ISOLATION AND STRUCTURE OF NOVEL AUTOREGULATORS FROM *STREPTOMYCES GRISEUS*

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Two novel autoregulators affecting the cyto-differentiation of anthracycline-producing *Streptomyces griseus* have been isolated from cultures of aerial mycelium-forming progenitor strains and blocked mutants. By spectral (IR, CD, MS, ^{1}H and ^{13}C NMR) methods, the autoregulators are shown to be the diastereomers of 4,5-dihydroxydecanoic acid-4-lactone.

Morphological differentiation and production of antibiotics in streptomycetes are known to be regulated by common cellular signals as, *e. g.*, the size of the metabolite pools, regulatory nucleotides, *etc.* and also by specific signals acting as triggers which are effective with a limited group of microorganisms only[1]. Known examples for specific signals are provided by the A-factor (**1**) which induces the formation of aerial mycelium and streptomycin production in certain strains of *S. griseus*[2], the C-factor and the antibiotic pamamycin provoking cyto-differentiation in the asporogenous strains of *S. griseus*[3] and *S. alboniger*[4], respectively*. Some less extensively explored phenomena[6,7] suggest that regulation by specific cellular signals (or autoregulators) constitute a fundamental feature of the cyto-differentiation, hence studies on related phenomena might yield useful informations on the control mechanisms of microbial development[8].

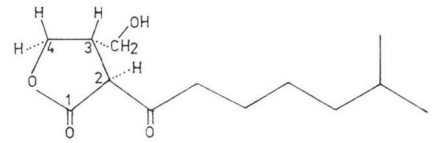
Earlier we have shown[9,10] that, in cultures of the blocked mutant of *S. griseus* JA 5142/86 (Amy$^-$ Lkm$^-$), culture liquids and surface mycelia of the progenitor strain JA 5142 (Amy$^+$ Lkm$^+$) induced the formation of aerial mycelium and the production of leukaemomycin, a daunomycin-type antibiotic[11]. Similar effects on the strain JA 5142/86 have exerted the culture samples of another leukaemomycin-producing *S. griseus* JA 3933 (Amy$^+$Lkm$^+$), the blocked mutant JA 5142/39 (Amy$^-$Lkm$^-$)[11,13], as well as the culture samples of the streptomycin-producing strain *S. griseus* HP (Amy$^+$Str$^+$) and its bald mutant, LM1 (Amy$^-$Str$^-$)[12]. These finding suggested the presence of specific signal substances which, owing to their effects on the biosynthesis of leukaemomycin, were termed 'L-factors' (**2** and **3**, see Fig. 1).

The indicator strain, JA 5142/86, was a prototrophic mutant obtained from the parent strain, *S. griseus* JA 5142, by the treatment of the latter with NTG[9]. Without the addition of either **2** or **3** to its surface or submerged cultures, the indicator strain proved incapable of producing aerial mycelium and/

* One of our referees has drawn our attention to the paper by YANAGIMOTO *et al.*[5] describing the isolation of a C_{12} γ- or δ-lactone inducing staphylomycin production in *Streptomyces virginiae*.

or leukaemomycin within 48 hours, the standard period of cultivation. Only after prolonged cultivation on agar surfaces (160~180 hours) could a weak formation of aerial hyphae and anthracyclines be detected. Samples of the 180-hour mycelia taken from the surface cultures of the indicator strain were shown to contain only small amounts of **2** which indicated that strain JA 5142/86 possesses a defect in the regulation of autoregulator biosynthesis. Details of the cultivation and the effects of the autoregulators thereupon have been published elsewhere[9]. The present paper deals with the isolation and structure elucidation of these novel hormonal regulation factors.

Fig. 1. Structure of A-factor (**1**)[16,17], proposed structure and suggested relative stereochemistry of autoregulators of anthracycline synthesis isolated from *S. griseus* JA 5142 (or JA 3933) (**2**) and *S. griseus* LM1 (**3**).



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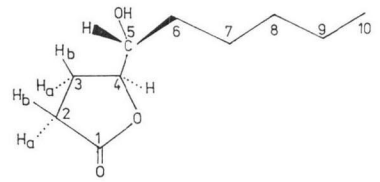

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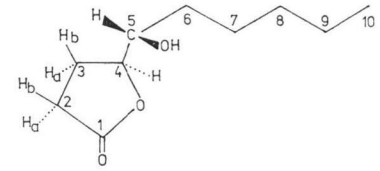

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Materials and Methods

Organisms and Conditions of Fermentation

S. griseus JA 5142, JA 3933, JA 5142/86[9,10,13] and *S. griseus* LM1[12] were cultivated according to ERITT *et al.*[9]. Inocula were prepared by the use of 3% D-glucose, 1% soya meal, 0.3% NaCl and 0.3% $CaCO_3$ (pH 7.0) as the medium (48 hours, shake flasks, 500 ml containing 80 ml of medium, 25°C). The same medium was used for cultivations under aerobic conditions in 400 liters fermentors for 96 hours[13].

Bioassays

Oatmeal agar consisting of 2% oatmeal, 1.5% agar in tap water (pH 7.8) was streaked with a stock of mycelium conserve containing the indicator strain JA 5142/86[9]. Appropriate samples of the autoregulator adsorbed on paper disks (5 mm diameter) or chromatograms were placed on the agar surface at zero time. After 48~72 hours (25°C), zones of aerial mycelium developed on the agar surface with concomitant production of leukaemomycin (reddish color) around the sample. The diameter of zones was used as the measure of the concentration of L-factor[9].

Chromatography

For preparative chromatography, a 8×150 cm column was filled with Sephadex LH-20 in methanol. Cellulose chromatography was performed with a column (1.5×100 cm) filled with cellulose powder (Schleicher & Schuell, No. 123, Dassel, FRG). A similar column was used for chromatography on silica gel (60, 0.063~0.2 mm, Merck, Darmstadt, FRG) using benzene - ether (1:1, v/v) as the solvent. Before use, the silica gel was washed with 0.1 M phosphate buffer, pH 7.0, and dried at 110°C. Preparative TLC was carried out on Silufol sheets precoated with silica gel (Kavalier, CSSR; see Fig. 2) using the above solvent system.

Instruments

^1H and ^{13}C NMR spectra were recorded with a Bruker WP 200/SY and a disk-augmented Varian XL-100/15 Fourier transform spectrometer operating at 200.13 and 25.16 MHz, respectively. Mass spectrometric analysis was carried out on a JEOL JMS- D 100 instrument at 75 eV, direct inlet, 55°C. The IR spectra were obtained with a Nicolet 7000 Fourier transform instrument in dilute $CDCl_3$ solutions. A dichrographe model III (JOBIN-YVON-ROUSSEL-JOUAN) was used to record CD spectra at room temperature (95% ethanol, spectral grade, as the solvent). GC was performed with a Varian

Aerograph model 705 instrument equipped with 0.6×1500 cm glass column filled with 2% OV 101 on Chromosorb W (a.w., DMCS, 80~100 mesh) and a flame ionization detector. The column was operated isothermically at 120°C. Argon was used as the carrier gas (90 ml/minute).

Results and Discussion

Isolation of Autoregulators

Displayed in Fig. 2 are the purification steps for autoregulators **2** and **3** isolated, respectively, from

Table 1. Physico-chemical properties, IR, UV, CD and MS data of the autoregulators **2** and **3**.

Properties	**2**	**3**
Appearance	Slightly yellow oils at room temperature	
Molecular formula	$C_{10}H_{18}O_3$	$C_{10}H_{18}O_3$
Molecular weight (MS)	186.1253 (M^+)	186.1276 (M^+)
Characteristic MS fragments	*m/z* 158.1308 (M^+-CO), $C_9H_{18}O_2$ *m/z* 115.0404 (M^+−side chain except for C5), $C_5H_7O_3$ *m/z* 101.0967 (side chain), $C_6H_{13}O$ *m/z* 86.0370 (butyrolactone moiety), $C_4H_6O_2$	
IR ($\lambda_{max}^{CDCl_3}$) cm^{-1}	895, 905, 990, 1030, 1187, 1420, 1774, 2930, 3610	995, 1030, 1120, 1180, 1422, 1782, 2960, 3580
UV (λ_{max}^{EtOH}) nm (ε)	212 (1300)	212 (1300)
CD (λ_{max}^{EtOH}) nm ($\Delta\varepsilon$)	261 (+0.03) 243 (0) 209 (−0.17)	260 (+0.02) 240 (0) 209 (−0.33)
Rf values (Silufol)		
Benzene - ether (1:1, v/v)	0.2	0.25
Benzene - ether (3:1, v/v)	0.1	0.12
Benzene - acetone (5:3, v/v) (paper Schleicher & Schuell, 2043b Mgl)	0.8	0.85
H_2O	0.65	0.70

Fig. 2. Isolation procedure for **2** and **3**.

Culture liquid (pH 5~7), 300 liters
↓ extraction with butyl acetate (0.2 vol.)
neutralization of extract with $NaHCO_3$
evaporation of solvent
Residue (200 g)
↓ gel chromatography (Sephadex LH-20, methanol)
Active fractions (40 g)
↓ chromatography on cellulose powder (H_2O)
extraction of active fractions with ether
evaporation of solvent
Crude autoregulators (2 g)
↓ chromatography on neutral silica gel (benzene - ether, 1:1, v/v)
2 or **3** (300 mg, 80% purity)
↓ preparative TLC, Silufol sheets
2 or **3** (110 mg, 90% purity)
↓ preparative GC (Chromosorb W/OV 101, 120°C)
pure **2** or **3** (100% purity, 6 mg, yield will depend on the effectivity of collector system)

Table 2. ^{13}C NMR data.*

Carbon	**2**	**3**
1	177.71	177.53
2	28.74	28.68
3	21.14	24.09
4	83.13	83.02
5	71.54	73.05
6	32.49	32.29
7	25.73	25.69
8	32.13	32.15
9	22.96	23.03
10	14.25	14.31

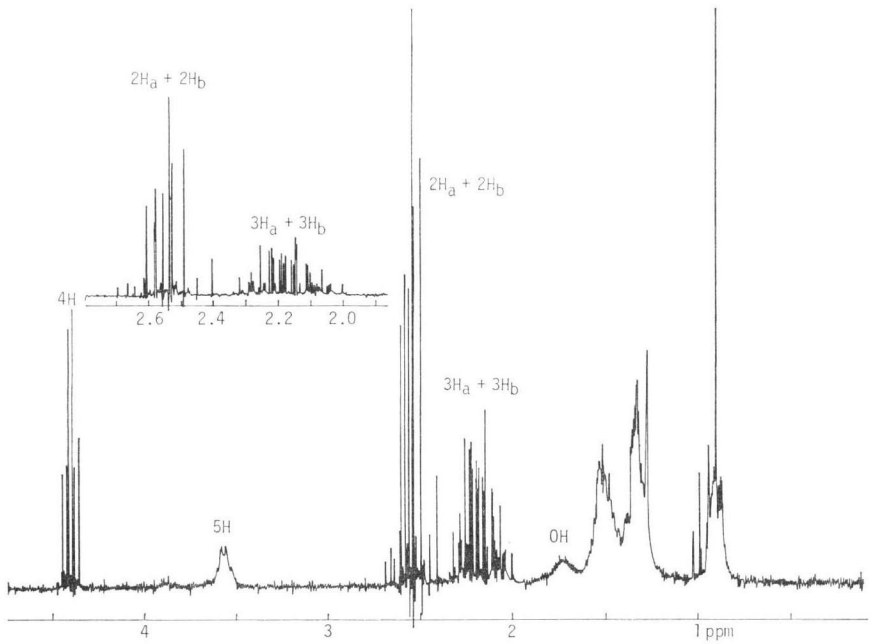
* In C_6D_6 solutions. Chemical shifts (in ppm) are relative to internal TMS. Assignments are based on variable frequency off-resonance and single frequency selective decoupling experiments.

S. griseus JA 5142 (or JA 3933) and from *S. griseus* LM1. The residue of the butyl acetate extract of a 96-hour culture liquid was submitted to gel chromatography in order to remove anthracyclines and other by-products. Active fractions detected by bioassay were pooled, evaporated to dryness *in vacuo* (40°C), dissolved in ether, mixed with 20 g of dry cellulose powder and the ether was removed *in vacuo*. Subsequently, the mixture was filled into a column containing a bed of 10 cm of dry cellulose powder. Elution with H_2O, reextraction with ether and evaporation of solvent yielded the crude autoregulators. According to bioassay performed on the chromatograms, these products contained at least two further active fractions in minor amounts. Purification of the autoregulators to approximately 90% purity was achieved either by column chromatography on buffered silica gel or by preparative TLC on Silufol sheets. Here, again, the autoregulators were spotted through bioassay, their slight fluorescence in UV light and reddish-violet color which developed after spraying with 1% vanillin in conc. H_2SO_4. Final purification was carried out by preparative GC yielding the single substances with retention times of approximately 16 minutes (**2**) and 14 minutes (**3**), respectively.

Structure and Stereochemistry

The isolated and purified products were characterized through their physico-chemical and spectral properties. The relevant data are summarized in Tables 1 and 2. Despite differences in their Rf values and gas-chromatographic retention times, substances **2** and **3** have very similar spectral data suggesting isomeric relationship. They have similar absorptions in their UV spectra and nearly identical CD curves with negative COTTON effect at 209 nm. They possess the same elemental composition $C_{10}H_{18}O_3$ (M^+: *m/z* 186.1253; 186.1276; Calcd. 186.1256). Their IR spectra show the presence of a saturated five-membered lactone ring (**2**: 1774 and 1420 cm^{-1}, **3**: 1782 and 1422 cm^{-1}) and a hydroxyl group (**2**: 3610 cm^{-1}: **3**: 3580 cm^{-1}). ^1H NMR spectra (see Fig. 3) attest to the presence of a six-spin system with re-

Fig. 3. 200 MHz ^1H NMR spectrum of **3** (1.5 mg in 0.4 ml $CDCl_3$, 325K).



sonances between 2 and 4.5 ppm, an exchangeable (OH) proton and an unbranched C_5H_{11} aliphatic side chain. ^1H-^1H double resonance experiments disclosed that the protons of the six-spin system of both molecules are arranged in a ring-C(1)O–C(2)H_aH_b–C(3)H_aH_b–C(4)H(O-ring)–C(5)H(OH)–C(6)H_2- sequence which, on the basis of chemical shifts and coupling constant considerations ($\delta_H^{CDCl_3}$ ppm, **2**: $2H_a$ 2.60, $J_{2a2b}=-17.8$, $J_{2a3a}=9.8$, $J_{2a3b}=6.0$; $2H_b$ 2.52, $J_{2b3a}=8.4$, $J_{2b3b}=9.6$; $3H_a$ 2.27, $J_{3a3b}=-12.5$, $J_{3a4}=7.3$; $3H_b$ 2.15, $J_{3b4}=6.2$; 4H 4.45, $J_{45}=3.4$; 5H 3.91, J_{56} 6.5. **3**: $2H_a$ 2.58, $J_{2a2b}=-17.8$, $J_{2a3a}=9.9$, $J_{2a3b}=5.8$; $2H_b$ 2.52, $J_{2b3a}=8.3$, $J_{2b3b}=9.6$; $3H_a$ 2.25, $J_{3a3b}=-12.4$, $J_{3a4}=7.3$; $3H_b$ 2.15, $J_{3b4}=6.6$; 4H 4.38, $J_{45}=4.4$; 5H 3.55, $J_{56}=6.5$) are readily accomodated in structures **2** and **3** featuring butyrolactone moiety. (Of relevance in this respect is the −17.8 Hz measured for geminal J_{ab}, a value which located the carbonyl group in the above partial structure). Carbon-13 chemical shifts (see Table 2) provided corroboration for the proposed formulation.

Informations regarding the nature and site of isomerism could be gleaned from ^1H and ^{13}C spectral comparison. ^1H NMR data (*vide supra*) show that the stereochemical difference between **2** and **3** leaves most proton chemical shifts and all but vicinal $J_{4H,5H}$ coupling constants unchanged. This suggests that the preferred conformation of the butyrolactone ring and the relative configuration at C-4 are unaffected by the isomerism. On the other hand, the variation in the value of $J_{4H,5H}$ (and also in that of δ 5H) may be accounted for by considering an altered configuration at C-5 and concomitant changes in the preferred side chain conformations. The finding that carbon-13 chemical shift changes are confined to C-3, C-5 and C-6 (see Table 2) also suggests that molecules **2** and **3** are diastereomers with different configurations at C-5. Support for this proposal was obtained from CD results (see Table 1). These show that the COTTON effect at 209 nm attributable to the π–π^* transition of the saturated γ-lactone moiety[14] has the same, negative, sign in both **2** and **3**. Since the sign of this COTTON effect is known to reflect the chirality of the substituted lactone ring (independent of the configuration of the side chain OH group)[14,15], the diastereomeric relationship between **2** and **3** requires that the molecules differ in their configuration at C-5. By considering the preferred conformations of the lactone ring in **2** and **3** and applying the empirical rules for substituted γ-lactones[14,15], one can furthermore conclude that the observed negative sign of the COTTON effect is compatible with an *S* absolute configuration at C-4 (see Fig. 1).

Biological Activity

The minimum amount of either **2** or **3** required to induce the formation of aerial mycelium on surface cultures of the blocked strain *S. griseus* JA 5142/86 and initiate leukaemomycin production therein after 48 hours[9] was found to be 1×10^{-7} g (or 5.4×10^{-10} mole) under the conditions described in Material and Methods. Such low quantities suggest that molecules **2** and **3** exert regulatory effects on the cellular development of the microorganism rather than act as precursors in the biosynthesis of the antibiotic or, in a more general sense, in the differentiation-associated metabolism. Interestingly, the butyrolactone moiety is also a principal structural element of the A-factor (2-(6-methylheptanonyl)-3*S*-hydroxymethyl-4-butanolide, **1**)[16,17] (*vide supra*). Therefore, it was of interest to investigate the effects the latter molecule exerts on the cyto-differentiation of the same, JA 5142/86, strain. These experiments performed under conditions identical with those described above have disclosed that factor A affects the cyto-differentiation much in the same way as do compounds **2** and **3** and, even, the observed biological activity is by a factor of approximately 20 times higher than that found with **2** and **3**.*

* By careful GC analysis, the eventual occurrence of small amounts of the A-factor in the test samples of **2** and **3** could be unambiguously excluded.

It thus seems reasonable to assume that involved in the programmed performance of the differential gene expression there is, in streptomycetes, a receptor system which is specific for butyrolactone derivatives. That the two diastereomeric autoregulators (**2** and **3**) exhibit about the same biological activity may be interpreted by assuming that the receptor possesses moderate selectivity with respect to the side chain stereochemistry. Further studies concerned with the absolute stereochemistry of **2** and **3** and the structure biological activity relationship for various butyrolactone derivatives are now in progress in our laboratories.

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