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Novel Thiodiketopiperazine Fungal Metabolites As Epidermal Growth Factor Receptor Antagonists

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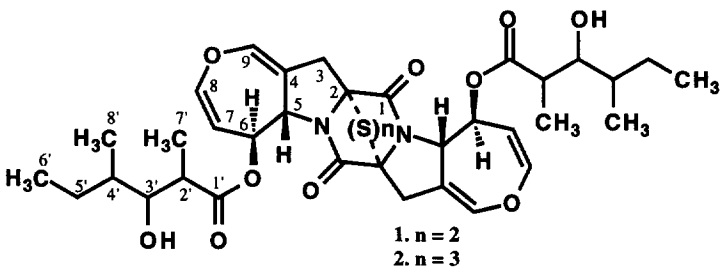
Abstract: The organic extract of the fermentation broth of an unidentified fungus was found to contain three epidermal growth factor (EGF) receptor antagonists: SCH 64874 (**1**), SCH 64875 (**2**), SCH 64877 (**3**). These compounds belong to a family of thiodiketopiperazine derivatives and are esters of an eight carbon aliphatic acid, 2,4-dimethyl-3- hydroxyhexanoic acid and a thiodiketopiperazine, aranotin. They exhibited potent EGF receptor antagonist activity. The IC_{50} in EGF receptor assays for compounds **1, 2** and **3** are 1.0, 1.0 and 1.25 μg/ml, respectively. © 1997, Elsevier Science Ltd. All rights reserved.

As part of our continuing investigation of natural products as lead compounds for new antitumor agents, we screened ethyl acetate extracts of fermentation broths. A broth was identified that displayed distinct activity in the epidermal growth factor receptor assay. Bioassay-guided fractionation of this extract led to the isolation of three compounds, the first examples of thiodiketopiperazines as EGF receptor antagonists.

A 3.4 liter fermentation broth was adjusted to pH 6.5 with dilute sulfuric acid and extracted twice with 7 L of ethyl acetate. The organic layers were combined, dried over anhydrous Na_2SO_4 and the solvent removed. The solids from this gummy extract containing oil were precipitated by dissolving the extract in dichloromethane and adding the solution to hexane. The resulting precipitate was filtered and dried to yield 412 mg of solid containing the EGF receptor antagonists. This precipitate (250 mg) was loaded on a silica gel column (1 X 10") packed in toluene and eluted with 1% methanol in toluene. The elution of the active compounds was monitored by activity in the EGFR assay. The fractions containing mixtures of EGF receptor active compounds were collected and dried to yield 62 mg of enriched complex. Separation of these compounds was achieved by reverse phase preparative HPLC on a Water's Deltapak C-18 silica column (1.9 X 30 cm), eluting with a mixture of acetonitrile and water (1:1 v/v). Acetonitrile was removed from the individual peak eluates, and the aqueous solutions were freeze dried to yield 25, 3.5 and 3.5 mgs of **1**, **2** and **3**, respectively.



All of the isolated EGF receptor active compounds were thiodiketopiperazines with a bridge containing multiple sulfur atoms across the piperazine ring. The FAB mass spectrum of SCH 64874 (**1**) showed m/z 641, 481, and 319. However Cz+ ion liquid secondary ionization mass spectrum in the presence of KCl displayed a molecular ion at m/z 743 $(M+K)^+$, revealing the molecular weight to be 704. The

molecular formula of **1** was established as $C_{34}H_{44}N_2O_{10}S_2$ by HRMS.[1] The UV spectrum (MeOH) displayed maxima at 205 and 230 nm and the IR spectrum in KBr showed peaks at 3515, 2965, 1719 and 1652 cm^{-1}, suggesting the presence of -OH or -NH, ester and amide groups. Evaluation of the spectral information in conjunction with published literature suggested the presence of a thiodiketopiperazine moiety in the molecule. ^1H and ^{13}C NMR chemical shifts of **1- 3** are tabulated in Table 1.

Table 1

^1H and ^{13}C NMR Chemical Shifts for **1-3**

C #	1		2			3	
	^{13}C	^1H	^{13}C		^1H	^{13}C	^1H
1	162.2		163.5	164.2		165.9	
2	75.2		77.2			75.5	
3	33.4	2.96 (dt, J=1.8, 18.2 Hz) 4.12 (dq, J=18.3, 1.2 Hz)	40.7	39.3	3.7 (d, J = 18 Hz) 2.9 (d, J=18 Hz)	41.5	3.28 (dd,J=15,1 Hz) 3.11 (d, J=15 Hz)
4	112.6		109.6	109.2		108.0	
5	62.4	5.12 (dq, J= 8.5 Hz)	62.2	60.9	5.4 (s)	61.0	5.30(s)
6	69.4	5.72 (dt, J=8.6, 2 Hz)	70.8	71.9	5.78 (d, J=8, 1 Hz, 0.5H) 5.05 (dd, J=8,1 Hz, 0.5H)	70.9	5.30(s)
7	104.6	4.56 (dd, J=1.7, 8.2 Hz)	106.6	105.7	4.6 (dd, J=8, 1 Hz)	105.8	4.6 (d, J=10 Hz)
8	141.0	6.34 (dd, J= 2, 8.3 Hz)	140.5		6.3(d, J=8 Hz)	139.9	6.28 (d, J= 8 Hz)
9	139.1	6.65 (q, J= 2 Hz)	139.3	138.6	6.55 (s)	138.9	6.60(s)
1'	175.0		175.6			175.8	
2'	41.1	2.67 (dq, J=7.1Hz, 1.8 Hz)	42.0	41.7	2.72 (dt, J= 8, 2 Hz)	41.9	2.8 (dq, J= 8,2 Hz)
3'	73.3	3.72 (dd, J=1.8, 9.6 Hz)	73.7		3.85 (dd)	73.9	3.81 (dd, J=10,1 Hz)
4'	36.0	1.49 (m)	36.9	36.5	1.5 (bs)	36.8	1.5(m)
5'	25.0	1.15-1.24 (m) 1.84 (m)	25.4	25.4	1.15-1.3 (m) 1.83 (m)	25.3	1.10-1.13(m) 1.85 (dq, J= 8,2 Hz)
6'	10.4	0.94 (t, J=7.4Hz)	10.9		0.94 (t, J=8 Hz)	10.9	0.95 (t, J=8 Hz)
7'	8.3	1.17 (d, J=7.2 Hz)	9.1		1.2 (d, J=8 Hz, 1.5H) 1.24 (d, J=8 Hz, 1.5H)	9.1	1.28 (d, J=8Hz)
8'	14.4	0.83 (d, J=6.7 Hz)	14.9		0.83 (d, J=8 Hz)	14.9	0.84 (d, J=8 Hz)

The ^1H NMR spectrum in $CDCl_3$ showed only 22 proton signals and the ^{13}C NMR also showed only 17 carbon signals revealing that the compound has two-fold symmetry. The molecular formula showed 14 degrees of unsaturation. ^1H, ^{13}C NMR and 2D(^1H-^1H), HETCOR and NOESY spectra were analyzed and indicated the presence of the aranotin moiety.[2-5] The NMR spectrum also showed the presence of an aliphatic chain. The identity of the piperazine portion of this molecule with that of aranotin was established from the chemical shifts and splitting patterns of the proton signals. The quartet signal at δ6.65(H-9) showed allylic coupling with the H-5 (δ5.12) methine and the H-3 (δ 2.96 & 4.12) methylene protons. Whereas the peak at δ6.34 (H-8) showed vinylic coupling with H-7 proton (δ 4.56) and allylic coupling with H-6 proton (δ5.72). These assignments were

Table 2:
SINEPT NMR Correlation Spectral Data for Compound 1

#	Irradiation of the Peak at	Peak signals observed
1	4.56 (H-7)	C-5, C-6, C-8
2	5.12 (H-5)	C-2, C-4, C-6, C-9
3	5.72 (H-6)	C-4, C-5, C-7, C-8, C-1'
4	6.34 (H-8)	C-6, C-7, C-9
5	6.65 (H-9)	C-4, C-5, C-8
6	4.12 (H-3)	C-1, C-2, C-4, C-9
7	3.72 (H-3')	C-1', C-4', C-5', C-7'
8	2.96 (H-3)	C-1, C-2, C-4, C-9
9	2.67 (H-2')	C-1', C-7'
10	1.49 (H-4')	C-3', C-5', C-6', C-8'
11	1.17 (H-7')	C-1', C-2', C-3', C-4', C-8'
12	0.83 (H-8')	C-3', C-4', C-5'

further supported by the 2D(^{1}H-^{1}H) (Figure 1) & HETCOR correlation studies and SINEPT experiments (Table 2). ^{13}C NMR also suggested C-6 must be an oxygenated carbon with -OH or an ester. The ^{1}H chemical shifts of the dithiodiketo- piperazine moiety were also similar with that of aranotin.[2]

The structure of aliphatic portion was very straightforward. The remainder of the molecular formula $C_8H_{15}O_2$, obtained by deducting the aranotin portion, indicated one unsaturation which was accounted for by the ester carbonyl. The structure of the aliphatic portion was established by coupling patterns of the adjacent proton signals and was further supported by the 2D(^{1}H-^{1}H) correlation spectrum (Figure 1). ^{1}H and ^{13}C long range correlations observed in the SINEPT experiments confirmed the structure as **1**. Table (2) shows the significant ^{1}H and ^{13}C long range correlations observed in the SINEPT spectrum and the ^{1}H-^{1}H COSY correlation for **1**. Thus **1** is an ester of aranotin and 2,4-dimethyl-3-hydroxyhexanoic acid. The attachment of the aliphatic portion to the dithio-diketopiperazine must be at the hydroxy group on C-6 as shown in **1**. This structure accounts for the fragment ions observed in the mass spectrum (Figure 2).

The molecular formula obtained from HRMS for **2**[1] showed an additional sulfur atom compared to **1**. The ^{13}C NMR indicated several extra ^{13}C signals (Table 1) probably due to the destruction of symmetry by the additional sulfur atom in the bridge. Compound **3**[1] contained two additional sulfur atoms compared to **1**, as determined from HRMS, revealing a four sulfur atom bridge. The spectral data of this compound was similar to that of **1**.

Compounds **1-3,** showed inhibition in the EGF receptor assay with IC_{50} of 1.0, 1.0, and 1.2 μg/ml respectively.

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1. Spectral data for SCH 64874 (**1**): $[\alpha]_D^{21.5}$: -301.1°; IR (KBr) ν max : 3515, 2965, 2935, 2877, 1719, 1652, 1456, 1351, 1141 cm^{-1}; FAB: m/z 743 $(M+K)^+$; HRMS: m/z 743. 2118, calc for $C_{34}H_{44}N_2O_{10}S_2K$ 743.2075.

SCH 64875 (**2**): $[\alpha]_D^{21.5}$: -232.4°; UV (MeOH) λ max : 230 nm; IR (KBr) ν max: 3530, 3425, 2964, 1702,

1650, 1365, 1135 cm^{-1}; FAB : m/z 775 $(M+K)^+$; HRMS : m/z 775.1820 calc for $C_{34}H_{44}N_2O_{10}S_3K$ 775.1795.

SCH 64877 (**3**): $[\alpha]_D^{21.5}$: -165°; UV (MeOH) λ max : 230 nm; IR(KBr) ν max : 3515, 3429, 2964, 1722, 1692, 1651, 1371, 1135 cm^{-1}.; FAB: m/z 807 $(M+K)^+$; HRMS: m/z 807.1529 calcd for $C_{34}H_{44}N_2O_{10}S_4K$ 807.1516.

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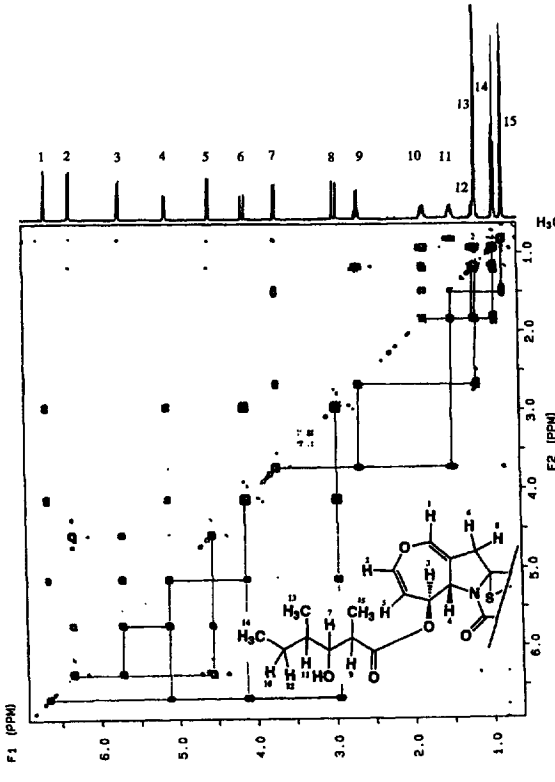
Figure 1

2D(^{1}H-^{1}H) correlation spectrum of **1**

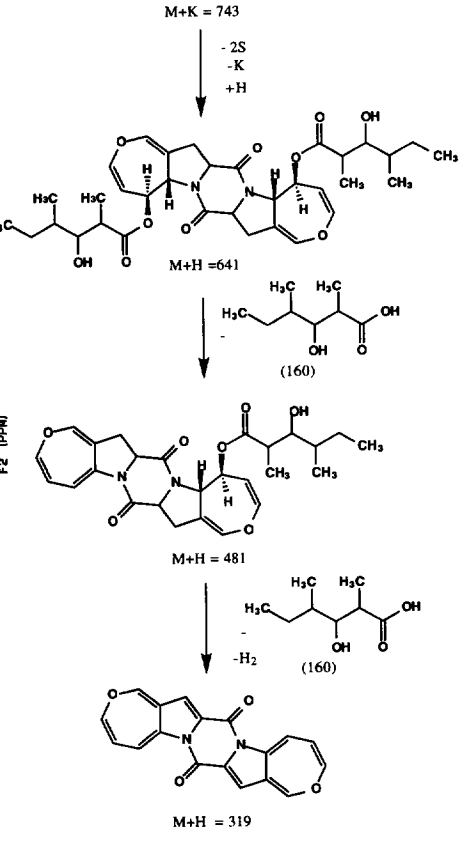


* #s on protons in the structure refers to signals

Figure 2

Interpretation of Mass Spectral Fragments

1



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