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Two Antiviral Compounds from the Plant *Stylogne cauliflora* as Inhibitors of HCV NS3 Protease

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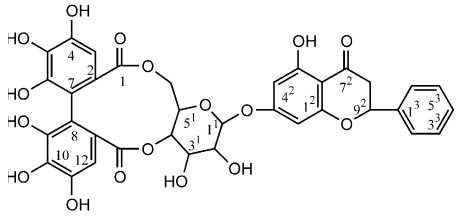
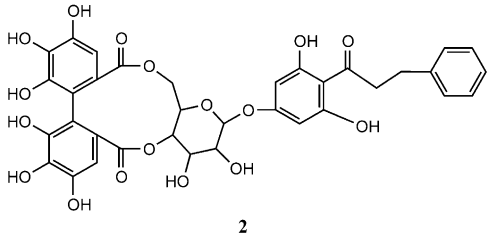
Abstract—The 70% aq methanolic extract of the Peruvian plant *Stylogne cauliflora* was found to contain two novel oligophenolic compounds SCH 644343 (**1**) and SCH 644342 (**2**), which were identified as inhibitors of HCV NS3 protease. The structure of **1** and **2** was established based on high-resolution NMR studies. Compound **1** inhibited HCV NS3 protease with an IC_{50} of 0.3 μM, while compound **2** showed an IC_{50} of 0.8 μM.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide, often leading to liver cirrhosis, hepatic failure, and hepatocellular carcinoma.[1–4] The current approved therapy is long term treatment with interferon-α (IFN α), either alone, or in combination with ribavirin. Many patients do not respond to treatment, however, and there is no preventive vaccine available. Hence there remains an important need to find additional therapeutic agents. HCV is an enveloped virus with a positive-strand RNA genome encoding a polyprotein of about 3010 amino acids. Cleavage of the polyprotein at four cleavage sites in the nonstructural part of the protein, an essential process, is carried out by the NS3 serine protease, together with its cofactor, NS4A.[5] The NS3/4A protease has been well-characterized biochemically, and the crystal structure has been solved by several groups,[6–9] making it an attractive target for antiviral drug discovery and drug design.

As part of our continuing investigation of natural products as leads for inhibiting HCV infection, we screened several semi-purified fractions of aqueous methanolic extracts of many plants. One of these fractions, which was derived from a plant identified as *Stylogne cauliflora* sp, was active in NS3/4A protease assay. Bioassay-guided fractionation of this extract led to the isolation of two oligo-phenols **1**, and **2**.



1



2

The detanninized aqueous methanolic extract (0.83 g) was loaded on a CHP-20 (2.5×30 cm) column equilibrated with water and chromatographed on the column using a water and methanol gradient system. These fractions were screened for their activity in NS3/4A assay. The active fractions was collected and dried to yield 55.8 mg of enriched complex. Separation of the active compounds were achieved by reverse-phase preparative HPLC on a Phenomenex Luna C-18 silica column (21.2×250 mm), eluting with a mixture of acetonitrile and water (25:75 v/v). Acetonitrile was removed from the active peak eluate and the aqueous

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solution was freeze-dried to yield 7.5 and 5.7 mg of **1** and **2**, respectively.

SCH 644343 (**1**) showed a sodiated ion *m*/*z* 721 $(M+Na)^+$ in the FAB mass spectrum suggesting a molecular weight of 720 daltons. The molecular formula of **1** was established as $C_{35}H_{28}O_{17}$ by HRMS (high-resolution mass spectrum),[10] indicating 22 unsaturations in the molecule. The UV spectrum (MeOH) showed absorption maxima at 210, 281 and 335 nm and the IR spectrum in KBr showed peaks at 1645, 1580, 1472, 1178 and 1078 cm^{-1}, suggesting the presence of ester functionality. ^1H and ^{13}C NMR chemical shifts of **1** and **2** are listed in Table 1. The ^1H NMR indicated the presence of several aromatic protons, and the presence of one sugar. D_2O exchange revealed seven phenolic–OH's and at least two hydroxyl groups. The ^{13}C NMR also showed 35 carbon signals in agreement with the number of carbons revealed by HRMS. APT ^{13}C NMR identified them as three >C=O, 24 olefinic (nine =CH–, 15 =C<), one anomeric methine (O–CH–O), five >CH–O, one O–CH_2, one >CH_2. This suggests that the compound contains four aromatic rings and one sugar. When the three carbonyl functionalities were included, we can account for 20 unsaturations. The phenolic nature, high oxygen content and absence of nitrogen suggested the compound must be a tannin precursor. Using contemporary 2D NMR techniques (COSY, HMQC, HMQC-TOCSY and HMBC) the structure was established as shown in **1.** This structure was further confirmed by degradation. The compound was hydrolyzed with 6 N HCl overnight in a sealed tube, and then the contents were diluted with water and dried and finally purified on HPLC. Two compounds **3** and **4** were isolated from this degradation mixture. The mass, ^1H and ^{13}C NMR spectral data of **3** suggested an oligo phenolic compound with molecular formula $C_{14}H_{10}O_{10}$. A search of the literature found that this compound was identical to elagic acid.[11] The identity **3** was further confirmed by HPLC comparison with authentic sample of elagic acid. Compound **4** showed molecular ion *m*/*z* 257 in Electro Spray MS suggesting molecular weight 256. ^1H and ^{13}C NMR chemical shifts of **4** are tabulated in Table 2. Both ^1H and ^{13}C NMR chemical shifts are similar to those of flavanone part of compound **1**. Based on spectral data the structure of this fragment was established as **4**.[12] This was further confirmed by COSY, and HMBC spectral analysis. The sugar in **1** appears to

Table 1. ^1H and ^{13}C NMR chemical shifts for **1** and **2**

Compd	**1**	**1**	**2**	**2**
1		167.8		167.9
2		124.6		124.4
3	6.32 (s)	105.5	6.31 (s)	105.4
4	9.28 (–OH)	144.2		143.8
5	8.69 (–OH)	134.9		135.1
6	8.14 (–OH)	144.4		145.1
7		115.2		115.4
8		115.5		115.7
9	8.14 (–OH)	144.5		145.2
10	8.74 (–OH)	135.3		135.4
11	9.23 (–OH)	144.2		143.8
12	6.51 (s)	106.1	6.49 (s)	106.0
13		124.4		124.5
14		167.0		167.1
1^1	5.11 (d, J = 7.8 Hz)	99.6	5.03 (d, J = 8 Hz)	99.7
2^1	3.38 (m) 5.65 (s, –OH)	73.6	3.35 (m)	73.6
3^1	3.51 (m), 5.36 (d, –OH)	73.7	3.55 (m)	73.7
4^1	4.58 (t, J = 10 Hz)	71.4	4.60 (t, J = 10 Hz)	71.4
5^1	4.16 (dd, J = 6, 10 Hz)	70.9	4.11 (dd, J = 6.5, 10 Hz)	71.0
6^1	4.94 (dd, J = 6, 13.5 Hz)			
	3.7 (d, J = 13.5 Hz)	62.7	4.97 (dd, J = 6.5, 13.5 Hz)	
	3.71 (d, J = 13.5 Hz)	62.6		
1^2		162.6		163.8
2^2		103.4		105.4
3^2	12.04 (–OH)	163.0	12.05 (–OH)	163.8
4^2	6.19 (d, J = 2 Hz)	96.5	6.08 (s)	95.0
5^2		164.9		163.1
6^2	6.24 (d, J = 2 Hz)	95.3	6.08 (s)	95.0
7^2		196.9		204.8
8^2	2.84 (dd, J = 3, 17.2 Hz)	42.2	3.33 (t, J = 7.5 Hz)	45.2
	3.37 (m)			
9^2	5.67 (m)	78.7	2.89 (t, J = 7.5 Hz)	30.0
1^3		138.4		141.5
2^3	7.53 (d, J = 7.5 Hz)	126.8	7.25 (m)	128.3
3^3	7.43 (t, J = 7.5 Hz)	128.6	7.25 (m)	128.3
4^3	7.39 (t, = 7.5 Hz)	128.7	7.18 (dd, J = 7.5 Hz)	125.8
5^3	7.43 (t, J = 7.5 Hz)	128.6	7.25 (m)	128.3
6^3	7.53 (d, J = 7.5 Hz)	126.8	7.25 (m)	128.3

NMR's were run in DMSO-d_6; similar chemical shifts may be interchanged.

Table 2. ^{1}H and ^{13}C NMR chemical shifts for **4** and **5**

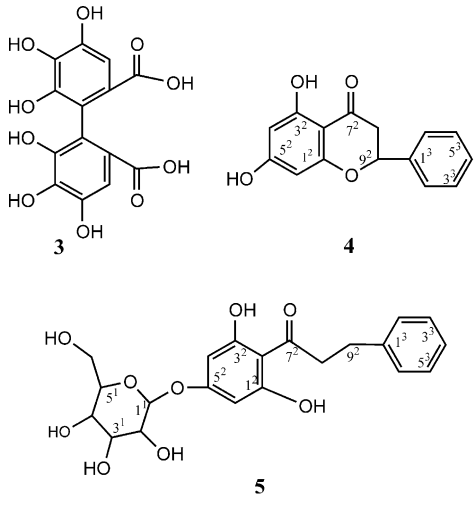
Compd	4		5	
	^{1}H	^{13}C	^{1}H	^{13}C
1^{2}		162.7	12.25 (–OH)	163.7
2^{2}		101.8		105.2
3^{2}	12.10 (–OH)	163.5	12.25 (–OH)	163.7
4^{2}	5.89 (d, $J=2$ Hz)	95.9	6.05 (s)	95.1
5^{2}	10.85 (-OH)	166.7		163.4
6^{2}	5.92 (d, $J=2$ Hz)	95.0	6.05 (s)	95.1
7^{2}		196.0		204.7
8^{2}	2.77 (dd, $J=3$, 17 Hz) 3.25 (dd, $J=12.5$, 17 Hz)	42.1	3.3 (m)	45.2
9^{2}	5.58 (d, $J=3$, 12.5 Hz)	78.4	2.9 (t, $J=7.5$ Hz)	30.0
1^{3}		138.7		141.5
2^{3}	7.5 (d, $J=7.5$ Hz)	126.6	7.25 (m)	128.3
3^{3}	7.42 (t, $J=7.5$ Hz)	128.6	7.25 (m)	128.3
4^{3}	7.38 (t, $J=7.5$ Hz)	128.6	7.16 (m)	125.8
5^{3}	7.42 (t, $J=7.5$ Hz)	128.6	7.25 (m)	128.3
6^{3}	7.5 (d, $J=7.5$ Hz)	126.6	7.25 (m)	128.3
1^{1}			4.86 (d, $J=7.5$ Hz)	99.5
2^{1}			3.18 (m)	73.0
3^{1}			3.25 (m)	76.4
4^{1}			3.16 (m)	69.4
5^{1}			3.29 (m)	77.1
6^{1}			3.47 (m), 3.67 (d, $J=11.5$ Hz)	60.4

NMR's were run in DMSO-d_6.

be glucopyranose based on the relative stereochemistry established from NMR chemical shifts and ^{1}H–^{1}H coupling studies. However the absolute structure has not been established.

SCH 644342 (**2**) showed a sodiated ion m/z 723 $(M+Na)^{+}$ in the FAB mass spectrum suggesting the molecular weight of 722 daltons. The molecular formula of **1** was established as $C_{35}H_{30}O_{17}$ by high-resolution mass spectrum (HRMS).[10] Compound **2** showed UV maxima (MeOH) 215, 281 335 nm. The ^{1}H and ^{13}C NMR chemical shifts of **2**, are tabulated in Table 1, revealing close structural similarities between **1** and **2.** Comparison of individual ^{1}H and ^{13}C NMR chemical shifts of elagic acid, sugar and unsubstituted phenyl ring portion revealed that in compound **2** this portion is intact. However the flavanone portion showed some changes at C-8 and C-9 carbons and protons. The ^{1}H and ^{13}C NMR chemical shifts of these carbons indicate that the pyran-4-one ring is opened and the C-8 carbon is a methylene and does not appear to be attached to oxygen. The structure **2** was proposed and further confirmed by HMBC. During fractionation of the extract we have also isolated another related but known compound 5.[13] The proton and carbon chemical shifts of compound **5** is tabulated in Table 2 and shows a very close similarity to this portion of compound **2**. This lends further support to structure **2**.

Recently, compounds with similar structures (**1**[14] and **2**[15]) have been reported in the literature; however, we could not confirm the exact identity because of the lack of material. No hydrolysis experiments were performed to identify the sugars separately, due to the lack of material. Their structures were deduced mainly from the 2D NMR experiments.



Compounds **1** and **2** were active in the HCV NS3 Protease Activity Assay,[16] with IC_{50} values of 0.3 and 0.8 μM, respectively. Subsequent studies showed that compound **1** was also active in the HCV Protease Binding Assay[17] ($IC_{50}=2.8$ μM). This assay measures displacement of a radiolabeled product-like hexapeptide from the active site of the protein.

Acknowledgements

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10. Spectral data for SCH 644343 (**1**): FABMS *m*/*z* 721 (M + H); HR-FABMS for $C_{35}H_{28}O_{17}$ (obsvd 743.1252 $(M + Na)^+$, calcd 743.1224); IR (neat): 3306, 2960, 1715, 1636, and 1540 cm^{-1}. SCH 644342 (**2**): FABMS *m*/*z* (M + H); HR-FABMS for $C_{35}H_{30}O_{17}$ (obsvd 745.1398 $(M + Na)^+$, calcd 745.1381).
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16. **HCV NS3 protease activity assay**: Inhibition of proteolytic activity of HCV NS3 protease was determined by scintillation proximity assay (SPA). The recombinant single chain HCV protease $NS4A_{21–32}$-GSGS-$NS3_{3–181}$, purified to 95% homogeneity from *Escherichia coli* (9), was incubated with the peptide substrate Biotin-Asp-Thr-Glu-Asp-Val-Val-Pro-Cys-Ser-Met-Ser-Tyr-Thr-Trp-Thr-Gly-Lys-[^{3}H] (Syn-Pep, Dublin, CA, USA) derived from the HCV NS5A/5B cleavage site. After stopping the reaction, cleavage of the Cys-Ser bond is detected by the addition of streptavidin-coated SPA beads (Amersham-Pharmacia, Piscataway, NJ, USA), which can bind to the biotin-labeled peptide. Cleavage is indicated by reduction in signal caused by separation of the radiolabeled fragment from the bound biotinylated fragment (measured in CPM) which is proportional to proteolytic activity. Inhibitor assays were run in 96-well microtiter plates by incubating 2 uM substrate and 0.6 nM HCV protease with synthetic compounds in a 50-μL reaction volume containing 50 mM 3-[*N*-Morpholino]propane-sulfonic acid (MOPS), pH 7.5, 0.1 M NaCl, 0.0.05% *n*-dodecyl-β-D-maltoside, 0.25 mM DTT, 10% glycerol. After incubating for 2 h at room temperature (21 °C), reaction was stopped with the addition of 50 μL of 10% phosphoric acid and 50 μL of 10 mg/mL of streptavidin-coated SPA beads. Plates were shaken for 15 min on a plate shaker and radioactivity in each well was counted using a TopCount NXT Microplate Reader (Pachard, Meriden, CT, USA).
17. **HCV protease binding assay**: The assay measures binding of a radiolabeled non-hydrolyzable HCV product hexapeptide (P6-P1) derived from the NS5A/5B cleavage site to HCV protease. The recombinant single-chain His-tagged HCV protease, His–$NS4A_{21–32}$-GSGS–$NS3_{3–181}$, purified to 95% homogeneity from *E. coli* (**9**) is incubated with peptide Asp-Glu-Leu-Ile-Leu-Nva-OH,[3-Leucyl-4,5-3H] (NEN Life Science Products, Boston, MA, USA). Binding of the labeled peptide is detected by capture of the His-tagged protease onto the nickel chelate scintillant-coated microplate plate (FLASHPlate PLUS 96-well Nickel Chelate, NEN Life Science, Boston, MA, USA). Inhibitor assays were run in a reaction mix containing 50 mM MOPS, pH7.0, 25 mM NaCl, 25% glycerol, 0.0015% lauryl maltoside, 1 μM peptide and 100 nM enzyme, incubating at room temperature (21 °C) for 1 h with shaking. Radioactivity was counted in each well using a TopCount NXT Microplate Reader (Pachard, Meriden, CT, USA).