

Prefluostatin and New Urauchimycin Derivatives Produced by *Streptomyces* Isolates*

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Guided by chemical screening, two new members of the antimycin group, urauchimycin C (**1b**) and D (**1a**), were isolated from the marine-derived *Streptomyces* sp. isolate B1751 and from a terrestrial strain AdM21, together with prefluostatin (**2**), a new natural derivative of isoprekinamycin (**4**). Their structures were established on the basis of NMR data and by comparison with known compounds. In the agar diffusion test, urauchimycin C (**1b**) and D (**1a**) were antibiotically inactive against some bacteria and fungi at a concentration of 25 µg per paper disk, while the isoprekinamycin derivative **2** showed weak cytotoxicity and moderate activity in the agar diffusion test against *Bacillus subtilis*, *Mucor miehei*, *Escherichia coli* and *Staphylococcus aureus* at concentrations of 20 µg per paper disk.

Key words: Marine Streptomyces, Urauchimycin, Antimycin, Kinamycin, Prefluostatin

Introduction

The antimycins are forming a group of about 30 closely related lactolide antibiotics with the skeleton of **1**. Their common feature is a 9-membered ring of an α -substituted β,γ -dihydroxyvaleric acid and threonine, wherein the nitrogen is acylated with 3-formyl-aminosalicylic acid. The antimycins differ in the nature of their alkyl residue at C-7 and of the oxygen substituent at C-8: Acylation of the 8-hydroxy group modulates the strong antifungal, antiviral and antitumor activities. These acylated antimycins inhibit the mitochondrial ATP-production and are therefore not effective against bacteria that do not have mitochondria. Antimycins have also been reported to inhibit the oxidation of NADH [1]. They are occasionally used in fruit cultivation as antifungals and can be applied in fish-breeding to kill sick specimens. The relatively low

stability of the agent allows new stock to be used after just a few days [2].

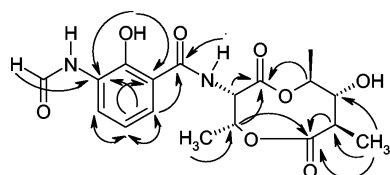
In the course of our screening for new metabolites from bacteria, we have isolated two new members of the urauchimycin group, which we named urauchimycin C (**1b**) and D (**1a**), from the extracts of the marine streptomycete isolate B1751 and from a terrestrial strain AdM21. Urauchimycins are antimycin derivatives with a free 8-OH group.

Results and Discussion

The extract of the streptomycete isolate B1751 exhibited UV absorbing spots on TLC, which developed a characteristic greenish colour reaction upon spraying with anisaldehyde/sulphuric acid. The strain was fermented on a 20 l scale on a yeast extract/malt extract/glucose (YMG) medium and worked up under usual conditions [3]. By silica gel column chromatography and reversed phase HPLC, *cis-cyclo*(leucyl-propyl) [4] and N-(2-phenylethyl)-acetamide [5] were obtained and easily identified by comparison of their NMR data with the literature. Fractionation of the

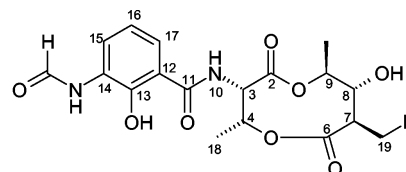
* Art. No. XXXIII on Marine Bacteria. XXXII: S. Fotso, S. J. Wu, S. Qin, H. Laatsch: 5,7-Dihydroxy-5,6,7,8-tetrahydro-1*H*-azocin-2-one from a Marine-derived *Streptomyces* sp. Nat. Prod. Comm. accepted 11/2005.

Position	¹ H (200 MHz); δ (J [Hz])			¹³ C (125 MHz)		¹³ C (75 MHz)
	1a	1b	1d	1a	1b	1d
2				170.1	170.1	170.1
3	5.22 t (7.3)	5.25 t (7)	5.25 t ^a (7) ^b	53.5	53.7	53.7
4	5.70 quint (6.5)	5.71 quint (7)	5.71 quint (7)	70.4	70.7	70.7
6				174.1	173.8	174.0
7	2.40 m	2.31 d (7)	2.33 dt (10, 4)	45.3	53.7	52.3
8	2.5 m	3.61 t (10)	3.61 t (10)	77.9	77.3	77.1
9	4.83 m	4.95–4.80 m	4.96–4.80 m	77.4	77.0*	77.0*
9-CH ₃	1.45 d (6.3)	1.46 d (6)	1.46 d (7)	18.4	18.3	18.4
10 (NH)	7.08 d (8.2)	7.10 d (7)	7.10 d (7)			
11				169.4	169.4	169.4
12				112.5	112.6	112.6
13				150.6	150.6	150.6
13-OH	12.60 s	12.65 s	12.66 s			
14				127.4	127.4	127.4
14 (NH)	7.91 s	7.93 s	7.94 s			
14 (NHCHO)	8.48 d (1.6)	8.51 d (1)	8.51 d (2)	158.9	159.1	159.2
15	8.53 dd (1.3, 7.7)	8.55 dd (8, 1)	8.55 dd (8, 2)	124.8	124.8	124.8
16	6.90 t (8.3, 16.4)	6.93 t (8)	6.93 t (8)	118.9	119.0	119.0
17	7.80 d	7.25–7.22 m	7.26 dd (8, 2)	120.1	120.2	120.2
18	1.25 d (6.5)	1.31 d (7)	1.32 d (7)	14.5	15.0	15.0
19		1.80–1.60 m	1.84–1.62 m		22.1	26.8
20	1.29 d (6.5)	0.94 t (7)	1.24–1.08 m	14.1	11.6	36.2
21			1.61–1.51 m			28.0
21-(CH ₃) ₂			0.89 d (7)			22.6
			0.89 d (7)			22.2

Table 1. ¹H and ¹³C NMR data of urauchimycins B (**1d**), C (**1b**) and D (**1a**) in CDCl₃.^a Multiplicity; ^b coupling constants in [Hz]; * under solvent peak.Fig. 1. Selected HMBC (→) and H,H COSY (↔) correlations of urauchimycin D (**1a**).

more polar part by PTLC and RP HPLC yielded three main components. Two of them were identified as deisovalerylblastmycin (**1c**) [6] and urauchimycin B (**1d**) [7] by comparison of their spectroscopic properties with AntiBase data [8].

Compound **1b** was obtained as a yellowish solid which gave a greenish colour reaction with anisaldehyde/sulphuric acid and a molecular ion peak at m/z 408.1532 (EI HRMS), corresponding to the molecular formula C₁₉H₂₄N₂O₈. Colour reaction and the ¹H NMR data (Table 1) in the range of $\delta > 1.0$ were identical with those of **1c** suggesting that this compound belonged to the group of urauchimycins/anti-mycins as well. At high field, three methyl signals were visible, two doublets ($\delta = 1.46, 1.31$) and a triplet ($\delta = 0.94$). According to the H,H COSY and HMBC data, there was an ethyl group at C-7 resulting in **1b**, instead of a butyl group as in **1c**. Compound **1b** is a new



R	R
1a: H	1e: CH(CH ₃)CH ₂ CH ₃
1b: CH ₃	1f: (CH ₂) ₄ CH ₃
1c: (CH ₂) ₂ CH ₃	1g: (CH ₂) ₂ CH(CH ₃) ₂
1d: CH ₂ CH(CH ₃) ₂	

member of the urauchimycin family and was named urauchimycin C.

In a similar way, a 25 l shaker culture of a terrestrial streptomycete strain AdM21 delivered a complex mixture of antimycin A components [9] with masses between m/z 436 and 578, *cis-cyclo*(leucylprolyl) [10], a mixture of palmitic acid and homologues, polyhydroxybutyric acid (SPHB) [11], and the new urauchimycin D (**1a**). Additionally, a new derivative of isoprenkinamycin identified as 1,6,7-trihydroxy-3-methylbenzo[a]fluoren-11-one (**2**) [12] is reported here for the first time from a natural source.

The (+)-ESI mass spectrum of urauchimycin D (**1a**) gave a *pseudo* molecular ion at m/z 417 ([M+Na]⁺), which delivered the molecular formula C₁₈H₂₂N₂O₈

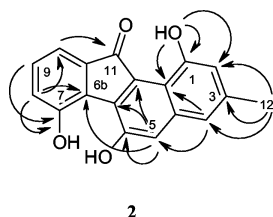


Fig. 2. Structure and selected HMBC correlations of prefluostatin (**2**).

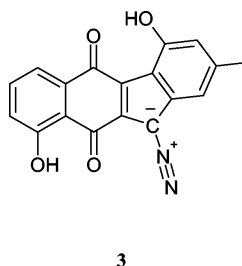
by ESI HRMS. The ^1H NMR data of **1a** (Table 1) were very similar to those of **1b** and **1c**, the major difference being a third methyl doublet instead of the methyl triplet of C-20 in **1b**. The H,H COSY and HMBC data (Fig. 1) indicated a correlation of the methyl group at $\delta = 1.29$ (C-19) with the methine carbon at $\delta = 45.3$ (C-7), the carbonyl signal of C-6 at $\delta = 174.1$ and the methine at $\delta = 77.9$ (C-8), confirming the methyl to be in position C-7. Further HMBC correlations (Fig. 1) confirmed the structure of urauchimycin D as a further member **1a** in the series of homologous urauchimycins.

Compound **2** was obtained as a purple solid, which showed on TLC a brown colour after spraying with anisaldehyde/sulphuric acid. The ^1H NMR spectrum of **2** exhibited in $\text{DMSO}-d_6$ a singlet at $\delta = 11.46$ attributed to a chelated hydroxy group, however, the expected typical colour change of *peri*-hydroxyquinones with diluted sodium hydroxide was very weak. In the aromatic region, a triplet and two doublets were attributed to an 1,2,3-trisubstituted aromatic system and confirmed by H,H COSY couplings, two further doublets indicated protons in *meta*-position. In the aliphatic region, only a 3H signal of an aromatic methyl group was present. The ESI mass spectra showed *pseudo* molecular ions at m/z 606 ($[\text{2M}+\text{Na}]^+$) and 291 ($[\text{M}-\text{H}]^-$) for a molecular mass of m/z 292, and ESI HRMS delivered the molecular formula $\text{C}_{18}\text{H}_{13}\text{O}_4$. The ^{13}C NMR spectrum exhibited 18 signals including a carbonyl at $\delta = 197.9$, 10 quaternary carbons, 6 sp^2 methines and the methyl carbon.

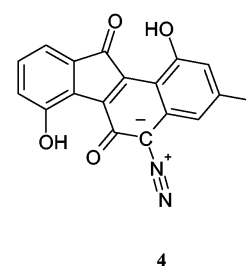
The HMBC spectrum showed a correlation of the methyl group (C-12) with the quaternary ($\delta = 137.8$, C-3) and the methine carbons at $\delta = 113.4$ (C-2) and 117.4 (C-4), respectively, confirming that both 1H singlets at $\delta = 6.98$ (4-H) and 6.80 (2-H) were due to protons in *meta* position (Fig. 2). Overlapping correlations with cross signals of 5-H indicated finally a 1,6-dihydroxy-naphthalene. Among others, the HMBC cor-

Table 2. ^1H and ^{13}C NMR data of prefluostatin (**2**) in DMSO.

Position	^1H (300 MHz)		^{13}C (75.5 MHz)	
	2	ref. [12]	2	ref. [12]
1			153.1	154.8
1-OH	11.42	11.42		
2	6.80 s	6.41 s	113.4	111.7
3			137.8	138.7
3-CH ₃	2.30 s	2.25 s	20.1	21.0
4	6.98 s	6.82 s	117.4	117.1
4a			127.5	127.2
5	7.38 s	7.08 s	121.1	121
6-OH		3.34 br	148.3	—
6a			135.7	136.7
6b			125.8	126.2
7-OH		3.34 br	150.7	153.1
8	7.01 d (8.1)	6.78 d (8.1)	126.1	126.9
9	7.19 t (7.1)	7.01 t (7.6)	131.1	130.7
10	7.15 d (7.0)	6.9 d (6.9)	117.3	115.1
10a			133.7	133.6
11			197.5	198.4
11a			137.8	138.3
11b			115.8	114.9



3



4

relation between the proton signal at $\delta = 7.15$ (10-H) and the carbonyl signal at $\delta = 197.9$ indicated a 4-hydroxyinden-1-one. Two ways to connect both fragments are possible, one yielding the skeleton of prekinamycin (**3**) [13] or momofulvenone A [14], the other delivering structure **2**. A long-range coupling between 5-H and C-6b confirmed the latter, for a 4J coupling in **2** is more plausible than the corresponding 5J coupling in a benzo[b]fluorenone of type **3**.

Compound **2** was previously obtained during synthetic studies on kinamycins [12] by rhodium acetate induced deazotisation of isoprekinamycin (**4**), a minor metabolite from *Streptomyces murayamaensis* [15], however, it is found here for the first time in nature. Our ^{13}C NMR data are identical with the reported values within the error limits [12], although the proton shifts show some deviations (Table 2).

The kinamycins and the aromatized prekinamycin (**3**) are in the same relation, as the fluostatins [16] and **2**, for which therefore the name prefluostatin is suggested. Isoprekinamycin (**4**) or other kinamycins

have not been found in *S. sp.* AdM21 so far, however, a search by systematic variations of the fermentation conditions may make sense.

Biological activity

For the urauchimycins A (**1e**) and B (**1d**) [17] and the related kitamycins A (**1f**) and B (**1g**) [18], only weak antifungal properties were reported due to the free hydroxy group at C-8 [17]. Correspondingly, the urauchimycins C (**1b**) and D (**1a**) were inactive as well against *Candida albicans* and *Mucor miehei* in the agar diffusion test at concentrations of 25 µg per paper disk. Expectedly, they were also inactive against *Escherichia coli* and *Staphylococcus aureus*.

Although the diazo group is believed to be responsible for the antibacterial and weakly antitumoral properties [19] of kinamycins, compound **2** showed as well moderate activity in the agar diffusion test against *Bacillus subtilis* and *B. brevis* (11 mm inhibition zone), *Mucor miehei* (11 mm), *Escherichia coli* (11 mm) and *Staphylococcus aureus* (18 mm) at concentrations of 20 µg per paper disk. Weak cytotoxic activities towards MCF-7 cells and HeLa S3 cells were observed with LD₅₀ ranging between 20 and 30 µg/ml. Jurkat and Hep G2 cells were only affected at higher concentrations (LD₅₀ 50 µg/ml).

Experimental Section

General experimental procedures were used as reported previously [20].

Assays for cytotoxic activity

Cytotoxic activity was assayed as described previously [21] with slight modifications. Jurkat cells (DSMZ ACC 282) were grown in RPMI 1640 medium (GIBCO, BRL), HeLa S3 (ATCC CCL 2.2) MCF-7 (ATCC HTB-22) and Hep G2 (DSMZ ACC 180) cells in D-MEM (GIBCO, BRL), supplemented with 10% fetal calf serum (GIBCO, BRL), 65 µg/ml of penicillin G and 100 µg/ml of streptomycin sulphate. The assays contained 1×10^5 cells/ml medium. The concentration at which cell proliferation was reduced by 50% is given as IC₅₀ value.

Taxonomy

Strain B1751 has been isolated from sediment taken north east of the Shetland Islands (59°00'N, 00°00'W) using chitin agar [22] containing 50% natural seawater. The reference culture of B1751 is kept on yeast extract-malt extract agar [7] in the Collection of Marine Actinomycetes at the Alfred-Wegener-Institute for Polar and Marine Research in Bremer-

haven. Strain AdM21 was isolated from a tropical soil sample and is stored in the Department of Organic and Biomolecular Chemistry (ID number 2421).

The almost complete 16S rDNA gene sequence of the strain B1751 shows 99% similarity with *Streptomyces caviscabies* (accession no. AF112160). The strain forms a beige substrate mycelium and a grey aerial mycelium with straight to flexuous spore chains (*Rectiflexibiles*). The spores are oval with a spiny sometime hairy surface. Melanin pigment is neither produced on peptone-yeast extract-iron agar nor on tyrosine agar [23]. The optimum growth temperature is at about 30 °C. The strain does not grow at 10 °C and at 45 °C. Good growth occurs in media from 0% up to 10% sea-water salinity. Chitin, starch, casein, and gelatine are degraded. Cellulose and esculin are not hydrolyzed. The strain is catalase positive. Nitrate reductase and H₂S are not produced. The use of carbon sources was tested with SFN2-Biolog (Hayward, CA, USA) using BMS-N without agar as basal medium [24]. The following organic compounds can be utilized for growth: N-acetyl-D-glucosamine, γ-amino butyric acid, L-arabinose, D-arabitol, L-asparagine, L-aspartic acid, bromosuccinic acid, cellobiose, citric acid, dextrin, D-fructose, D-galactose, gentibiose, D-gluconic acid, D-glucosaminic acid, glucose, L-glutamic acid, glycerol, glycogen, L-histidine, β-hydroxy butyric acid, α-D-lactose, maltose, D-mannitol, D-mannose, proline, propionic acid, L-rhamnose, succinic acid, L-threonine, tween 40, tween 80.

Fermentation and work-up of strain B1751

The marine isolate *Streptomyces sp.* B1751 was cultivated in 1 l Erlenmeyer flasks containing 200 ml of marine YMG medium [3] at 28 °C for 72 h. Of this culture, 2 l were used as inoculum for a 20 l-fermentor (Meredos, Goettingen, Germany) with the same YMG medium starting at pH 7.0, 28 °C with agitation of 200 rpm. The pH remained between 5.75 and 8.25.

After 72 h, the culture broth was homogenized with an Ultraturrax and filtered with the aid of diatomaceous earth. The culture filtrate and the cell material were exhaustively extracted with ethyl acetate. The combined organic phases were evaporated under vacuum at 40 °C and the extract (3.7 g) was subjected to silica gel chromatography (stepwise chloroform/methanol gradient, 100 : 0, 99 : 1, 97 : 3, 95 : 5, 90 : 10), and five fractions were collected. RP HPLC (acetonitrile-H₂O azeotrope/H₂O 35:65; flow rate 10 ml/min) of fraction 3 yielded cyclo(L-leucyl-L-prolyl) (R_f = 0.47, CHCl₃/CH₃OH 9 : 1; t_R = 10.02 min; 9.0 mg) and N-(2-phenylethyl)-acetamide (R_f = 0.50, CHCl₃/CH₃OH 9 : 1; t_R = 20.14 min; 7.1 mg). PTLC of fraction 4 (2 PTLC plates 20 × 40 cm, ethyl acetate/cyclohexane 9 : 1) and subsequent RP HPLC (acetonitrile-H₂O azeotrop/H₂O, gradient 10 : 90 to 100 : 0 within 15 min, remaining at 100 : 0 for 10 min; flow rate 10 ml/min) of the yellow zone afforded urauchimycin C

(**1b**, t_R = 22.58 min, 1.9 mg), deisovalerylblastmycin (**1c**, t_R = 25.06 min, 3.7 mg), and urauchimycin B (**1d**, R_f = 0.39, $\text{CHCl}_3/\text{CH}_3\text{OH}$ 90 : 10; t_R = 25.73 min, 2.1 mg).

Fermentation and work-up of strain ADM21

For the terrestrial streptomycete strain AdM21, 100 × 1 l Erlenmeyer flasks each containing 250 ml of M_2 medium were inoculated from agar plates and grown for 3 days at 30 °C. The culture broth was mixed with *ca.* 1 kg Celite and separated by pressure filtration. The mycelial cake was extracted three times with ethyl acetate and acetone. Multiple separations of the combined extracts (5.1 g) delivered 80 mg of antimycin A complex, and 100 mg of aliphatic fatty acids (mainly palmitic acid).

The water phase was extracted with XAD-16 (column 96 × 32 cm) and the resin washed with water and extracted with methanol. The methanol phase was concentrated and the aqueous residue extracted with ethyl acetate. Chromatography of this extract (1.5 g) on Sephadex LH-20 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 6 : 4) delivered fractions A–D, which were successively separated by PTLC ($\text{CHCl}_3/10\%$ MeOH). RP HPLC ($\text{MeCN}/\text{H}_2\text{O}$ gradient) of fraction B yielded urauchimycin D (**1a**) (t_R = 18.20 min, 45.7% azeotrope MeCN , 2.5 mg). Fraction C gave 5 mg of the violet fluorenone **2** (R_f = 0.51, $\text{CH}_2\text{Cl}_2/5\%$ MeOH).

Urauchimycin D (**1a**)

Yellow solid, R_f = 0.81 ($\text{CH}_2\text{Cl}_2/5\%$ MeOH). – $[\alpha]_D^{20}$ = +53° (*c* 0.1, acetone). – UV/vis (MeOH): $\lambda_{\text{max}}(\lg \epsilon)$ = 201 nm (4.02), 221 nm (3.77), 348 nm (2.99). – IR (KBr): ν = 3790, 3662, 3420, 2926, 2855, 2242, 1663, 1583, 1414, 1385, 1206, 1139, 657 cm^{-1} . – ^1H , ^{13}C NMR see Table 1. – (+)-ESI MS: m/z (%) = 417 ($[\text{M}+\text{Na}]^+$, 100), 810.8 (28) $[\text{2M}+\text{Na}]^+$. – (–)-ESI MS: m/z (%) = 393.1 (100) $[\text{M}-\text{H}]^-$, 809.1 (98) $[\text{2M}-2\text{H}+\text{Na}]^-$. – (+)-ESI HRMS: m/z = 417.126886 $[\text{M}+\text{Na}]^+$, 395.14492 ($[\text{M}+\text{H}]^+$) ($\text{C}_{18}\text{H}_{22}\text{N}_2\text{O}_8\text{Na}$, calcd. 417.126850).

Urauchimycin C (**1b**)

Faint yellow solid, R_f = 0.67 ($\text{CHCl}_3/10\%$ CH_3OH). – UV/vis (MeOH): $\lambda_{\text{max}}(\lg \epsilon)$ = 278 nm (3.46), 321 (3.30); IR (KBr): ν = 3424, 2928, 2362, 1650, 1541, 1382, 1197 cm^{-1} ; ^1H , ^{13}C NMR see Table 1. – MS (EI, 70 eV):

m/z (%) = 408.5 (61) $[\text{M}^+]$, 220 (82), 164 (65), 136 (70), 135 (100). – MS (DCI, NH_3): m/z (%) = 426 (100) $[\text{M}+\text{NH}_4]^+$, 409 (82) $[\text{M}+\text{H}]^+$. – MS (HREI): m/z = 408.1532 ($\text{C}_{19}\text{H}_{24}\text{N}_2\text{O}_8$, calcd. 408.1526).

Deisovalerylblastmycin (**1c**)

Faint yellow solid, R_f = 0.60 ($\text{CHCl}_3/10\%$ CH_3OH). – ^1H NMR (CDCl_3 , 200 MHz): δ = 12.65 (s, 1H, 13-OH), 8.55 (d, J = 8 Hz, 1H, 15-H), 8.50 (d, J = 1 Hz, 1H, 14-NHCHO), 7.92 (s br, 1H, 14-NH), 7.25–7.22 (m, 1H, 17-H), 7.09 (d, J = 7 Hz, 1H, 10-NH), 6.92 (t, J = 8 Hz, 1H, 16-H), 5.70 (quint, J = 7 Hz, 1H, 4-H), 5.25 (t, J = 7 Hz, 1H, 3-H), 4.92–4.82 (m, 1H, 9-H), 3.60 (t, J = 10 Hz, 1H, 8-H), 2.36 (dt, J = 10 Hz, J = 4 Hz, 1H, 7-H), 1.81 (s, 1H, 8-OH)*, 1.70–1.60 (m, 2H, 19- CH_2)*, 1.46 (d, J = 6 Hz, 3H, 9- CHCH_3), 1.34–1.28 (m, 2H, 21-CH), 1.30–1.27 (m, 2H, 20- CH_2), 1.31 (d, J = 7 Hz, 3H, 18- CH_3), 0.90 (t, J = 7 Hz, 3H, 22- CH_3); *under water peak. – MS (DCI, NH_3): m/z (%) = 890.9 (0.01) $[\text{2M}+\text{NH}_4]^+$, 454.5 (100) $[\text{M}+\text{NH}_4]^+$, 437.4 (18) $[\text{M}+\text{H}]^+$. – MS (HREI): m/z = 436.1847 ($\text{C}_{21}\text{H}_{28}\text{N}_2\text{O}_8$, calcd. 436.18456).

1,6,7-Trihydroxy-3-methylbenzo[*a*]fluoren-11-one; prefluostatin (**2**)

Purple solid, R_f = 0.47 (5% $\text{MeOH}/\text{CH}_2\text{Cl}_2$). – UV/vis (MeOH): $\lambda_{\text{max}}(\lg \epsilon)$ = 225 (4.54), 258 (4.52), 301 (4.23), 477 (3.75) nm. – IR (KBr): ν = 3225, 2955, 2918, 2850, 2350, 1711, 1667, 1614, 1585, 1464, 1390, 1366, 1263, 1216, 1160, 1096, 761, 669 cm^{-1} . – ^1H and ^{13}C NMR data see Table 2. – (+)-ESI MS: m/z (%) = 293 (10) $[\text{M}+\text{H}]^+$, 606.8 (4) $[\text{2M}+\text{Na}]^+$. – (–)-ESI MS: m/z (%) = 291.5 (100) $[\text{M}-\text{H}]^-$, 583 (80) $[\text{2M}-2\text{H}]^-$. – (+)-ESI HRMS: m/z = 293.08096 $[\text{M}+\text{H}]^+$ ($\text{C}_{19}\text{H}_{13}\text{O}_4$, calcd. 293.08138).

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