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Antiviral compounds from traditional Chinese medicines *Galla Chinese* as inhibitors of HCV NS3 protease

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Abstract—Under the guidance of bioassay, the EtOAc extract fraction of the Traditional Chinese Medicine (TCM) *Galla Chinese* was found to be efficient in inhibiting the NS3 protease of HCV and purified the fraction to get three polyphenol compounds 1,2,6-tri-*O*-galloyl-β-D-glucose (**1**), 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**2**), and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**3**), which were identified as inhibitors of Hepatitis C Virus (HCV) NS3 protease. Compounds **1**, **2**, and **3** inhibited HCV NS3 protease with IC_{50} of 1.89, 0.75, and 1.60 μM, respectively.

1. Introduction

HCV infection is a major cause of chronic liver disease worldwide, often leading to liver cirrhosis, hepatic failure, and hepatocellular carcinoma.[1] The number of carriers has reached the millions, while current treatments have only limited success and preventative vaccination or an effective anti-HCV drug have not been available.[2] HCV is a positive-stranded RNA virus, its genome is about 9.6kb and encodes the structural proteins C, E1, E2, and the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The N-terminal domain of the NS3 protein is a serine protein, therefore, the NS3 protein has become one of the most attractive targets for drug development of anti-HCV. In recent researches, HCV NS3-NS4A protease has been well-characterized biochemically, and the crystal structure has been solved by several groups,[3,4] and has been used as target enzyme for screening anti-HCV drugs.[5,6]

We screened more than one hundred kinds of TCMs. Bioassay-guided results (ELISA) suggested that the EtOAc extract fraction from *Galla Chinese* was efficient in inhibiting the NS3 protease activity of HCV.[7] Under direction of bioassay we isolated the fraction further and get three polyphenol compounds **1**, **2**, and **3**, which inhibited HCV NS3 protease with IC_{50} of 1.89, 0.75, and 1.60 μM respectively.

2. Results and discussion

Powdered *Galla Chinese* was extracted three times with acetone–H_2O at room temperature, and the concentrated filtrate was fractionated into petroleum, chloroform-, EtoAc-, and *n*-BuOH-soluble portions. The EtoAc extract was separated by successive column chromatography over Polyamide and Sephadex LH-20 to give 1,2,6-tri-*O*-galloyl-β-D-glucose (**1**), 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**2**), and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**3**) and are illustrated in Figure 1.[8–11]

Compound (**1**) was obtained as an off-white amorphous powder. The negative ESI-TOF-MS of **1** showed an $[M-H]^-$ ion peak at *m*/*z*: 635, which corresponds to the molecular formula $C_{27}H_{24}O_{18}$. The ^1H NMR spectrum of **1** was characteristic of a polyphenol, as revealed by its close similarity to that of 1,2,6-tri-*O*-galloyl-β-D-glucose. The spectrum exhibited three one-proton singlets (δ 7.07, 7.09, 7.15). The presence of a β-D-glucopyranose residue were conformed by its special ^{13}C NMR data, its ^1H NMR, and ^{13}C NMR data (Tables 1 and 2) are consistent with those in the literature.[8b,11]

Compound (**2**) was obtained as an off-white amorphous powder. The negative ESI-TOF-MS of **2** showed an

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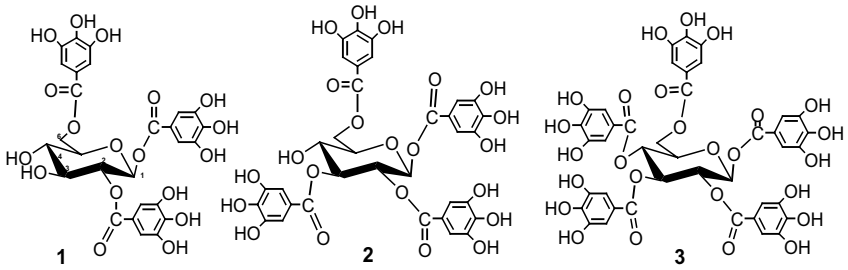

Figure 1. Three polyphenol compounds **1**, **2**, and **3** purified from *Galla Chinese*.

Table 1. ^{13}C NMR data of compounds ($\delta_c(CD_3)_2CO$)

	Compound						
	1		**2**		**3**		
Glucose							
1	93.52		93.47		93.44		
2	73.81		71.79		71.69		
3	75.48		75.89		75.49		
4	71.07		69.33		69.28		
5	75.99		76.04		75.99		
6	63.83		63.63		63.59		
Galloyl							
1′	120.21	121.38	120.04	120.69	120.04	120.69	
2′	109.89	110.07	110.00	110.09	109.98	110.08	
3′	145.83	145.98	145.88	145.96	145.85	145.88	
4′	138.82	138.87	138.96	138.96	138.96	138.96	
5′	145.83	145.98	145.88	145.96	145.85	145.88	
6′	109.89	110.07	110.00	110.09	109.98	110.08	
C=O	165.92	165.92	165.02	165.78	164.96	165.64	
1″	121.56		121.29	121.52	121.25	121.37	121.50
2″	110.19		110.16	110.30	110.07	110.10	110.25
3″	145.99		146.04	146.08	146.00	146.01	146.05
4″	139.48		139.18	139.72	139.12	139.48	139.70
5″	145.99		146.04	146.08	145.99	146.01	146.05
6″	110.19		110.16	110.30	110.07	110.10	110.25
C=O	166.58		166.20	166.63	166.16	166.60	166.64

Table 2. ^{1}H NMR data of compound (δ_H in $(CD_3)_2CO$)

	Compound		
	1	**2**	**3**
Glucose			
1	5.99, d	6.19, d	6.20, d
2	5.25, t	5.49, dd	5.49, t
3	5.74, t	5.70, t	5.70, t
4	3.93, m	4.12, t	4.07, dd
5	3.98, m	4.16, m	4.18, m
6	4.48, m	4.62, dd	4.65, m
	4.58, m	4.58, dd	4.58, m
Galloyl			
	7.07, s	7.02, s	6.99, s
	7.09, s	7.09, s	7.01, s
	7.15, s	7.11, s	7.06, s
			7.11, s
		7.19, s	7.19, s

$[M-H]^-$ ion peak at *m/z*: 787, which corresponds to the molecular formula $C_{34}H_{28}O_{22}$. IR spectral cm^{-1}: 3394, 1701, 1615, 1536, 1450, 1321, 1213, 1209, 1033, 960, 872, 805. The ^{1}H NMR spectrum of **2** was characteristic of a polyphenol, as revealed by its close similarity to that of 1,2,3,6-tetra-*O*-galloyl-β-D-glucose. The spectrum exhibited four one-proton singlets (δ 7.02, 7.09, 7.11, 7.19). The presence of a β-D-glucopyranose residue were conformed by its special ^{13}C NMR data, its ^{1}H NMR, and ^{13}C NMR data (Tables 1 and 2) are consistent with those in the literature.[8b,10,11]

Compound (**3**) was obtained as an off-white amorphous powder. The negative ESI-TOF-MS of **3** showed an $[M-H]^-$ ion peak at *m/z*: 939, which corresponds to the molecular formula $C_{41}H_{32}O_{26}$. The ^{1}H NMR spectrum of **3** was characteristic of a polyphenol, as revealed by its close similarity to that of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose. The spectrum exhibited five one-proton singlets (δ 6.99, 7.01, 7.06, 7.11, 7.19). The presence of a β-D-glucopyranose residue were conformed by its special ^{13}C NMR data, its ^{1}H NMR, and ^{13}C NMR data (Tables 1 and 2) are consistent with those in the literature.[8b,11]

Compounds **1**, **2**, and **3**, which were identified as inhibitors of HCV NS3 protease. Compounds **1**, **2**, and **3**

inhibited HCV NS3 protease with IC_{50} of 1.89, 0.75, and 1.60 μM, respectively. Natural products could play a great role as sources for anti-HCV agents. We reported compound **2** belongs to galloytannin or polyphenols. Compound **2** has been reported to have inhibitory effects on some tumor strain, lipid peroxidation, and blood platelet aggregation in vitro[10] and compound **2** and **3** have reported inhibitory effects on anti-hypertensive activity in vitro.[12]

Bioassay-guided fractionation methods provided direct information of the role of small molecule and enzyme. Our results showed compound **2** has specifical action against HCV NS3 protease with its high inhibiting role. HCV NS3 protease requires its NS4A cofactor peptide for optical binding,[4] our focused compound **2** may have a more effective role with them. The effects of galloytannin on NS3 serine protease and other proteases are worthy of further investigation. The search for novel candidates that can serve as medicines or as core entities for the design of potent and specific inhibitors of HCV as well as its essential enzymes is encouraging.[13,14] Efforts in the continued search for natural products especially polyphenols with anti-HCV activity should be emphasized.

3. Experimental

^1H and ^{13}C NMR spectra were determined on a Bruker ARX400 spectrometer at 400 and 100 MHz, respectively, in $(CD_3)_2CO$, with $SiMe_4$ as internal standard; chemical shifts are given as (ppm) values. IR spectra were recorded on a Nicolet Magana-IR750. Electrospray ionization and time of flight mass spectra (ESI-TOF-MS) were taken on a Mariner Biospectrometry workstation. TLC plates were made with polyamide film (China), and spots were detected by ultraviolet (UV) and by spraying with 2% ethanolic $FeCl_3$ reagent. Polyamide (100–200 mesh, China) and Sephadex LH-20 (Amersham Pharmacia Biotech) were used for column chromatography (CC). The solvent was evaporated under reduced pressure at below 40 °C.

Galla Chinese were purchased from the hospital of the Peking university (Beijing, China).

Powdered *Galla Chinese* (500 g) was extracted three times with acetone–H_2O (1:1, 1000 mL × 3) at room temperature for 24 h. The solvent was evaporated under reduced pressure and then partitioned between water (500 mL) and petroleum (60–90 °C, 500 mL × 4), chloroform (500 mL × 4), ethyl acetate (500 mL × 4), and *n*-butylol (500 × 4), successively. Bioasssay-guilded results indicated that the ethyl acetate fraction was efficient in inhibiting NS3 protease. A part of this mixture (20.0 g) in ethanol was applied on a polyamide column chromatography using a solvent system of EtoAc–methanol–water–acetone (EtoAc–EtoAc/methanol 10:1–EtoAc/methanol/water 10:1:0.5–ethanol/water 6:4–ethanol/water 8:2–ethanol–acetone/water 6:4–acetone/water 8:2–acetone), flow rate 3 mL/min, and on a Sephadex LH-20 column using a solvent system of water–methanol–acetone (water–methanol/water 2:8–methanol/water 4:6–methanol/water 6:4–methanol/water 8:2–methanol–acetone), flow rate 0.5 mL/min. On repeated chromatography on polyamide and Sephadex LH-20, tri(G3,**1**)-, tetra(G4,**2**)-, penta(G5,**3**)-galloyglucoses were obtained; hexa(G6)-, hepta(G7)-, and octa(G8)-galloyglucoses as a mixture based on its ESI-TOF-MS data and TLC results.

HCV NS3 protease was purified from an expression of a recombination plasmide pMANS34N3 (offered by Professor Shimotohno's lab) in *E. coli* JM109,[5a] HCV NS3 protease activity was determined by using ELISA.[5b] A peptide substrate with an acetyl-group at N-terminus and a biotin at C-terminus was hydrolyzed by NS3 protease into product with a free amino moiety at N-terminus. The product was immobilized and the free amino moiety was analyzed.

To gain more chemical insight of the structure–activity relationship (SAR), we have generated a model of the system by docking the inhibitors onto the active site of HCV NS3. The structure of HCV NS3 is taken from Protein Data Bank (PDB entry 1dy8), and the structure of inhibitors was minimized using molecule mechanics method with MMFF94 force field. The FlexX module of Sybyl software was used to do the molecular docking job. The residues around the inhibitor in the range of 8 Å are defined as the active site, and then inhibitors are docking into the active site. The work was done on a Sybyl software package distributed by Tripos Inc.

The predicted binding mode between the inhibitor and HCV-NS3 is illustrated in Figure 2, there are five hydrogen bond interactions between the inhibitor and the enzyme, with ASP81, LYS136, ALA157, ARG155, and CYS159, and there is a hydrophobic interaction between the glucose ring and VAL158. In this model, compound **2** makes two H-bond interactions with the enzyme backbone from the galloy OH. Because of the correct alignment of the OH, coupled with rigidity of the glucose ring, we believe this scaffold could be effective as a general structure that will be used in protease inhibitor design.

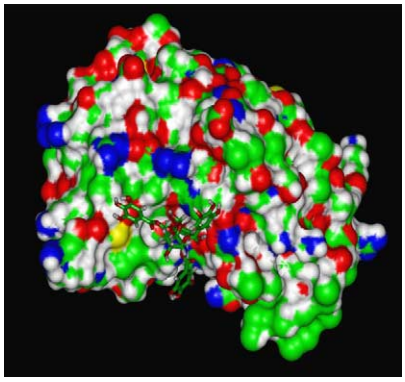

Figure 2. Compound **2** modeled into the active site of HCV NS3.

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