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Structure of Sch 68631: A New Hepatitis C Virus Proteinase Inhibitor from *Streptomyces* sp.

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Abstract: A novel hepatitis C virus (HCV) proteinase inhibitor, Sch 68631 (**1**), was isolated from the fermentation culture broth of *Streptomyces* sp. The structure of **1** was elucidated by analyses of spectroscopic data and shown to be a new member of the phenanthrenequinone family of compounds.

Hepatitis C virus (HCV) is identified as the major etiological agent of non-A non-B (NANB) hepatitis, which causes chronic liver disease and hepatocellular carcinoma (HCC) worldwide.[1-4] The viral infection accounts for more then 90% of transfusion associated hepatitis in United States and it is the predominant form of hepatitis in adults over 40 years of age. Interferon is the only available therapy for treatment of HCV infection, however the response rate is limited.[5] Therefore, the development of an effective therapeutic agent is important to control this public health problem by complementing α-interferon therapy.

HCV contains a positive strand RNA genome comprising approximately 9500 nucleosides that encodes a single large polyprotein of ~3000 amino acids which undergoes proteolysis to form mature viral protein in infected cells.[6,7] The polyprotein is cleaved at multiple sites by cellular and viral proteinases to produce the putative structural and nonstructural (NS) proteins. Experiments further suggest that the proteolytic processing of the nonstructural proteins is carried out by the viral proteinase NS3 along with another proteinase NS2-3 contained within the viral polyprotein.[8,9] Since the HCV NS3 proteinase, a member of the serine class of enzymes, plays an important role in cleaving the nonstructural HCV proteins which are necessary for HCV replication, the NS3 proteinase is considered an attractive target for the development of therapeutic agents against HCV infections.

During the search for new HCV proteinase inhibitors as part of our antiviral natural product research program, a novel secondary metabolite, Sch 68631 (**1**), was discovered from microorganisms identified as a *Streptomyces* sp. (culture 94-02747), which was isolated from a loam soil in a wooded area of Nepal with RP soil isolation media.[10]

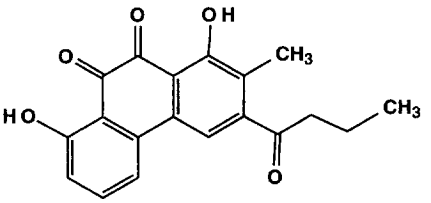


Figure 1 Structure of Sch 68631 (1)

The fermentation broth of culture 94-02747 (4L) was extracted with ethyl acetate at harvest pH (7.2-7.5) followed by a solvent partition with hexane:EtOAc:MeOH:H_2O (1:1:1:1) biphasic system. The active complex from the upper-phase was further chromatographed on normal phase HPLC (YMC PVA-Sil 20 x 250 mm semi-preparative column, S-5, 25-90% EtOAc in hexane with a linear gradient in 30 min, 12 mL/min, UV = 280 nm) to afford ~22 mg of pure **1** as a deep red solid with m.p. 185-188°C (dec).

Table 1. NMR Assignment and HMBC data of **1**[a]

Position	^{13}C (δ)	^{1}H (δ)	HMBC
1	166.2 s[b]	12.30 (s, OH)	C-1, C-2, C-10a
2	119.6 d	6.99 (d, 8.0[c])	C-1, C-3, C-4, C-10a, C-10 (weak)
3	139.4 d	7.60 (t, 8.0)	C-1, C-4a, C-10a
4	113.2 d	7.45 (d, 8.0)	C-1, C-2, C-4b, C-8a, C-10a, C-10 (weak)
4a	135.0 s	---	---
4b	132.8 s	---	---
5	115.9 d	7.36 (s)	C-4a, C-9 (weak), C-7, C-8a, C-11
6	150.0 a	---	---
7	126.3 s	---	---
8	165.1 s	12.78 (s, OH)	C-7, C-8, C-8a
8a	114.8 s	---	---
9	182.6 s	---	---
10	182.2 s	---	---
10a	115.3 s	---	---
11	205.3 s	---	---
12	44.9 t	2.84 (t, 7.3)	C-11, C-13, C-14
13	17.4 t	1.80 (m)	C-11, C-12, C-14
14	13.8 q	1.05 (t, 7.3)	C-12, C-13
15	12.0 q	2.26 (s)	C-6, C-7, C-8

a. Recorded in $CDCl_3$ on 400 MHz instrument
b. Multiplicity was determined by APT data
c. Coupling constants in Hz

The molecular weight of **1** was determined as 324 based on FAB mass spectral data by observing a molecular ion cluster at m/z 325 $(M+H)^+$, 326 $(M+2H)^+$ and 327 $(M+3H)^+$. This was also confirmed by CIMS showing a protonated ion peak at m/z 325 $(M+H)^+$. The molecular formula was established as $C_{19}H_{16}O_5$ by HRFABMS (Calcd: 325.1076. Found: 325.1079 for $C_{19}H_{17}O_5$) and ^{13}C NMR spectral data.

The UV spectrum of **1** indicated absorptions at 234, 283, 397 and 506 nm, which is a typical UV profile for anthraquinone compounds. The IR absorptions at 3436, 1700, 1627, 1578 and 1447 cm^{-1} revealed the presence of hydroxyl, conjugated carbonyl, quinone carbonyl and aromatic functionalities, respectively. In ^{13}C NMR spectrum (Table 1) two distinctive quinone resonances at δ 182.6 and 182.2 were observed in addition to the carbonyl signal at δ 205.3. Four aromatic methine and eight aromatic quaternary carbons were identified in combination with APT data analysis suggesting the presence of two benzene rings. The observation of two additional methylene and two methyl carbon signals matched a total of nineteen carbons for **1**. ^{1}H NMR data (Table 1) were consistent with all spectroscopic data by showing four aromatic protons at δ 7.38 (singlet), 7.60 (triplet), 6.99 and 7.45 (doublets), two methylenes at δ 2.84 (triplet) and 1.80 (multiplet), and one methyl group at δ 1.05 (triplet), respectively. The remaining methyl group at δ 2.26 (singlet) was clearly attached to an aromatic ring. As shown in Figure 2, partial structures A and B were established based on COSY experiments indicating coupling between three aromatic protons in a trisubstituted phenolic ring A, as well as correlations between two methylene groups and methyl protons in a n-butyl ketone chain B. This was also supported by NOE data (see Figure 3) due to observation of similar correlations.

Figure 2 COSY Correlations of **1**

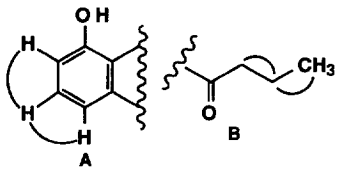
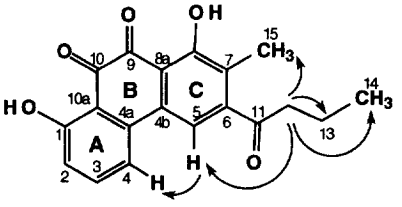


Figure 3 Important Difference NOE Data for **1**



Analyses of NOE spectral results together with HMBC data (Table 1) permitted the establishment of an anthraquinone tricyclic ring frame and the regiochemistry for all functional groups. NOE correlation of CH_2-12 to H-5 and CH_3-15 suggested that the n-butyl ketone group was located at position-6 on the C-ring. The proton singlet at δ 7.36 was assigned to position-5 due to an NOE correlation between H-5 and H-4, therefore, one of the hydroxyl group was attached to position-8. Since H-3 coupled to H-2 and H-4, and H-4 coupled to H-5, the remaining hydroxyl group could only be linked to position-1. The detailed assignments of each proton and carbon were finally accomplished by HMBC data analyses of long range couplings from protons to the adjacent carbons as summarized in Table 1. Thus, structure of **1** was proposed as a phenanthrenequinone, which is closely related to piloquinone[11,12] and haloquinone.[13,14]

Compound **1** demonstrated an inhibitory activity of 2.5 μg/mL in the *in vitro* assay for HCV NS3 serine proteinase.[15] Since quinones are fairly common entities in various natural products, several quinone analogues were also tested in the HCV proteinase assay along with **1**. However, quinones such as sakyomicin A, phenicin and fumigatin were inactive in the HCV assay. Further studies of the mechanism of inhibition for **1** will be required to identify the key functionalities which are responsible for the HCV proteinase inhibitory activity.

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10. RP media is composed of glucose 0.1 g sea salt (Sigma) 1g, rice protein RE70M (Deltown) 1 g, $NaNO_3$ 1 g, agar 15 g and one liter of tap water adjusted to pH 8.
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15. Cell-free Translation Assay for HCV NS3 Protease.
    Translated substrate was prepared as follows: The plasmid pTS102 was linearized with EcoRl and transcribed with T7 RNA polymerase (Promega) to produce RNA encoding HCV polyprotin ΔNS5A/Δ5B from amino acid residue 2312-2621 to produce RNA encoding HCV polyprotein ΔNS4A/Δ4B from a residue 1693-1903). The *in vitro* transcribed RNA was translated in rabbit reticulocyte lysates (Promega) in the presence of {35S}-methionine. Translation reactions wre terminated by adding DNAse-free RNAse (Boehringer Mannheim) and cyclohexamide (Sigma) to 10 ug/ml followed by incubation at 30°C for 15 min.
    Standard protease assays were initiated by the addition of 35 nM (10 nM) partially purified HCV protease to 2 ul {35S}-labeled translated substrate in a 20 ul volume containing 10 mM Tris pH 7.5, 120 mM NaCl, 5 mM DTT, 0.5% EDTA, 0.1% Tween 20, and 12% glycerol followed by incubation at 30°C for 30 min. Inhibitors were added to standard assay mixtures prior to incubation, and appropriate solvents were also added to standard assay mixes as controls. Cleavage reactions were terminated by addition of an equal volume of 2X Laemmli sample buffer, and boiling 3 min. Cleavage products were analyzed by SDS/ 15% PAGE gel electrophoresis and autoradiography.

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