Biosynthesis of the Antibiotic Echinosporin by a Novel Branch of the Shikimate Pathway

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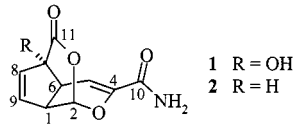
Echinosporin (**1**), a known antibiotic with a unique tricyclic acetal-lactone structure, is produced by *Streptomyces erythraeus* (strain Tü 4015), together with the novel 7-deoxyechinosporin (**2**) as minor compound, which was fully characterized. The biosynthesis of **1** was established by feeding experiments with ^{13}C-labelled precursors. The results revealed that **1** was formed by the shikimate pathway, with chorismate as a biosynthetic intermediate. The proposed mechanism for the conversion of chorismate into **1** represents a new branch of the shikimate pathway, producing a nonaromatic metabolite. In addition, the influence of aromatic amino acids and of glyphosate, an inhibitor in the shikimate pathway of plants, was investigated.

Introduction

Echinosporin (**1**), first isolated in 1982 from *Streptomyces echinosporus,*[1] is an antibacterial and antitumour antibiotic[1,2] with a unique tricyclic acetal-lactone structure.[3] The highly functionalized skeleton was a challenge for synthetic studies,[4,5] resulting in one total synthesis.[6] Nothing has been reported about the biosynthesis of this comparably small microbial secondary metabolite. From the structure itself it is not possible to predict the biosynthetic pathway by which the skeleton of **1** is formed (Figure 1). Such an ambiguous starting point may lead to new biosynthetic transformation steps.



1 R = OH
2 R = H

Figure 1. Structural formulae of echinosporin (**1**) and 7-deoxyechinosporin (**2**)

During an extended chemical screening[7] program, the new echinosporin-producing strain Tü 4015 was isolated from a soil sample collected in Thailand and identified as *Streptomyces erythraeus.*[8] With this strain available, we were able to begin biosynthetic investigations that revealed an unexpected branch of the shikimate pathway.

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Results and Discussion

Culture Conditions and Isolation

The first fermentation of *Streptomyces erythraeus* (strain Tü 4015) with standard culture media produced echinosporin (**1**) in yields of only 2 mg/L. For biosynthetic studies, it was a prerequisite to increase the production rate by optimisation of the culture conditions.[8] Hence, sodium gluconate was used as an additional carbon source in place of glucose, fructose or mannitol. Furthermore, the yields of **1** could be enhanced dramatically by the addition of sodium bromide or chloride (7 g/L) and by pH-static (pH = 7.0) fermentation with HEPES or phosphate buffer solutions. In shake flasks under these optimized conditions, the production of **1** started after 48 h and reached a maximum after 72 h. The yields of **1** were reproducibly in the range of 50−80 mg/L. In order to isolate **1**, the culture filtrate was extracted with ethyl acetate (pH = 4.0) and the crude product obtained was subjected to silica gel column chromatography. This was followed by gel permeation chromatography on Sephadex LH-20, with methanol as eluent.

The chemical and spectroscopic properties of the isolated **1** were identical with the data given in the literature. As a minor compound (1−2 mg/L), 7-deoxyechinosporin (**2**) was isolated. It could be identified as the direct precursor of **1** by shortening the fermentation period: the yield of **2** after 60 h was increased to 28 mg/L. Compound **2** was more lipophilic than **1** and gave an intensive violet colour reaction with anisaldehyde/H_2SO_4 as staining reagent. Its empirical

formula, $C_{10}H_9NO_4$, was established by HREI-MS (m/z = 207.0532 [M^+]) and its structure by comparison of its NMR spectroscopic data with those of **1** (Table 1). In particular, the quaternary C-7 signal (δ_C = 85.3) of **1** was shifted towards δ_C = 53.3, indicating a methine group (δ_H = 3.39).

Table 1. ^{1}H NMR and ^{13}C NMR signals of echinosporin (**1**) and 7-deoxyechinosporin (**2**) (CD_3OD)

C atom	**1** δ_H	**1** δ_C	**2** δ_H	**2** δ_C
1	3.01 (m)	42.1	3.18 (m)	43.1
2	6.00 (dd, 1.5, 1.5)	98.4	5.97 (dd, 1.5, 1.5)	97.4
4		144.2		145.0
5	6.27 (d, 5.5)	107.6	6.27 (d, 5.5)	109.0
6	3.14 (ddd, 5.5, 5.5, 1.5)	49.5	2.82 (m)	40.0
7		85.3	3.39 (dd, 4.5, 3.0)	53.3
8	6.22 (d, 6.0)	143.3	6.40 (dd, 5.5, 3.0)	137.8
9	6.44 (ddd, 6.0, 3.5, 0.5)	134.3	6.50 (dd, 5.5, 3.0)	136.2
10		165.1		165.2
11		173.6		170.8

Echinosporin (**1**) and deoxyechinosporin (**2**) show remarkable growth inhibition of different human cancer cell lines[9] (Table 2); the activity of **2**, with GI_{50} values between 0.1 and 1.6 μmol/ml, was significantly higher than that of **1**.

Table 2. Cytotoxic activity of **1** and **2** against different tumor cell lines (GI_{50} and TGI in μmol/ml) (see ref.[9])

Compound	**MCF7**		**Huh7**		**HepG2**	
	GI_{50} [a]	TGI[b]	GI_{50}	TGI	GI_{50}	TGI
1	6.4	> 10	6.4	> 10	0.92	> 10
2	0.4	> 10	1.6	> 10	0.085	5.0

[a] GI_{50} = concentration, which results in a 50% inhibition of the cell growth. [b] TGI = concentration, which results in a complete inhibition of the cell growth.

Feeding Experiments

Feeding experiments were performed with ^{13}C-labelled precursors, which were added to the growing cultures of strain Tü 4015 five times over 10 h (pulse feeding), starting 48 h after inoculation. After 72 h, echinosporin (**1**) was isolated from the culture filtrate as described, and analysed by ^{13}C NMR spectroscopy. Specific incorporations were calculated according to Scott et al.,[10] ^{13}C-^{13}C coupling constants are given, where possible (Table 3).

The experiments started with feeding of sodium [1-^{13}C]-acetate. The resulting echinosporin (**1**) showed no incorporation, and so the polyketide pathway could be ruled out. In a follow-up experiment, [1,3-$^{13}C_2$]glycerol was fed, and this was incorporated, with enriched signals (3.5−5%) being seen for C-2, C-5, C-6, C-8, C-10 and C-11 (Table 3, Figure 2). To establish whether glycerol was incorporated intact or after metabolic cleavage, [U-$^{13}C_3$]glycerol was fed. The ^{13}C NMR spectrum of **1** showed intact incorporation of two C_3 units (C-5/C-4/C-10 and C-6/C-7/C-11) and of one C_2 unit (C-8/C-9). The intensity of the C-2 signal was increased, but no coupling was detectable. The origin of C-1 could not be clarified unequivocally by this experiment, because only a small increase in its signal intensity was evident. To rule out derivation of C-1 from C_1 sources, [methyl-^{13}C]methionine and sodium [^{13}C]formate were fed, but no significant enrichment of any carbon atom of **1** could be seen. The glycerol feeding experiments suggested that the whole carbon skeleton derived from glucose by the pentose-phosphate pathway. Confirmation of this was obtained by experiments with [1-^{13}C]glucose, which gave enrichments at C-5, C-6 and C-8 (Figure 2). The significant enrichment at C-5 suggested direct incorporation of [1-^{13}C]glucose as phosphoenolpyruvate (PEP) generated by glycolysis (Scheme 1). The weaker incorporation of [1-^{13}C]glucose at C-6 and C-8 was consistent with participation of a further PEP unit and of erythrose 4-phosphate (E4P), both derived from metabolism of glucose but involved in an intermediate biosynthesis that caused considerable dilution relative to C-5. The participation of PEP was confirmed by feeding of sodium [2-^{13}C]pyruvate; enrichment of C-4 and C-7 was detected.

To provide further information, additional experiments were conducted with [U-$^{13}C_6$]glucose. Its metabolism to PEP and E4P gave rise to a labelling pattern in **1** identical with that observed in the [U-$^{13}C_3$]glycerol experiment. Thus, C-5/C-4/C-10, C-6/C-7/C-11 and C-8/C-9 were found to be enriched and coupled. The origin of C-1 and C-2 from this source was also confirmed by this experiment, although

Table 3. ^{13}C-NMR analysis of enriched **1** after feeding of individual ^{13}C-labelled precursors (% specific incorporations (calculated according to Scott et al.[10]) or ^{13}C-^{13}C coupling constants)

	C-1	C-2	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-11
[1,3-$^{13}C_2$]Glycerol	0.94	3.58	0	5.04	3.5	0.48	3.42	0.80	4.86	4.76
[U-$^{13}C_3$]Glycerol (J [Hz])	[a]	[a]	74, 78	78	34	56, 34	68	68	74	56
[1-^{13}C]Glucose	0.14	0.12	0.08	3.21	1.81	0	1.66	0.14	0.22	0.24
[4-^{13}C]Glucose	0.01	3.33	0	0	0	0.16	0.09	0.14	2.15	2.35
[U-$^{13}C_6$]Glucose (J [Hz])	[a]	[a]	75, 80	5, 80	34	57, 34	68	68	5, 75	57
[1,7-$^{13}C_2$]Shikimic acid	0.45	0.02	0.66	0.08	0	1.81	0.06	0.34	0.76	1.41
J [Hz]	–	–	–	–	–	57	–	–	–	57
[2-^{13}C]Pyruvate	0.09	0.11	0.97	0	0.11	1.13	0.07	0.37	0.12	0.01

[a] Incorporation, but no coupling.

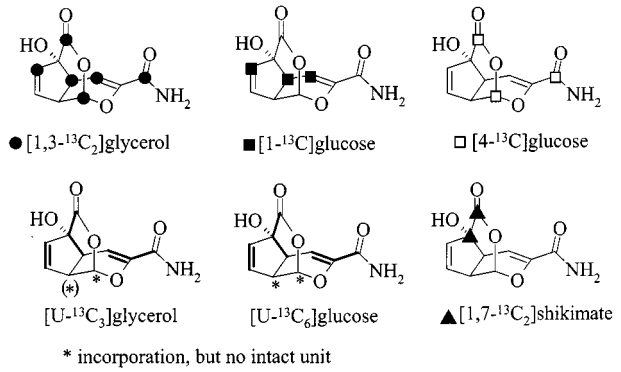
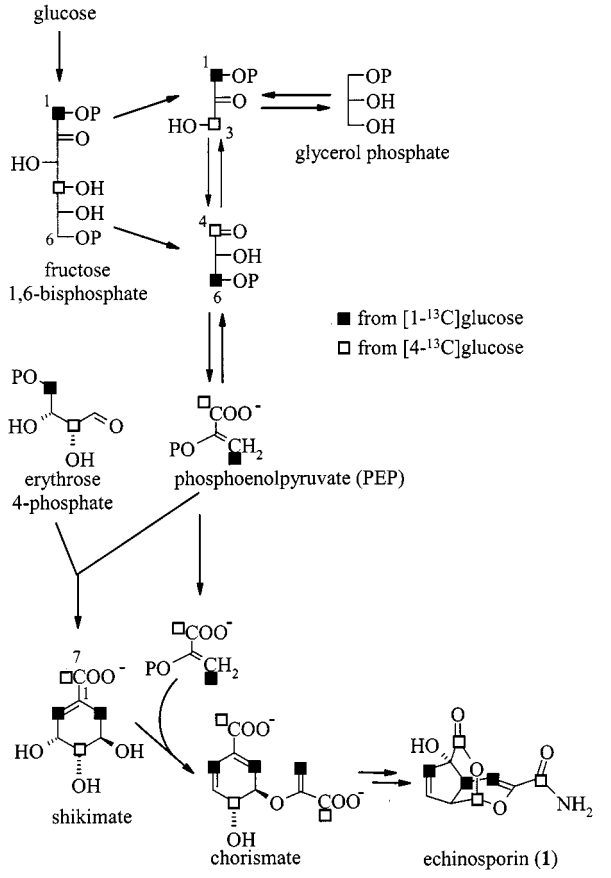


Figure 2. ^{13}C-labelling pattern of echinosporin (**1**) after feeding of various precursors



Scheme 1. Incorporation of [1-^{13}C]glucose and [4-^{13}C]glucose into echinosporin (**1**) via shikimate and chorismate

the levels of incorporation of [U-$^{13}C_6$]glucose into these positions were not high and no coupling was visible for these carbon atoms. The results suggested that echinosporin (**1**) could be a product of the shikimate pathway. This was corroborated by feeding [1,7-$^{13}C_2$]shikimic acid to the growing culture of strain Tü 4015. As expected, C-7 and C-11 were found to be enriched and coupled (Table 3, Figure 2).

The reported data unequivocally demonstrated that echinosporin had been produced by the shikimate pathway, from two molecules of PEP and the four-carbon unit erythrose 4-phosphate (Scheme 1). The incorporation of a second PEP into **1** implied that chorismic acid was a late intermediate in the biosynthesis. Feeding of [G-$^{13}C_{10}$]chorismic acid, which was isolated from the culture broth of *Klebsiella pneumonia* 62-1 after feeding of [U-$^{13}C_6$]glucose,[11] resulted in very low, not analysable enrichment. Chorismic acid probably did not penetrate into cells of strain Tü 4015, or it decomposed during the fermentation because of instability.

The erythrose 4-phosphate derived part of shikimic acid was not incorporated as an intact unit, because only C-8/C-9 of **1** retained their coupling after feeding of [U-$^{13}C_6$]glucose or [U-$^{13}C_3$]glycerol. This indicated that a rearrangement had taken place during a ring contraction from a six- to a five-membered carbocycle. To obtain more information about this unexpected reaction, we performed a further experiment with [4-^{13}C]glucose. Besides enrichment at positions C-10 and C-11, expected following glycolysis, high incorporation for C-2 was detected. This suggested the conclusion that C-2 in **1** corresponded to C-2 of erythrose 4-phosphate, and thus was derived from C-4 of shikimic acid (Scheme 1).

Feeding of Amino Acids and Glyphosate

The aromatic amino acids phenylalanine, tyrosine and tryptophan, as well as anthranilic acid, are products of the shikimate pathway with chorismate as their biosynthetic precursor.[12] Addition of these substances might make it possible to inhibit their de novo biosynthesis and thereby to accumulate chorismate in the culture broth. A higher concentration of chorismate as the potential precursor of echinosporin (**1**) should result in a higher yield of **1**. Addition of tryptophan resulted in a decreased yield of **1**, with solely an accumulation of anthranilic acid being observed. After feeding of 1 g/L anthranilic acid, the growth of Tü 4015 decreased and no **1** was detected, whereas addition of only 0.1 g/L anthranilic acid resulted in a slight increase in echinosporin production. A change in the metabolic pattern was observed after feeding of 1 g/L tyrosine. In addition to 60 mg/L **1** (as much as under standard conditions), 100 mg/L 7-deoxyechinosporin (**2**) were isolated. The addition of tyrosine is therefore an effective means to direct the biosynthesis towards **2** and to enhance the biosynthesis of the acetal-lactone skeleton twofold.

The enzyme inhibitor glyphosate inhibits plant EPSP synthase, which catalyses the conversion of shikimate 3-phosphate into 5-enolpyruvyl shikimate 3-phosphate (EPSP), the precursor of chorismate.[13] Complete inhibition of the microbial shikimate pathway is achieved by concentrations of 2 mM glyphosate, but some cyanobacteria tolerant to the inhibitor up to a concentration of 20 mM are known.[14]

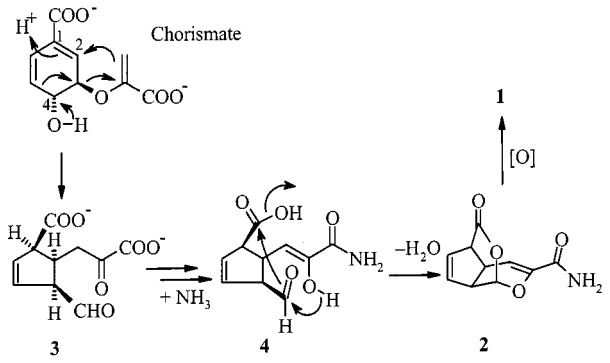
If echinosporin is formed through chorismate, inhibition of EPSP synthase should block the biosynthesis of **1**. Glyphosate was added to our standard culture medium in concentrations between 0.01 and 3 mM either at the beginning of the cultivation or after 30 h. Cell growth was not influenced by the inhibitor at all, and echinosporin production did not decrease. The addition of 0.06 mM surprisingly

enhanced the echinosporin yield twofold. Thus, our strain Tü 4015 seems to be tolerant to glyphosate. The reason for the increased yield of **1** is not understood.

Conclusion

From the data of our feeding experiments, the biosynthesis of echinosporin (**1**) obviously follows the shikimate pathway. The biosynthesis of **1** represents a new branch of this pathway, affording a nonaromatic secondary metabolite. There are only a few examples of nonaromatic compounds elaborated by this pathway. The participation of chorismate as an intermediate in the biosynthesis could not be proved unambiguously, but the incorporation of shikimic acid and of an additional PEP unit are strong hints in this direction.

From the results of the feeding experiments it is possible to propose a mechanism for the conversion of chorismate into 7-deoxyechinosporin (**2**). The absence of ^{13}C-^{13}C coupling between C-1 and C-9 in the echinosporin derived from [U-$^{13}C_3$]glycerol and [U-$^{13}C_6$]glucose, and the appearance of label from [4-^{13}C]glucose at C-2, indicate a rearrangement resulting in a ring-contraction from the six- to the five-membered carbocycle. The feeding experiments revealed a bond cleavage between C-3 and C-4 of shikimate, corresponding to C-4 and C-5 of chorismate. The proposed mechanism from chorismate to **2** is shown in Scheme 2. The process is probably highly concerted. Protonation at C-1 initiates a Michael addition of enolpyruvate at the C-1/C-2 double bond of chorismate. The enolpyruvyl moiety of chorismate simultaneously serves as the nucleophile for the stereocontrolled attack of C-3′ at C-2 and as the leaving group initiating the ring contraction by migration of C-5 to the backside of C-3, accompanied by deprotonation of 4-OH. The enzyme catalysing this reaction probably mediates the deprotonation/protonation steps and binds the chorismate molecule in a conformation favouring this rearrangement over other reaction paths. Thus, we propose a new type of chorismate mutase, using chorismate as a source of microbial secondary metabolites. A comparable reaction has been described as part of vitamin B_{12} biosynthesis.[15]



Scheme 2. Tentative mechanism for the conversion of chorismate to echinosporin (**1**)

Methylation of precorrin 3 hydroxylactone triggers a ring contraction through a pinacol-type rearrangement to precorrin 4.

The unstable intermediate **3** of the echinosporin pathway (Scheme 2) may be aminated in the next step. The enol **4** is the starting point for a cyclisation cascade resulting in 7-deoxyechinosporin (**2**). The hydroxylation at C-7 probably involves a monooxygenase that uses atmospheric oxygen. It would be desirable to isolate the echinosporin synthase, since it uses chorismate as substrate and thus represents the branchpoint from primary metabolism. Further intermediates should be helpful for clarification of the biosynthesis of **1** in detail.

Experimental Section

General: Melting points: Reichert hot-stage microscope (not corrected). ^1H and ^{13}C NMR spectra: Bruker AMX 300 (300 MHz), Varian Inova 500 (500 MHz). Chemical shifts are expressed in δ values (ppm), with the solvent as internal reference. The multiplicities of the ^{13}C NMR signals were assigned by the attached proton test (APT). EI-MS: Finnigan MAT 311 A, 70 eV, direct insert, high resolution with perfluorokerosine as standard. IR spectra: Perkin−Elmer Model 298 (KBr discs). UV spectra: Varian Cary 3E. Optical rotations: Perkin−Elmer 343. Fermentation: Braun BS4 (180 rpm, 28 °C). Column chromatography: Silica gel ICN SiliTech 32-63 (0.032−0.063 mm, ICN Biomedicals GmbH), Sephadex LH-20 (Pharmacia).

Labelled Precursors: Sodium [1-^{13}C]acetate (99% ^{13}C), sodium deuterio[^{13}C]formate (99% ^{13}C), L-[methyl-^{13}C]methionine (99% ^{13}C): Chemotrade; [1,3-$^{13}C_2$]glycerol (50% ^{13}C), [U-$^{13}C_3$]glycerol (99% ^{13}C), [1-^{13}C]glucose (99% ^{13}C), [U-$^{13}C_6$]glucose (99% ^{13}C), [4-^{13}C]glucose (99% ^{13}C), sodium [2-^{13}C]pyruvate (99% ^{13}C): Cambridge Isotope Lab (CIL). [1,7-$^{13}C_2$]shikimic acid (99% ^{13}C) was synthesised as described in the literature.[16] [G-$^{13}C_{10}$]chorismic acid (ca. 90% ^{13}C) was isolated from the culture broth of *Klebsiella pneumoniae* 62-1 after feeding of [U-$^{13}C_6$]glucose as described in the literature.[11]

Fermentation: *Streptomyces erythraeus* (Tü 4015) was grown on slants of medium A (malt extract 10 g/L, glucose 4 g/L, yeast extract 4 g/L, $CaCO_3$ 0.2 g/L, deionised water, pH = 7.0 prior to sterilisation). A 1-cm^2 piece of agar from 7 d old cultures was used to inoculate 100 mL of medium B [soybean meal 20 g/L, sodium gluconate 20 g/L, NaBr 7 g/L, buffer: KH_2PO_4 24 g/L or HEPES 23.8 g/L, pH = 7 (addition of NaOH) prior to sterilisation] in 300-mL Erlenmeyer flasks with three indentations. These cultures were incubated on a rotary shaker (180 rpm, 28 °C) for 72 h.

Feeding Experiments: Feeding experiments were carried out under the conditions described above. In general, precursors were administered to the fermentation as sterile aqueous solutions adjusted to pH = 7.0. Pulse feeding procedures were followed in all cases, with administration of the precursor at 48, 50, 52, 54 and 56 h. The following amounts were added: sodium [1-^{13}C]acetate, 10.8 mmol L^{-1}; sodium [^{13}C]formate, 4.3 mmol L^{-1}; L-[methyl-^{13}C]methionine, 1.2 mmol L^{-1}; [1,3-$^{13}C_2$]glycerol, 6.5 mmol L^{-1}; [U-$^{13}C_3$]glycerol, 6.7 mmol L^{-1}; [1-^{13}C]glucose, 5.8 mmol L^{-1}; [U-$^{13}C_6$]glucose, 5.8 mmol L^{-1}; [4-^{13}C]glucose, 3.6 mmol L^{-1}; [1,7-$^{13}C_2$]-shikimic acid, 0.58 mmol L^{-1}; sodium [2-^{13}C]pyruvate, 3.5 mmol L^{-1}, [G-$^{13}C_{10}$]chorismic acid, 2.0 mmol L^{-1}.

Isolation and Purification: The culture broths were separated from the mycelia by centrifugation (4500 rpm, 15 min). The mycelium was discarded. The solutions obtained were acidified to pH = 4.0 with acetic acid and extracted three times with equal volumes of ethyl acetate. The combined organic phases were concentrated to dryness. The obtained crude material was purified by chromatography on silica gel, with dichloromethane/methanol (19:1) as eluent. The main fractions were further purified on Sephadex LH-20 (methanol) to yield 50−80 mg/L of **1** and 1−3 mg/L of **2**.

Addition of Amino Acids and Glyphosate: The strain was cultivated as described above. The amino acids tryptophan and tyrosine were added to the culture medium after sterilisation in amounts of 1 g/L, anthranilic acid in amounts of 0.1 g/L and 1 g/L. Glyphosate (Fluka) was added as an aqueous solution after 24 h cultivation in amounts of 0.01 mmol L^{-1}, 0.06 mmol L^{-1}, 0.59 mmol L^{-1} and 2.96 mmol L^{-1}, and at the beginning of the cultivation in amounts of 0.06 mmol L^{-1} and 0.59 mmol L^{-1}.

Echinosporin (1): Colourless solid, m.p. 260 °C (ref.[2] 260 °C). R_f = 0.27 (CH_2Cl_2/MeOH, 9:1). $[\alpha]_D^{20}$ = −400 (*c* = 0.1, MeOH) (ref.[2] −400). IR (KBr): $\tilde{\nu}$ = 3435 cm^{-1}, 3313, 3171, 1743, 1689, 1652, 1603, 1406, 1324, 1181, 1003, 917. UV (CH_3OH): λ_{max} (log ε) = 220 (3.93). EI-MS: *m/z* (%) = 223 (20) $[M]^+$, 205 (9) $[M - H_2O]^+$, 195 (38) $[M - CO]^+$, 178 (32) $[M - CHO_2]^+$, 151 (26), 134 (30), 107 (100). HREIMS: found as calcd. for $C_{10}H_9NO_5$: 223.0481. ^1H and ^{13}C NMR spectroscopic data see Table 1.

7-Deoxyechinosporin (2): Colourless solid, m.p. 200 °C. R_f = 0.29 (CH_2Cl_2/MeOH, 9:1). $[\alpha]_D^{20}$ = −420 (*c* = 0.65, MeOH). IR (KBr): $\tilde{\nu}$ = 3440 cm^{-1}, 1752, 1683, 1650, 1400, 1319, 1199, 992, 965. UV (CH_3OH): λ_{max} (log ε) = 214 (3.98). EI-MS: *m/z* (%) = 207 (10) $[M]^+$, 151 (65), 106 (100). HREIMS: found as calcd. for $C_{10}H_9NO_4$: 207.0532. ^1H and ^{13}C NMR spectroscopic data see Table 1.

Acknowledgments

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