Pergamon

Tetrahedron Letters 40 (1999) 2489–2492

TETRAHEDRON LETTERS

Spiroxins, DNA Cleaving Antitumor Antibiotics from a Marine-Derived Fungus

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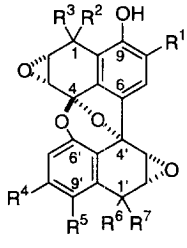
Received 10 December 1998; accepted 25 January 1999

Abstract: The spiroxins (**1-5**) were purified from the culture extract of a marine-derived fungus. Their unique bisnaphthospiroketal structures were established by NMR spectroscopy. In addition to cytotoxicity, these compounds showed antibiotic activity and were active in a mouse xenograft model against human ovarian carcinoma. The mechanism of action of these compounds was shown to be due, in part, to their effect on DNA. © 1999 Elsevier Science Ltd. All rights reserved.

Among the variety of biosynthetically related unusual bisnaphthospiroketal compounds appearing in the literature,[1,2,3] the most notable are the bisspiroketal-containing preussomerins, which have reportedly shown antibacterial/antifungal[4,5] and ras farnesyl-protein transferase[6] inhibitory activities. As part of our ongoing efforts to find novel bioactive natural products, we have discovered a series of related, apparently highly strained compounds from the extract of a marine-derived fungus. The extract showed activity *in vitro* in a 25 cell line cytotoxicity assay and subsequent bioassay-directed purification followed by structure elucidation revealed a series of active compounds with an unusual octacyclic ring system. Here we report the structures, activities, and mechanism of action of this class of natural product compounds possessing a unique bisnaphthospiroketal octacyclic ring system. The structure of one of these compounds (**2**) was recently published in a patent application.[7]

Fungal strain LL-37H248 was isolated from a soft orange coral collected from the waters of Dixon Bay, Vancouver Island, Canada. As the culture was non-sporulating, it could not be taxonomically classified.[8] Cultivation of the fungus in a liquid medium[9] containing suspended HP20 resin yielded high titers of the spiroxins. Production rates were approximately 35-fold greater in the presence of HP20 than in fermentations without the resin. Bioassay-directed purification led to spiroxins A (**1**), B (**2**), C (**3**), D (**4**), and E (**5**). The adsorbed compounds were eluted from the HP20 resin by acetone. The crude complex was subsequently chromatographed on silica gel and C_{18} reverse phase supports to yield pure compounds **1-5**. Spiroxin A was the major component produced in culture.



1: R^1=Cl; R^2+R^3=O; R^4=H; R^5=OH; R^6+R^7=O

2: R^1=Cl; R^2+R^3=O; R^4=Cl; R^5=OH; R^6+R^7=O

3: R^1=H; R^2+R^3=O; R^4=H; R^5=H; R^6+R^7=O

4: R^1=H; R^2=OH; R^3=H; R^4=H; R^5=H; R^6+R^7=O

5: R^1=Cl; R^2+R^3=O; R^4=Cl; R^5=OH; R^6=OH; R^7=H

PII: S0040-4039(99)00242-7

The structure of the spiroxins may be described as two partially saturated naphthalene rings joined together by spiroketal and carbon-carbon bond linkages. The saturated portion of each naphthalene ring is fused with an epoxide resulting in an unusual octacyclic ring system. These structural features are common to all naturally occurring spiroxins with the various analogs differing in their levels of halogenation or oxidation. Spiroxin A (**1**)[10] did not readily ionize in the positive electrospray MS, suggesting the presence of acidic functional group(s). Negative electrospray MS did, however, show a peak at *m/z* 410.9 $(M-H)^-$ with a corresponding 1/3 intensity peak at *m/z* 412.9 indicative of one chlorine atom. High resolution EI MS measurements allowed determination of the molecular formula as $C_{20}H_9O_8Cl$. Twenty carbon-13 and nine proton resonances corroborated the molecular formula which requires sixteen degrees of unsaturation. The presence of two epoxide rings in compound **1** was evident from the two pairs of coupled proton doublets resonating between 4.0 and 4.6 ppm and the four high field oxygen-bearing carbon resonances between 51 and 54 ppm in the DMSO-d_6 spectra. The presence of the chromophoric *o*-hydroxybenzoyl groups is supported by sharp, hydrogen-bonded, exchangeable proton singlets at 9.7 and 10.3 ppm and UV absorbances at 265 and 382 nm. IR bands at 3370 and 1683 & 1664 cm^{-1} lent additional support for the hydroxyl groups and conjugated ketones, respectively. The proposed structure for **1** is derived from these data and from extensive analysis of one- and two-dimensional NMR data including DEPT, HMQC, HMBC and NOESY. Figure 1 shows the HMBC correlations supporting the structure assignments for **1**.[10] Each arrow represents an observed ^1H-^{13}C correlation and account for the corresponding bonds drawn between the hydrogen and carbon atoms. Most notable is the correlation between H7 and C4' which establishes the bond between C4' and C6, the only through-bond correlation between the upper and lower ring assemblies. Two important NOESY correlations observed between the epoxide hydrogen atoms H2' & H3' and the aromatic singlet H7 established the relative stereochemistry of **1** (Fig. 2).

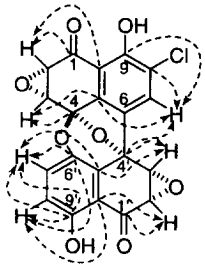


Fig. 1. Long-range ^{13}C-^1H coupling (HMBC ⋯→) correlations for spiroxin A (**1**)

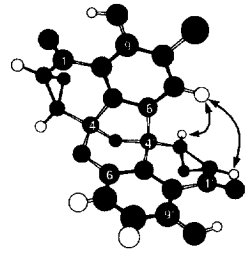


Fig. 2. Model of spiroxin A (**1**) showing NOE (↔) correlations and relative stereochemistry

Analogous interpretation of the physico-chemical data also allowed structure assignments of spiroxins B(**2**),[11] C(**3**),[12] D(**4**),[13] and E(**5**).[14] Spiroxin B(**2**)[11] shows a molecular ion at *m/z* 444.8. Its characteristic pattern indicates the presence of two chlorine atoms. Two aromatic singlets, four epoxide, and two exchangeable signals in the ^1H-NMR spectrum established the structure of **2**, which was completely supported by ^{13}C-NMR and other data. Spiroxin C(**3**)[12] produced a molecular ion at *m/z* 360.9 and showed

NMR signals of aromatic protons consistent with tetra- and tri-substituted aromatic rings where the substituents are contiguous. Additionally, only one exchangeable proton signal was observed in $CDCl_3$, indicating the presence of only one hydroxyl group. Spiroxin D(**4**)[13] differs from compound **3** only in the oxidation state of the C1 carbon. Carbon-13 NMR indicates that the C1 ketone (194.2 ppm) in **3** is reduced to a hydroxyl (67.5 ppm) in **4**. A COSY experiment defined a spin network consisting of two epoxide protons (3.92, d, J = 4.2 Hz and 3.82, dd, J = 4.2, 1.8 Hz) and a methine (5.48, d, J = 1.8 Hz) on an oxygen-bearing carbon. A weak NOE correlation between H1 and the C9-OH protons supported the placement of this moiety in the upper ring assembly (C1-C10). Additionally, similar to the observation made for compound **1**, both H2' and H3' protons show NOE correlations to H7, placing these epoxide protons on the lower ring assembly (C1'-C10'). Spiroxin E(**5**)[14] is the C1' carbonyl-reduced analog of **2**. Both the mass (*m/z* 446.8) and pattern of the molecular ion are consistent with two chlorine atoms. Correlations in the NOESY spectrum are observed between H7 (7.98, s), one of the two aromatic singlets, and both H3' (3.99, d, J = 4.3 Hz) and H2' (3.90, dd, J = 4.3, 1.4 Hz), thereby placing these epoxide protons in the lower ring assembly. The H2' epoxide proton, in addition to coupling to H3', shows COSY coupling to a methine proton (5.67 , dd, J = 1.9 Hz) on C1'.

Spiroxin A (**1**) showed some activity against Gram-positive bacteria but only marginal activity against Gram-negative bacteria. Compound **1** showed antitumor activity in nude mice against ovarian carcinoma (59% inhibition after 21 days) at 1 mg/kg/dose given IP on days 1, 5, and 9 post staging. In a cytotoxicity assay, **1** exhibited a mean IC_{50} value of 0.09 μg/mL against a panel of 25 diverse cell lines. Based on an algorithm to compare mean bar graph profiles,[15] **1** was similar to other quinone epoxides such as terreic acid and frenolicin.

In evaluating its probable mechanism of action, it was observed that in the presence of either DTT or 2-mercaptoethanol, **1** caused a concentration-dependent nicking of pBR322 DNA, suggesting that the compound partly exerts its cytotoxic effect through a single-stranded DNA cleavage. Cytotoxicity of quinones has been attributed to DNA modification, alkylation of essential protein thiol groups, oxidation of essential protein thiol groups by superoxide radicals or a combination of these mechanisms.[16] The oxidation state of the spiroketal carbon, a masked ketone, could allow the spiroxins to behave chemically as quinone epoxides, possibly causing DNA cleavage under reducing conditions via an oxidative stress mechanism involving the formation of thiol conjugates. LC/MS experiments have in fact demonstrated that **1** reacts with 2-mercaptoethanol and dithiothreitol forming conjugates. Thus, a variety of mechanisms may play a role in spiroxin-mediated cytotoxicity.

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8. This is due to the lack of teleomorph morphology. The culture would be included as a member of the class Deuteromycetes or Fungi Imperfecti.

9. Difco potato dextrose broth, pH 7.0.

10. Spiroxin A (**1**): $C_{20}H_9O_8Cl$; ES MS *m/z* 410.9 $(M-H)^-$; HR EI MS *m/z*411.9979 $(M)^+$; $[\alpha]^{25}_D$ -644° ± 4 (*c* 0.1828, MeOH); UV λ_{max} nm (ε) (MeOH) = 265 (14,087), 382 (5,597); IR: ν_{max} cm^{-1} (film) 3370, 1683, 1664(s), 1617, 1465, 1442, 1347, 1320, 1229; ^{1}H-NMR: (300 MHz DMSO-d_6) δ 8.22 (s, 1H, H7), 7.13 (d, 1H, *J* = 9.1 Hz, H7'), 6.91 (d, 1H, *J* = 9.1 Hz, H8'), 4.03 (d, 1H, *J* = 3.8 Hz, H2), 4.02 (d, 1H, *J* = 4.1 Hz, H2'), 4.59 (d, 1H, *J* = 3.8 Hz, H3), 4.44 (d, 1H, *J* = 4.1 Hz, H3'), 10.42 (s, 1H, 9'-OH), 10.73 (bs, 1H, 9-OH); ^{13}C-NMR: (75 MHz, DMSO-d_6, ppm downfield from TMS) 193.9 (1'), 190.5 (1), 154.2 (9'), 152.7 (9), 140.9 (6'), 137.6 (5), 133.8 (6), 127.9 (7), 125.7 (7'), 124.3 (8), 123.2 (5'), 118.8 (8'), 111.4 (10), 109.3 (10'), 103.5 (4), 83.7 (4'), 53.5 (2), 53.3 (2'), 51.5 (3), 51.4 (3').

11. Spiroxin B (**2**): $C_{20}H_8O_8Cl_2$; ES MS *m/z* 444.8 $(M-H)^-$; $[\alpha]^{25}_D$ -475° ± 4 (*c* 0.2104, MeOH); UV λ_{max} nm (ε) (MeOH) = 265 sh (11,627), 385 (6,220);IR: ν_{max} cm^{-1} (film) 1664, 1607, 1441, 1346, 1328, 1244; ^{1}H-NMR: (300 MHz DMSO-d_6) δ 8.21 (s, 1H, H7), 7.44 (s, 1H, H7'), 4.02 (d, 1H, *J* = 3.9 Hz, H2), 4.10 (d, 1H, *J* = 4.1 Hz, H2'), 4.59 (d, 1H, *J* = 3.9 Hz, H3), 4.49 (d, 1H, *J* = 4.1 Hz, H3'), 10.84 (bs, 1H, 9'-OH); ^{13}C-NMR: (75 MHz, DMSO-d_6, ppm downfield from TMS) 198.2 (1'), 193.8 (1), 153.0 (9'), 157.1 (9), 144.0 (6'), 137.0 (5), 140.7 (6), 131.0 (7), 128.0 (7'), 127.9 (8), 125.8 (5'), 125.0 (8'), 114.8 (10), 113.7 (10'), 103.20 (4), 86.5 (4'), 56.1 (2), 56.2 (2'), 54.2 (3), 54.0 (3').

12. Spiroxin C (**3**): $C_{20}H_{10}O_7$; ES MS *m/z* 360.9 $(M-H)^-$; $[\alpha]^{25}_D$ -706° ± 4 (*c* 0.256, MeOH); UV λ_{max} nm (ε) (MeOH) = 256 (13,184), 329 (4,688); IR: ν_{max} cm^{-1} (film) 3390, 1694, 1675, 1601, 1476, 1460, 1353, 1318, 1300; ^{1}H-NMR: (300 MHz $CDCl_3$) δ 7.30 (d, 1H, *J* = 8.4 Hz, H7), 6.94 (d, 1H, *J* = 8.4 Hz, H8), 7.02 (dd, 1H, *J* = 8.0, 1.2 Hz, H7'), 7.33 (dd, 1H, *J* = 8.4, 8.0 Hz, H8'), 7.46 (dd, 1H, *J* = 8.0, 1.2 Hz, H8'), 3.98 (d, 1H, *J* = 3.9 Hz, H2'), 3.82 (d, 1H, *J* = 3.5 Hz, H2), 4.29 (d, 1H, *J* = 3.5 Hz, H3), 4.13 (d, 1H, *J* = 3.9 Hz, H3'), 9.27 (s, 1H, 9-OH); ^{13}C-NMR: (75 MHz, $CDCl_3$, ppm downfield from TMS) 191.3 (1'), 194.2 (1), 119.8 (9'), 159.7 (9), 149.3 (6'), 139.2 (5), 134.1 (6), 127.1 (7), 122.5 (7'), 119.5 (8), 124.9 (5'), 130.5 (8'), 109.1 (10), 125.7 (10'), 103.6 (4), 84.7 (4'), 53.2 (2), 53.5 (2'), 52.4 (3), 51.7 (3').

13. Spiroxin D (**4**): $C_{20}H_{12}O_7$; Molecular Weight: CI MS = *m/z* 365.2 $(M+H)^+$; EI MS = *m/z* 364.0 $(M)^+$; ^{1}H-NMR: (300 MHz MeOH-d_4) δ 7.36 (dd, 1H, *J* = 7.8, 1.8 Hz, H9'), 7.32 (t, 1H, *J* = 7.8 Hz, H8'), 7.05 (d, 1H, *J* = 8.1 Hz, H7), 7.02 (dd, 1H, *J* = 7.8, 1.8 Hz, H7'), 6.75 (d, 1H, *J* = 8.1 Hz, H8), 5.48 (d, 1H, 1.8 Hz, H1), 4.15 (d, 1H, *J* = 4.2 Hz, H3'), 3.95 (d, 1H, *J* = 4.2 Hz, H2'), 3.92 (d, 1H, *J* = 4.2 Hz, H3), 3.82 (dd, 1H, *J* = 4.2, 1.8 Hz, H2); ^{13}C-NMR: (75 MHz, MeOH-d_4, ppm downfield from TMS) 193.4 (1'), 158.3 (9), 151.3 (6'), 136.0 (5), 135.8 (6), 131.0 (8'), 127.8 (10'), 127.0 (5'), 123.2 (7'), 120.2 (7), 119.8 (9'), 119.0 (8), 116.8 (10), 107.1 (4), 85.1 (4'), 67.5 (1), 56.4 (2), 54.6 (3'), 53.3 (2'), 51.5 (3).

14. Spiroxin E (**5**): $C_{20}H_{10}O_8Cl_2$; ES MS *m/z* 446.8 $(M-H)^-$; ^{1}H-NMR: (300 MHz DMSO-d_6) δ 7.98 (s, 1H, H7), 6.89 (s, 1H, H7'), 5.67 (d, 1H, *J* = 1.9 Hz, H1'), 7.59 (bs, 1H, 1'-OH), 3.90 (dd, 1H, *J* = 4.6, 1.9 Hz, H2'), 3.97 (d, 1H, *J* = 3.9 Hz, H2), 4.52 (d, 1H, *J* = 3.9 Hz, H3), 3.99 (d, 1H, *J* = 4.6 Hz, H3'), 9.59 (s, 1H, 9'-OH), 10.64 (s, 1H, 9-OH); ^{13}C-NMR: (75 MHz, DMSO-d_6, ppm downfield from TMS) 66.0 (1'), 190.7 (1), 146.4 (9'), 152.5 (9), 141.1 (6'), 137.6 (5), 134.2 (6), 127.0 (7), 124.2 (8), 120.4 (8'), 118.9 (5'), 117.2 (10'), 116.5 (7'), 111.5 (10), 103.1 (4), 84.8 (4'), 54.0 (2'), 53.4 (2), 51.5 (3), 49.9 (3').

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