] hydroxylation of aclacinomycins to produce rhodomycins,[7] and the change of the starter unit and stereochemistry in the *Streptomyces nogalater*/*S. galilaeus* hybrids.[8] A further step forward was taken with combinatorial gene techniques which created functional biosynthetic routes by combining genetic material from different sources to guide, and ultimately control, the biosynthetic pathway within the host.[9] In this work we report the combination of an anthracycline biosynthetic route with an actinorhodin biosynthetic route to produce novel compounds.

The production strain TK24/pSY15 consists of the host *S. lividans* TK24 carrying the plasmid pSY15 containing genetic information from *S. nogalater*. The role of pSY15 is to produce the first three rings of nogalamycin anthraquinone aglycone, corresponding to **8**. The structures of nogalamycin **1** and actinorhodin **2** are depicted in Fig. 1. The structures differ substantially from each other; actinorhodin is a dimeric aglycone with the base monomer constructed from eight acetate units, whereas the nogalamycin polyketide is built from ten acetate units and includes two sugar moieties. The cultivation of TK24/pSY15 leads to an accumulation of products which reflect, structurally, the activities of the genes cloned in the plasmid pSY15 and also those of the host, *S. lividans* TK24. *S. lividans* TK24 is generally used as a primary host in cloning experiments since it does not exhibit a restriction barrier for foreign DNA. It was the unexpected production of pigmentation after the introduction of pSY15 into *S. lividans* TK24 that initially aroused our interest in investigating the accumulated metabolites.



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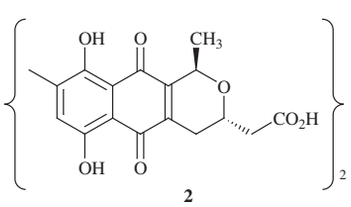

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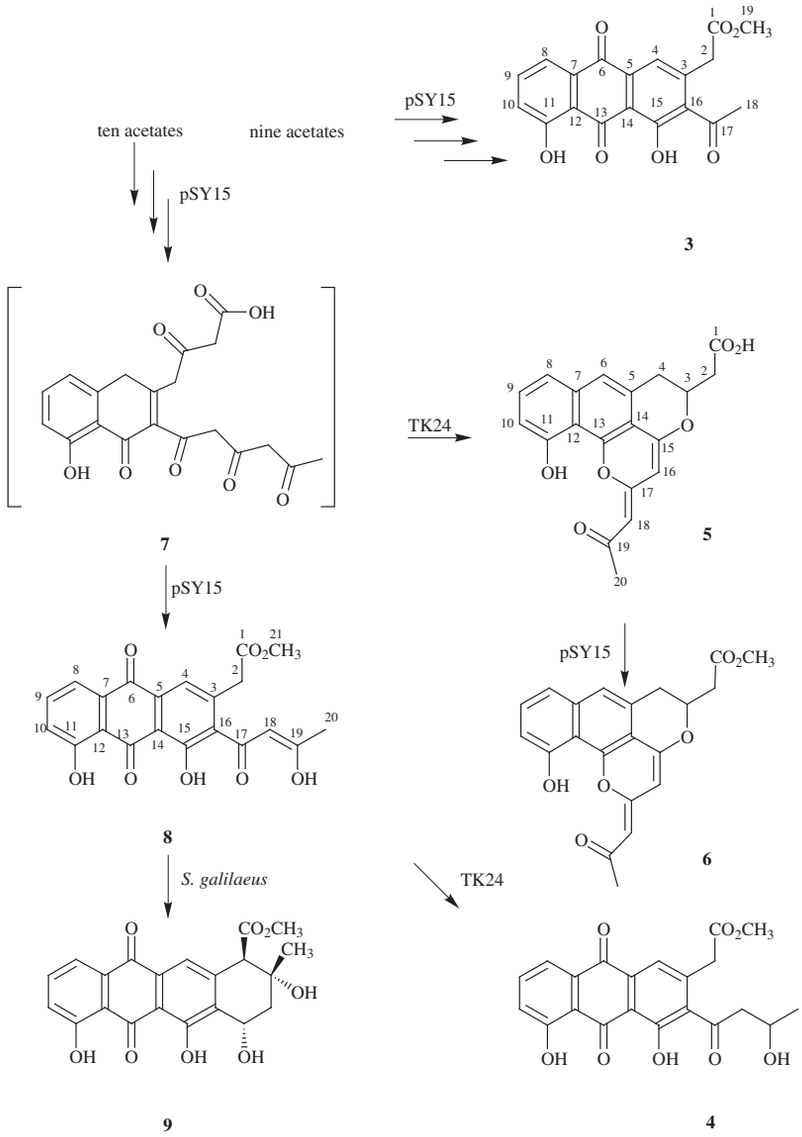
Fig. 1 Structures of nogalamycin **1** and actinorhodin **2**.

Results and discussion

HPLC analysis of the liquid culture extract of TK24/pSY15 revealed the presence of one major (50%) and three minor



Scheme 1 The proposed biosynthetic pathways for the hybrid compounds obtained from the strain TK24/pSY15.

products (10% each). They were classified into two pairs according to their similar UV spectra. Compounds **3** and **4** had characteristic anthraquinone chromophore absorptions at 280 and 430 nm, whereas **5** and **6** showed multiple absorptions from 260 to 500 nm. Bathochromic shifts of 70 nm were observed upon the addition of base to either **3** or **4**, indicating the presence of two phenolic hydroxys; similar addition of base to either **5** or **6** resulted in bathochromic shifts of 30 nm, indicating the presence of only one phenolic hydroxy in these compounds. For complete structural elucidation by NMR, large-scale fermentations permitted the extraction of suitable quantities of the metabolites which were subsequently purified by chromatography. Proton and carbon assignments were based on conventional DEPT, HSQC, HMBC, and NOE difference measurements. The connectivities providing the structural elucidation, in particular, relied heavily on the HMBC experiments. The stereochemistry of the sidechain in **5** and **6** was confirmed by a strong NOE (15%) between protons located on C-16 and C-18. Compound **4** has been reported previously,[10] whilst compounds **3**, **5**, **6** and **8** are novel compounds. Confirmation of the molecular weights of the new compounds was provided by HRMS.

The proposed biosynthetic pathway of the products from the strain TK24/pSY15 is depicted in Scheme 1. According to the structures and proposed biosynthetic pathways for actinorhodin[11] and anthracyclines,[10] intermediate **7** is generated by the action of the nogalamycin genes cloned in pSY15. Polyketide chain length, *i.e.* the number of building blocks incorporated, is determined by the *S. nogalater* multifunctional enzyme complex, polyketide synthetase (PKS).[12] It is somewhat flexible, as evidenced by the structure of **3** exhibiting one acetate unit less than the usual polyketide backbone of nogalamycin. A similar compound lacking only the methyl ester group has been reported earlier.[5] Compound **8** was not directly detected in TK24/pSY15, though its presence is implied by the shunt product **4** resulting from the reduction of **8**. However, **8** was isolated as the major product from a hybrid strain *S. argillaceus* carrying the same plasmid, pSY15, and thereby implicating the host strain TK24 as being responsible for the reduction. The proton spectrum of **8** indicated 89% enolisation of the diketo sidechain in $CDCl_3$. **8** reacted readily under aldol addition conditions (K_2CO_3, MeOH, RT) yielding a red compound, which most likely results from cyclisation occurring between C-2 and C-19 followed by water elimination and aromatisation. To convert **8** to auramycinone **9**, two additional enzymes that are absent in pSY15, auravic acid methyl ester cyclase and auraviketone reductase, are required. These are available in *S. galilaeus* and introduction of pSY15 into *S. galilaeus* yields the hybrid strain HO28/pSY15 which produces auramycinone **9** and two of its glycosides.[13]

The host strain TK24 occasionally produces **2** on ISP4 agar plates, but the production is suppressed in the liquid production

medium E1. However, this does not completely exclude its genetic abilities for actinorhodin biosynthesis and the high yield of hybrid product **5** readily shows that the enzymatic mechanism involved in actinorhodin biosynthesis is functional. The structure of **5** is clearly a hybrid since it exhibits the tetrahydropyran ring that is also present in actinorhodin.[5] The methylation of **5** that results in **6** is derived from the nogalamycin biosynthesis, and this is a further expression of the combination of the biosynthetic pathways. **6** was also isolated from the fermentation broth under neutral conditions using toluene extraction, thus excluding the possible methylation of **5** during workup as its origin. However, the question naturally arises whether the PKSs or the individual enzymes of TK24 and pSY15 act individually or collectively. Due to the reactive nature of the biosynthetic intermediates, the enzymatic interplay between the host and the plasmid enzymes is likely to be a highly intimate one. Thus, not only does this study demonstrate that the concomitant action of biosynthetic genes can readily result in hybrid structures, but that efficient collaboration is clearly established since **5** and **6** constitute 60% of the polyketide metabolites from TK24/pSY15.

Experimental

Bacterial strains and plasmid

Strain TK24/pSY15 was maintained on an ISP4 agar plate supplemented with thiostrepton (5 μg ml^{-1}). pSY15 is a pIJ486 based plasmid containing the genes for nogalamycin precursor: minimal PKS (*SnoA*), polyketidereductase (*Sno1-3*), aromatase (*SnoE*), oxygenase (*SnoB*), and methyl transferase (*SnoC*). *S. lividans* was originally obtained from Professor Sir David Hopwood (England).

NMR, UV and mass spectrometry

NMR spectra were acquired on a JEOL JNM-GX 400 spectrometer equipped with either a 5 mm normal configuration CH probe or a 5 mm inverse HX probe operating at 399.78 MHz for ^1H and 100.54 MHz for ^{13}C. The spectra were run at ambient temperature in the solvents indicated and both ^1H and ^{13}C were referenced internally to TMS, assigned as 0 ppm. 1D proton spectra were acquired with single-pulse excitation, 45° flip angle, and spectral widths of 7 kHz (digital resolution 0.11 Hz pt^{-1}). NOE difference measurements were acquired on samples flushed with dry, nitrogen gas and using saturation times of 6–8 s. Spectral widths were the same as for the normal proton spectra, but with the resolution reduced to 0.9 Hz pt^{-1}; 1 Hz of exponential weighting was usually applied prior to Fourier transformation. 1D carbon spectra were acquired with single-pulse excitation, 45° flip angle, spectral widths of 20 kHz (digital resolution 0.5 Hz pt^{-1}), and with 1 Hz of exponential weighting applied prior to Fourier transformation. DEPT spectra (90° and 135°) were acquired under similar conditions. HSQC and HMBC experiments were acquired with spectral widths appropriately optimised from the 1D spectra. Both sequences incorporated a pre-emptive BIRD sequence, the delay for which was optimised by minimisation of the incoming FID (*ca.* 0.4 s). Both spectra utilised a $^1J_{HC}$ coupling of 145 Hz, whilst the HMBC correlations were optimised for a long-range $^nJ_{HC}$ coupling of 5 Hz.

Electron impact high resolution mass spectrometry (EIHRMS) spectra were taken on a VG Analytical Organic mass spectrometry 7070 E spectrometer. UV spectra were recorded on a Pharmacia biochrom 4060 spectrophotometer in methanol and in methanol containing 0.1% NaOH.

Polyketide production and purification

The 10 litre scale fermentation was conducted for 5 days in E1 medium consisting of glucose, starch, Pharmamedia (Traders protein), yeast extract, calcium carbonate, sodium chloride, $MgSO_4$ and K_2HPO_4 in tap water supplemented with 5 μg ml^{-1} thiostrepton (28 °C, aeration 10 l min^{-1}, and stirring 400 rpm). The pH of the culture was adjusted to 3.0 with 1 M hydrochloric acid prior to harvest. The culture broth was centrifuged to separate the mycelia which were extracted with methanol. The methanol extracts and supernatant were combined and extracted with dichloromethane (2 × 750 ml). The combined dichloromethane extracts were dried with Na_2SO_4 and then vacuum-concentrated to yield a viscous residue, which was dissolved in ethyl acetate and then treated with hexane to effect precipitation. The precipitant was loaded onto a silica column and eluted with dichloromethane with increasing proportions of methanol. Polyketide metabolites were detected by TLC (Kieselgel 60 F_{254} glass plates) and HPLC on a Merck Hitachi instrument (L-6200A/L-4250) using a LiCHroCART RP-18 column (Lichrosphere, 5 μm, 4.6 × 250 mm) and eluent mixture consisting of MeCN–H_2O–HCO_2H (49 : 50 : 1).

Compound 3. Combined fractions were rechromatographed (SiO_2, 10 × 5 cm, eluent EtOAc–hexane–AcOH, 49 : 50 : 1). Pure fractions were combined, and after solvent removal, were taken up in chloroform whereby precipitation was effected by the addition of methanol affording 20 mg of **3**, 95% pure by HPLC. EIHRMS, M^+ calcd. for $C_{19}H_{14}O_7$ 354.0730, found 354.0735. ^1H NMR ($CDCl_3$) δ (area, multiplicity, $J_{H,H}$ in Hz) 12.54 (1H, s), 11.94 (1H, s), 7.85 (1H, dd, 7.4, 1.2), 7.73 (1H, dd, 8.5, 7.4), 7.71 (1H, s), 7.34 (1H, dd, 8.5, 1.2), 3.88 (2H, s), 3.72 (3H, s), 2.96 (3H, s). For ^{13}C NMR data see Table 1.

Compound 4. Pooled fractions were flash chromatographed (SiO_2, 10 × 5 cm, eluent hexane–AcOH–$CHCl_3$, 1 : 1 : 98). A pure fraction was collected, the solvent removed, and precipitation effected from methanol–water affording 25 mg of **4**, 96% pure by HPLC. EIHRMS, M^+ calcd. for $C_{21}H_{18}O_8$ 398.1001, found 398.1002. ^1H NMR ($CDCl_3$) δ (area, multiplicity, $J_{H,H}$ in Hz) 12.58 (1H, s), 11.91 (1H, s), 7.85 (1H, dd, 7.4, 1.2), 7.73 (1H, dd, 8.5, 7.4), 7.72 (1H, s), 7.34 (1H, dd, 8.5, 1.2), 4.42 (1H, dqtd, 9.1, 6.5, 2.7), 3.88 (2H, s), 3.73 (3H, s), 3.27 (1H, dd, 17.9, 2.7), 2.97 (1H, dd, 17.9, 9.1), 1.29 (3H, d, 6.5). For ^{13}C NMR data see Table 1.

Compound 5. Combined fractions were concentrated and applied to a sephadex LH-20 column (40 × 7 cm, eluent $CHCl_3$–AcOH–MeOH, 4 : 1 : 95). Pure fractions were combined, the solvent removed and precipitation effected from ethyl acetate–hexane affording 300 mg of **5**, 98% pure by HPLC. EIHRMS, M^+ calcd. for $C_{20}H_{16}O_6$ 352.0946, found 352.0947. ^1H NMR (d_6-DMSO) δ (area, multiplicity, $J_{H,H}$ in Hz) 11.16 (1H, s), 7.38 (1H, dd, 7.8, 7.9), 7.32 (1H, s), 7.18 (1H, dd, 7.8, 1.0), 6.82 (1H, dd, 8.4, 1.0), 5.90 (1H, s), 5.44 (1H, s), 4.69 (1H, m), 3.18 (1H, dd, 16.4, 3.1), 3.00 (1H, dd, 16.4, 10.6), 2.91 (1H, dd, 16.1, 4.9), 2.74 (1H, dd, 16.1, 7.8), 2.03 (3H, s). For ^{13}C NMR data see Table 1.

Compound 6. Pooled fractions were flash chromatographed (SiO_2, 10 × 5 cm, eluent hexane–$CHCl_3$–AcOH, 40 : 49 : 1). A pure fraction was collected, the solvent removed, and precipitation effected from methanol–water affording 25 mg of **7**, 96% pure by HPLC. EIHRMS, M^+ calcd. for $C_{21}H_{18}O_6$ 66.1105 found 366.1104. ^1H NMR ($CDCl_3$) δ (area, multiplicity, $J_{H,H}$ in Hz) 11.43 (1H, s), 7.45 (1H, dd, 7.8, 7.3), 7.28 (1H, s), 7.19 (1H, dd, 7.3, 1.0), 7.03 (1H, dd, 7.8, 1.0), 5.75 (1H, s), 5.38 (1H, s), 4.73 (1H, m), 3.77 (3H, s), 3.20 (1H, dd, 16.0, 3.0), 3.03 (1H, dd, 16.0, 10.0), 2.91 (1H, dd, 15.8, 7.2), 2.75 (1H, dd, 15.8, 5.8), 2.15 (3H, s). For ^{13}C NMR data see Table 1.

Compound 8. *S. argillaceus*/pSY15 was cultivated in a fermentator for 5 days under the same liquid broth conditions as TK24/pSY15. The broth was centrifuged and the cells were extracted with acetone (2 × 500 ml) followed by precipitation of

Table 1 ^{13}C (100 MHz) NMR data (chemical shift, multiplicity) from identified compounds. The carbon numbering is depicted in Scheme 1. The assignments of shifts marked A or B can be interchanged, introspectively

Assignment	**3**	**4**	**5**	**6**	**8**
1	169.7 (s)	170.5 (s)	170.8 (s)	170.0 (s)	170.1 (s)
2	38.3 (t)	38.8 (t)	39.2 (t)	39.6 (t)	39.3 (t)
3	141.1 (s)	141.9 (s)	74.2 (d)	74.0 (d)	142.6 (s)
4	121.9 (d)	120.4 (d)	31.2 (t)	32.2 (t)	122.2 (d)
5	133.0 (s)	133.2 (s)	125.5 (s)	124.7 (s)	133.1 (s)
6	180.4 (s)	180.8 (s)	121.7 (d)	122.1 (d)	180.8 (s)
7	132.7 (s)	133.7 (s)	136.5 (s)	137.1 (s)	133.5 (s)
8	119.8 (d)	120.4 (d)	118.1 (d)	118.2 (d)	120.2 (d)
9	137.2 (d)	137.7 (d)	130.1 (d)	130.9 (d)	137.6 (d)
10	124.6 (d)	125.1 (d)	112.5 (d)	112.6 (d)	125.0 (d)
11	162.1 (s)	162.7 (s)	154.3 (s)	155.5 (s)	162.5 (s)
12	115.1 (s)	115.6 (s)	111.5 (s)	113.7 (s)	115.5 (s)
13	192.1 (s)	192.6 (s)	149.9 (s)	150.0 (s)	192.4 (s)
14	114.8 (s)	115.4 (s)	106.8 (s)	106.9 (s)	115.0 (s)
15	159.5 (s)	160.1 (s)	157.4 (s)	157.6 (s)	160.0 (s)
16	135.5 (s)	135.2 (s)	96.8^{A} (d)	97.6^{B} (d)	132.1 (s)
17	202.2 (s)	205.7 (s)	161.7 (s)	162.2 (s)	184.4 (s)
18	31.3 (q)	52.6 (t)	96.6^{A} (d)	97.1^{B} (d)	103.8 (d)
19	51.9 (q)	64.3 (d)	192.8 (s)	194.3 (s)	191.3 (s)
20	—	22.8 (q)	30.0 (q)	30.4 (q)	24.9 (q)
21	—	52.6 (q)	—	52.2 (q)	52.3 (q)

the products by the addition of water. The yellow precipitant was chromatographed (SiO_2, 10×10 cm, eluent hexane–AcOH–$CHCl_3$, 50 : 1 : 49). Precipitation from appropriate fractions was effected by the addition of methanol affording 200 mg of **8**, 98% pure by HPLC. EIMS, *m*/*z* (relative intensity) 396 (30), 353 (40), 323 (100), 307 (55), 295 (35). ^{1}H NMR ($CDCl_3$) δ (area, multiplicity, $J_{H,H}$ in Hz) 12.71 (1H, s), 11.97 (1H, s), 7.85 (1H, dd, 8.6, 1.1), 7.77 (1H, s), 7.72 (1H, t, 8.6), 7.32 (1H, dd, 8.5, 1.1), 5.94 (1H, s), 3.86 (2H, s), 3.71 (3H, s), 2.20 (3H, s). For ^{13}C NMR data see Table 1.

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