# Isolation and Structure Elucidation of 7,8-Dideoxy-6-oxo-griseorhodin C Produced by *Actinoplanes ianthinogenes*

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A novel product, isolated from a culture broth of *Actinoplanes ianthinogenes* fermented for producing purpuromycin, was purified and its structure established on the basis of physico-chemical data and chemical reactions. The new product resulted to be structurally related to griseorhodins, a group of hydroquinonic antibiotics obtained from *Streptomyces californicus*.

This compound showed a weak activity against Gram-positive and resulted inactive against Gram-negative bacteria and *Candida albicans*.

During the purification of a crude sample of purpuromycin, obtained from the culture broth of the *Actinoplanes ianthinogenes* ATCC 20884[1,2], a novel compound belonging to the griseorhodin family was isolated and its structure was determined as 7,8-dideoxy-6-oxo-griseorhodin C (**1**, Fig. 1). Griseorhodins are a well known group of antibiotics obtained from *Streptomyces californicus*.[3] Compound **1** represents the first example of griseorhodin-like structure produced by an *Actinoplanes* strain.

In this paper is reported the production, isolation, structure elucidation, physico-chemical properties and biological activities of this compound and of two chemical derivatives (**2** and **3**) synthesized from compound **1**.

## Results

### Fermentation

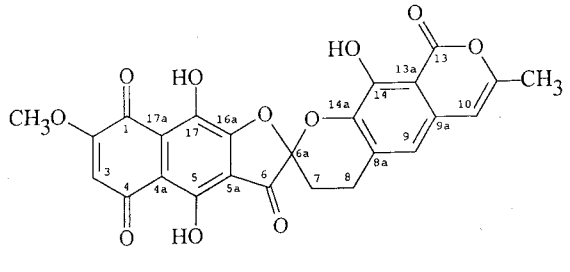
The producing strain *Actinoplanes ianthinogenes* ATCC 20884 was fermented in a 200 liter bioreactor at 32°C for 82 hours in order to produce purpuromycin. The HPLC analysis of the final broth showed the presence of another compound having a retention time higher than that of purpuromycin. After extraction of the mycelium cake and chromatographic purification of the crude material, performed as described under "Experimental", a total of 11.1 g of compound **1**, later identified as 7,8-dideoxy-6-oxo-griseorhodin C, and 270 g of purpuromycin were isolated.

### Physico-chemical Properties of Compound **1**

7,8-Dideoxy-6-oxo griseorhodin C (**1**) is an amorphous red powder insoluble in water and in the most common organic solvents. It is sligtly soluble in DMSO, THF and $CHCl_3$ under acidic conditions but practically insoluble, even in these solvents, in neutral or alkaline conditions. Its molecular weight of 492 Da was determined by mass spectrometry and the molecular formula $C_{25}H_{16}O_{11}$ was determined by elemental analysis. All physico-chemical properties of **1** are reported in Table 1.

The change of colour from red to blue, observed by alkalizing a DMSO or $CHCl_3$ solution of **1** containing traces of acetic acid suggested the presence in the molecule of phenolic moieties. The UV-Visible profile of **1** (Table 1) was similar to that of purpuromycin, and as purpuromycin compound **1** showed expected changes in acidic or neutral solution. A comparison of the light absorption spectrum of **1** with those of compounds containing a 5,8-dihydroxy-1,4-naphthoquinone moiety

Fig. 1. Structure of dideoxy-6-oxo-griseorhodin C (**1**).



(naphthazarin) showed a close similarity of **1** with griseorhodins C (Fig. 2).

## Structure Elucidation of 1

The structure of **1** was elucidated by IR, MS and NMR spectroscopies. The IR spectrum afforded some important pieces of information: i) a band at 1740 $cm^{-1}$ which is in agreement with the presence of a ketone group in a five membered ring,[4] ii) a band at 1683 $cm^{-1}$ due to the carbonyl of the lactone which is hydrogen-bound to the OH at position 14 and iii) the presence of an absorption between 1600~1640 $cm^{-1}$ showing the presence of the naphthazarin moiety and the absence of the band around 1730 $cm^{-1}$ which in the purpuromycin structure is attributed to the carbomethoxy group.

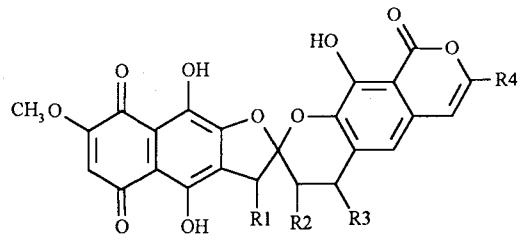
The $^1$H-NMR spectral pattern in DMSO solution of **1** suggested a purpuromycin-like compound,[5] while the presence of a $CH_3$ signal at $\delta = 2.23$ ppm and the absence of the purpuromycin carbomethoxy group signal at $\delta = 3.87$ ppm (Fig. 2) clearly demonstrated that **1** belonged to the griseorhodin family.

Comparison of the $^1$H-NMR spectra of **1** and **4**[6] in DMSO solution (Table 2) showed the lack of CHOH at position 6 in compound **1** and an upfield shift of the 7-H and 8-H protons from $\delta_H$ 4.56 to $\delta_H$ 3.09~3.32 ppm and from $\delta_H$ 4.25 to $\delta_H$ 2.24~2.45 ppm respectively passing from compound **4** to **1**. These shifts agreed with the lack

Table 1. Physico-chemical properties of compound **1**.

| | |
|---|---|
| Appearance | Red amorphous power |
| MP | >280°C |
| Molecular formula | $C_{25}H_{16}O_{11}$ |
| Molecular weight | 492.018 |
| NICI-MS (*m/z*) | 492.1 |
| UV $\nu_{max}$ nm (log $\varepsilon$) | |
| in $CHCl_3$ | 322.0 (3.86), 348.4 (3.84), 550.0 (3.61), 603 (sh), 670 (sh) |
| in $CHCl_3$/TFA | 317.2 (4.10), 351.2 (sh), 365.6 (sh), 508.4 (3.75), 550 (sh) |
| IR $\nu_{max}$ (nujol) $cm^{-1}$ | 3300 (OH), 1740 (ketone), 1683 (lactone ), 1640 (quinone), 1618 (hydrogen bonded quinone) |
| Solubility: | Poorly soluble DMSO, DMF, ACETIC ACID |
| | Insoluble $H_2O$, MeOH, $CHCl_3$ |
| TLC silica gel | Rf 0.40, $CHCl_3$/MeOH/AcOH 95:5:0.1 |
| | Rf 0.67, $CHCl_3$/MeOH/AcOH 90:10:0.1 |

Fig. 2. Structures of griseorhodin C, 8-methoxygriseorhodin C, purpuromycin and *gamma*-rubromycin.




| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| Griseorhodin C | OH | OH | OH | $CH_3$ |
| 8-Methoxy (**4**) | OH | OH | $OCH_3$ | $CH_3$ |
| Purpuromycin | H | H | OH | $CO_2CH_3$ |
| *gamma*-Rubromycin | H | H | H | $CO_2CH_3$ |

Table 2. $^1$H-NMR assignments, ($\delta$, ppm) of **1**, **2**, **3** and **4** (solvent: DMSO-$d_6$).

| Proton | 1[a] | 1[b] | 2[a] | 3 | 4[6] |
|---|---|---|---|---|---|
| 3 | 6.52 | 6.61 | 6.50 | 7.54 | 6.40 |
| 5 (OH) | n.d. | — | — | | 13.25 |
| 6 | — | — | — | — | 5.20 d |
| 6 (OH) | — | — | — | — | 6.39 d |
| 7 | 3.09~3.32 | 3.21~3.40 | 2.92~2.94 | 3.12~3.18 | 4.56 dd |
| 7 (OH) | — | — | — | — | 5.89 d |
| 8 | 2.24~2.45 | 2.35~2.48 | 2.52~2.57 | 2.20~2.40 | 4.25 |
| 9 | 6.91 | 6.90 | — | 7.37 | 7.10 |
| 10 | 6.54 | 6.45 | 6.73 | 6.52 | 6.57 |
| 14 (OH) | n.d. | — | n.d. | — | 10.91 br |
| 17 (OH) | n.d. | — | n.d. | — | 11.94 br |
| $CH_3$– | 2.23 | 2.35 | 2.29 | 2.20 | 2.22 |
| 2-$CH_3O$– | 3.92 | 4.05 | 3.91 | 3.94 | 3.88 |
| $CH_3CO$– | | | | 2.21 (two) | |
| | | | | 2.38 | |
| | | | | 2.45 | |
| | | | | 2.52 | |

[a] Solvent DMSO-$d_6$/TFA.
[b] Solvent $CDCl_3$/TFA.
**4** = 8-Methoxy griseorhodin C.
n.d. = Not determined.

of oxygenated groups in positions 7 and 8 of structure **1**. Of the two methylene proton signals the one at the lowest field (3.09~3.32 ppm) was assigned to position 7 due to the deshielding effect of the carbonyl group at position 6. The assignment of the second methylene signals at 2.24~2.45 ppm to position 8 was also confirmed by the comparison with the corresponding protons in dideoxygriseorhodin C (Fig. 2; R2=R3=H) which has signals at 2.43~2.51 ppm.[7]

The $^{13}$C-NMR spectrum of **1** was very similar to that of the reference compound **4** (Table 3) with a few exceptions. The presence in the carbon spectrum of **1** of a signal at 190.5 ppm, was attributed to the ketone group at position 6 in accordance with the above proton spectrum suggestion. The signals of carbons at positions 7 and 8 were attributed on the basis of the chemical shift obtained with a DEPT-135 and the Relay experiment based on carbon-proton correlation through one bond. Their upfield shift at 20.3 and 23.8 ppm, in respect to the C-7 and C-8 signals of compound **4** at 62.1 and 76.5 ppm, agreed with the absence of oxygen on these carbons in **1**. The DEPT-135 experiment confirmed the methylene nature of the two signals at 20.3 and 23.8 ppm in agreement with the values reported for the same carbons (21.3 and 23.2 ppm) in the similar dideoxygriseorhodin structure.[8] These carbon attributions were furtherly confirmed using the already known proton assignments at position 7 and 8; the carbons to which the protons were attached could in fact be deduced from the carbon-proton correlation of Relayed Coherence Transfer 2D experiment (Relay-AUR, Bruker Microprogram) carried out in $CDCl_3$/TFA. This experiment allowed also the assignment of the three proton singlets in the aromatic region. In fact, the C-3 carbon ($\delta_C$ 110.5 ppm) was coupled to the 3-H proton at $\delta_H$ 6.2 ppm, the C-10 ($\delta_C$ 103.7 ppm) to the 10-H at $\delta_H$ 6.53 ppm and the C-9 ($\delta_C$ 114.9 ppm) to the 9-H at $\delta_H$ 6.88 ppm.

Table 3. $^{13}$C-NMR assignments ($\delta$, ppm) at 150.76 MHz of **1** and **4**, (solvent DMSO-$d_6$).

| Carbon | 1[a] | 4[6] |
|---|---|---|
| 1 | 179.1 | 180.4 |
| 2 | 160.4 | 160.4 |
| 3 | 110.5 | 110.2 |
| 4 | 185.1 | 185.9 |
| 4a | 106.4 | 106.7 |
| 5 | 157.2 | 154.8 |
| 5a | 118.7 | 124.7 |
| 6 | 190.5 | 73.7 |
| 6a | 112.8 | 111.4 |
| 7 | 20.3 | 62.1 |
| 8 | 23.8 | 76.5 |
| 8a | 130.9 | 130.3 |
| 9 | 114.9 | 117.0 |
| 9a | 131.7 | 130.7 |
| 10 | 103.7 | 103.8 |
| 11 | 152.6 | 152.2 |
| 13 | 165.7 | 165.7 |
| 13a | 104.5 | 105.4 |
| 14 | 148.5 | 148.9 |
| 14a | 137.4 | 137.1 |
| 16a | 146.5 | 146.6 |
| 17 | 158.7 (b) | 156.5 |
| 17a | 113.6 (b) | 114.1 |
| $CH_3$– | 18.6 | 18.7 |
| $CH_3O$– | 57.1 | 57.1 |

[a] DMSO-$d_6$/TFA.
[b] Overlapped by TFA ($\delta_C$=111.5~118.4 and $\delta_C$=157.8~158.7 ppm) used to increase solubility.
**4**=8-Methoxygriseorhodin C.

The atmospheric pressure chemical ionization (APCI) mass spectrum of **1** showed only an intense ion corresponding to the protonated molecule at *m/z* 493. The MS/MS experiment, carried out on the $[MH]^+$ ion, showed some diagnostically important fragments at *m/z* 289, 261, 205 and 177. These ions arise from the naphthazarin (289 and 261) and isocumarin (205 and 177) moieties and match well with the fragmentation described for the antibiotic FCRC-57-G[9].

Therefore, all the above results confirmed for **1** the 7,8-dideoxy-6-oxo-griseorhodin C structure reported in Fig. 1.

## Chemical Derivatives

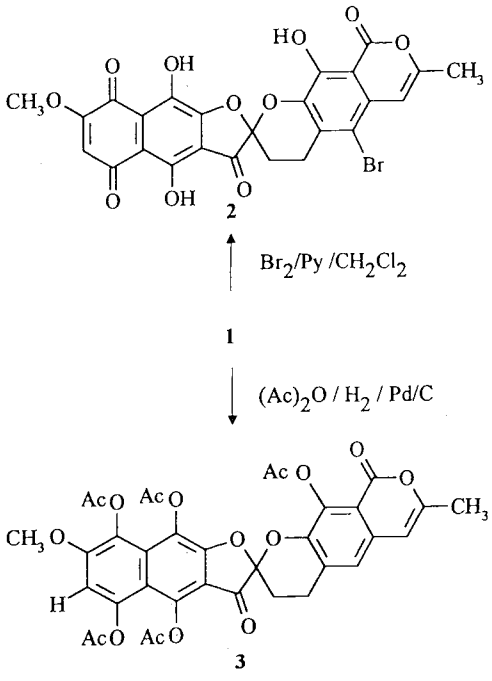
The structure of **1** was also confirmed by chemical derivatisation. In fact, by analogy with γ-rubromycin[10] (Fig. 2), the reaction of **1** with bromine (see Experimental part) gave the 9-bromo derivative **2** (Fig. 3), whose structure was confirmed by positive FAB mass spectrometry (*m/z* 571; $[MH]^+$) and by the absence of the 9-H signal in the $^1$H-NMR spectrum as reported in Table 2.

The acylation of **1** under reductive condition gave the pentaacetate **3** (Fig. 3) in a high yield. Compound **3** is a yellow fluorescent crystalline solid, more soluble in organic solvents than its parent compound. In respect to **1**, a down field shift of the signals of proton 3-H, from $\delta$=6.52 to 7.54 ppm, attributed to the reduction of the quinonic structure, and of proton 9-H, from $\delta$=6.91 to 7.37 ppm, were observed in the $^1$H-NMR as reported in Table 2.

## Antibacterial Activity

The biological activity data of **1** and **2**, tested against a panel of standard microorganisms, are summarized in Table 4. Compounds **1** and **2** showed weak inhibitory activity against Gram-positive bacteria and resulted inactive against Gram-negative bacteria. Both com-

Fig. 3. Chemical derivatives of compound **1**.




pounds showed weak activity against *Nesseria gonorrhoeae* (MIC 2 and 4 respectively) while against *Haemophilus influenzae* compound **1** had MIC 16 and **2** was inactive. In addition, **1** and **2** were inactive against *Candida albicans* the only fungus tested. No data are reported for derivative **3**, which resulted completely inactive in all the tests performed.

The inhibitory activity of **1** and **2** against *Staphylococcus aureus* is comparable with those reported for 8-methoxygriseorhodin C, **4**[6] and appears to be about 1/10 of that of dideoxygriseorhodin C, **5**[7]. They resulted also lower than that of purpuromycin whose data is in the same Table 4.

## Discussion

The new compound **1** has clearly a structure similarity with purpuromycin, the main fermentation product, but the biogenetic relationship between the two products has not been investigated. However, it is possible that **1** is a precursor of purpuromycin since it has been demonstrated[11] by $C^{13}$ labeled acetate incorporation that the carboxy group (R4 in Fig. 2) of purpuromycin derives, in the biosynthetic pathway, from oxidation of a methyl group.

Table 4. Antimicrobial activity of **1** and **2** in comparison with purpuromycin[a].

| Organism | MIC (μg/ml) | | |
|---|---|---|---|
| | **1** | **2** | Purpuromycin[5] |
| *Staphylococcus aureous* Tour | 2 | 8 | 0.03 |
| *S. epidermidis* ATCC 12228 | 1 | 8 | 0.03 |
| *S. haemolyticus* clinical isolate | 1 | 4 | 0.03 |
| *Streptococcus pyogenes* C203 | 8 | 8 | 0.03 |
| *S. pneumoniae* UC41 | 4 | 4 | 0.03 |
| *S. faecalis* ATCC 7080 | 4 | 32 | 0.06 |
| *Escherichia coli* SKF 12140 | >128 | >128 | 4 |
| *Proteus vulgaris* X19H ATCC 881 | >128 | >128 | 4 |
| *Pseudomonas aeruginosa* | >128 | >128 | >128 |
| *Neisseria gonorrhoeae* L997 | 2 | 4 | 0.06 |
| *Haemophilus influenzae* type b | 16 | >128 | 0.03 |
| *Candida albicans* SKF2270 | >128 | 128 | 0.25 |

[a] Broth microdilution method (see Experimental).

## Experimental

Melting points were determined on a Buchi apparatus and were uncorrected. The isolation of **1** and the preparation of derivatives **2** and **3** were followed by HPLC using a Hewlett-Packard 1090 L instrument equipped with a 4 × 150 mm RP-18 column (5 μm, C. Erba), a UV detector at 254 nm and a HP 3390 integrator. The mobile phases were: (A) 0.025 M $NaH_2PO_4/CH_3CN$ in the ratio 95:5 (v/v); (B) 0.025 M $NaH_2PO_4/CH_3CN$ in the ratio 30/70 (v/v). Elution was a linear gradient of phase B in phase A from 8% to 75% in 35 minutes at the flow rate of 1.5 ml/minute. HPLC retention time of compound **1** was 30.6 minutes and that of purpuromycin was 25.9 minutes. The UV spectra were recorded with a Perkin-Elmer spectrophotometer mod. Lambda 16. The IR spectra were obtained using a IFS 48 Fourier Transform Bruker spectrophotometer and the NMR spectra were recorded on a Bruker AM 600 spectrometer at 30°C using TMS, $\delta$ 0.00 ppm, as internal standard. Fast atom bombardment (FAB) spectra were obtained using a Finnigan TSQ700 mass spectrometer equipped with a xenon saddle field atom gun. APCI-MS and MS/MS spectra were recorded on a PE-SCIEX triple quadrupole instrument equipped with an heated nebulizer source (solvent acetonitrile : methanol : water (1:1:1) at 1 ml/minute, probe temperature 400°C, discharge current 3 μA, collision gas Ar).

Thin layer chromatography (TLC) was carried out on silica gel 60 $F_{254}$ (Merck 5 × 10 cm) plates.

### Antibacterial Activity

MICs were determined by a broth microdiluition. The media used were: Todd-Hewitt broth (Difco) for streptococci, Iso-sensitest (Oxoid) for staphylococci, *Enterococcus faecalis* and *Escherichia coli*, G.C base broth (Difco)+1% (v/v) Isovitalex (BBL) for *Nesseria gonorrhoeae*, buffered yeast nitrogen base broth (Difco) supplemented with glucose (1%) and L-asparagine (0.15%) for *C. albicans*. Inocula were about $10^4$ cfu/ml for bacteria and *C. albicans*. All organisms were cultured at 37°C. *N. gonorrhoeae* was incubated in 15% $CO_2$ atmosphere. MICs were read at 20~24 hours except for *N. gonorrhoeae* (read at 40~48 hours).

### Fermentation of the Producing Strain

A mature slant of *Actinoplanes iantinogenes* ATCC 20884 was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of AF/MS medium consisting of glucose 2%, soybean meal 0.8%, yeast extract 0.2%, NaCl 0.1% and $CaCO_3$ 0.4% prepared in distilled water and adjusted to pH 7.3 with 40% aq NaOH and sterilized at 121°C for 30 minutes. The culture was shaken at 200 rpm, 28°C for 144 hours. A 150-ml aliquot of the seed culture was transferred into a bioreactor containing 6 liters of the same medium and fermented at 28°C (900 rpm, air flow 0.5 vvm) for 48 hours. Part of this culture was then transferred into a fermenter containing 200 liters of the same medium and cultured at 28°C (180 rpm, air flow of 0.5 vvm) for 48 hours. Fermentation was continued in a tank bioreactor containing 2,000 liters of the E1 production medium consisting of glucose 5%, soybean meal 1%, yeast extract 0.1%, peptone 0.4%, meat extract 0.4%, NaCl 0.25% and $CaCO_3$ 0.5% in tap water, pH adjusted to pH 7.5 with 40% aq. NaOH and sterilized at 121°C for 30 minutes. Fermentation was carried out at 32°C, 150 rpm, air flow 0.5 vvm, for 85 hours.

At harvest time the pH of the culture was 6.7, the biomass expressed as packed mycelium volume (PMV) was 23% and the glucose in the medium was completely exhausted. Purpuromycin was the major product of the fermentation. However the HPLC analysis of the final broth showed another peak, at a retention time higher than that of purpuromycin, which corresponded to compound **1**.

### Isolation and Purification of 7,8-Dideoxy-6-oxy-griseorhodin C (1)

The harvest broth was filtered on a rotating drum filter and the mycelium cake (570 kg) was extracted at pH 1.5~1.8 with a MeOH/$CH_2Cl_2$ 1:1 mixture (900 liters × 2). The combined organic phases were concentrated under reduced pressure to a small volume and the solid obtained was filtered. This material was stirred in methanol (180 liters) for two hours and filtered again obtaining 1.5 kg of crude product. This material was splitted into three parts and each part was separately subjected to a column chromatographic purification.

Each portion of 500 g of crude material was dissolved in 45 liters of THF. The solutions obtained were filtered and chromatographed on 22 kg of silica gel (Meck 70~230 mesh ASTM) containing about 14%[††] of water in a 30 cm (i.d.) glass column. The columns were eluted with $CH_2Cl_2$ - AcOH 97:3 (v/v) and the fractions containing compound **1** (29 liters) were pooled and concentrated to 300 ml on a rotary evaporator. After filtration about 3.8 g × 3 of a red precipitate was recovered. It was stirred in 30 ml of AcOH and filtered, stirred in 100 ml of ethyl ether and filtered again yielding 11.1 g of pure **1**.

*Anal* Calcd for $C_{25}H_{16}O_{11}\cdot H_2O$: C 58.8, H 3.5.
Found: C 58.9, H 3.3.

### 9-Bromo-7,8-dideoxy-6-oxo-griseorhodin C (2)

415 mg of pyridinium hydrobromide perbromide (1.29 mmol) was added to a solution of **1** (300 mg, 0.609 mmol) in $CH_2Cl_2$ (100 ml) and anhydrous pyridine (120 ml). The mixture was stirred at room temperature for 3 hours to complete the reaction, after which the solvents were removed under vacuum (50°C) and the residue chromatographed on silica gel (50 g). The column was eluted with $CHCl_3$ - MeOH - AcOH, 98:2:0.1 (v/v) and the fractions containing the reaction product were pooled and concentrated to a small volume under vacuum. The precipitate was collected, yielding 60 mg (17.2%) of pure **2** as a red solid. Compound **2** was slightly soluble in DMF, DMSO and $CHCl_3$ and did not melt up to 275°C. TLC; Rf=0.35 ($CHCl_3$ - MeOH - AcOH, 90:10:0.1 (v/v/v). FAB-MS; *m/z* 571 $[MH]^+$. IR; $\nu_{max}$ (Nujol) $cm^{-1}$ 1744 (ketone), 1691 (lactone, C=O), 1617, 1595 (quinone), 1269, 1191, 1029.

### Pentaacetate of 1 (3)

Acetic anhydride (2 ml) and 10% Pd/C (10 mg) were

[††] In order to have reproducible separations the commercial stationary phase was maintained in a humidity controlled room until it reached the desired water content.

added to a solution of **1** (150 mg, 0.304 mmol) in pyridine (0.6 ml) and anhydrous THF (20 ml). The mixture was hydrogenated overnight at room temperature and at atmospheric pressure. During the hydrogenation the colour of the solution turned from red to green. The solvent was finally removed under vacuum and the product was precipitated with water (50 ml). The crude material was then dissolved in $CHCl_3$ and chromatographed on silica gel (50 g). The column was eluted with $CHCl_3$ and the fractions containing the pure acetate were pooled and concentrated under reduced pressure at room temperature. The solid residue was stirred with ethyl ether, filtered and dried obtaining 140 mg (65.3%) of **3**.

An analytical sample was obtained from $CH_2Cl_2$-MeOH, 10:1 (v/v) as a yellow crystalline compound, fluorescent in organic solvent solutions. MP 239~242°C. TLC: Rf 0.75 ($CHCl_3$/MeOH/acetic acid 95:5:0.1). FAB-MS: *m/z* 705 $[MH]^+$. IR $\nu_{max}$ ($CHCl_3$) $cm^{-1}$: 1775 (acetyls), 1735 (ketone and lactone), 1192 (CO–O–C). UV $\lambda_{max}$ ($CHCl_3$): 261, 286, 338 (sh), 354, 405 nm.

*Anal* Calcd for $C_{35}H_{28}O_{16}$: C 59.66, H 3.98.
Found: C 59.78, H 4.00.

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