Hepatitis C Virus NS3-NS4A Protease Inhibitors from the Endophytic *Penicillium chrysogenum* Isolated from the Red Alga *Liagora viscida*

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Hepatitis C virus (HCV) NS3-NS4A protease is an attractive target for anti-HCV agents because of its important role in replication. In this work, we demonstrated that the ethyl acetate extract of the endophytic fungus *Penicillium chrysogenum* exhibited a potent activity against HCV NS3-NS4A protease with an IC_{50} value of 20 μg/ml. The fungus was isolated from the red alga *Liagora viscida* and identified by its morphology and 18S rDNA. Large-scale fermentation of the fungus in Czapek's peptone liquid medium followed by chromatographic purification of the active extract from the liquid medium allowed the isolation of twelve known metabolites. The biological properties of the isolated compounds were explored for anti-HCV protease as well as antimicrobial and anticancer activities. A computational docking study of the active isolated compounds against HCV protease was used to formulate a hypothetical mechanism for the inhibitory activity of the active compounds on the tested enzymes.

Key words: Red algae, *Liagora viscida*, *Penicillium chrysogenum*, HCV NS3-NS4A Protease

Introduction

Chronic hepatitis C virus (HCV) infection is a global problem due to both the lack of an effective therapy and the difficulties in developing a protective vaccine. Thus, there is an urgent need for new, specifically targeted therapies for the treatment of chronic HCV infection. Hepatitis C virus protease (HCV PR) inhibition is considered to be one of the important targets for designing drugs for HCV treatment (Tsantrizos, 2008). HCV NS3-NS4A is a heterodimeric serine protease responsible for the proteolytic processing of four out of five junctions between nonstructural protein regions along the HCV polyprotein (De Francesco and Carfi, 2007). It also plays a role in silencing the host's antiviral immune response by interfering with interferon production as a natural defence against infections (Gale and Foy, 2005).

In recent years, numerous metabolites possessing uncommon structures and potent bioactivities have been isolated from strains of fungi collected from diverse environments, such as soils, animals, plants, and sediments (Laatsch, 2006). *Penicillium chrysogenum* is an important filamentous fungus because of its ability to produce large amounts of penicillin (Elander, 2003).

The secondary metabolites of *P. chrysogenum* include various penicillins, chrysogine, xanthocillins, secalonic acids, sorrentanone, and PR toxin (De Hoog *et al.*, 2000). Recently, many bioactive metabolites were reported from different cultures of *P. chrysogenum* which exhibited broad biological activities, such as xanthoviridicatins E and F (HIV-1 integrase inhibitory activity) (Singh *et al.*, 2003), sorbicillinoid alkaloids (sorbicillactones A and B; anti-HIV and strong cytotoxic activity) (Bringmann *et al.*, 2005), chrysogenamide A

(neuroprotective effect on SH-SY5Y cells) (Lin *et al*., 2008), glycerol derivatives, and penicitides A–C with cytotoxic activities against the human hepatocellular liver carcinoma cell line (Gao *et al*., 2011), as well as 16*β*-acetoxy-tetrahydroxy-22-ergostene and hypocrellin B or C as antifungal agents (Meng *et al*., 2011).

In the course of a program aiming at the isolation of bioactive natural products from marine endophytic fungi, *P. chrysogenum* was isolated from the inner tissues of the Egyptian Red Sea alga *Liagora viscida* (Forsskål) C. Agardh. Here, we describe the isolation and structure elucidation of the secondary metabolites from the culture medium of the isolated fungus. The organic extract and the isolated pure compounds were evaluated for their antimicrobial and anticancer activities, as well as for their inhibitory effect on HCV NS3-NS4A protease using a SensoLyte™ 520 HCV protease assay kit.

Results and Discussion

Characterization of isolated compounds

The fungus isolated from the Red Sea alga *Liagora viscida* was identified as *Penicillium chrysogenum* based on its morphology and authenticated by the molecular analysis of the internal transcript spacer (ITS) region of rDNA containing ITS1 and ITS4, and the intervening 5.8S rDNA gene. The fungus was grown in a static liquid medium. The culture broth was extracted with ethyl acetate, and the organic extract was submitted to a combination of silica gel column chromatography (CC), preparative thin-layer chromatography

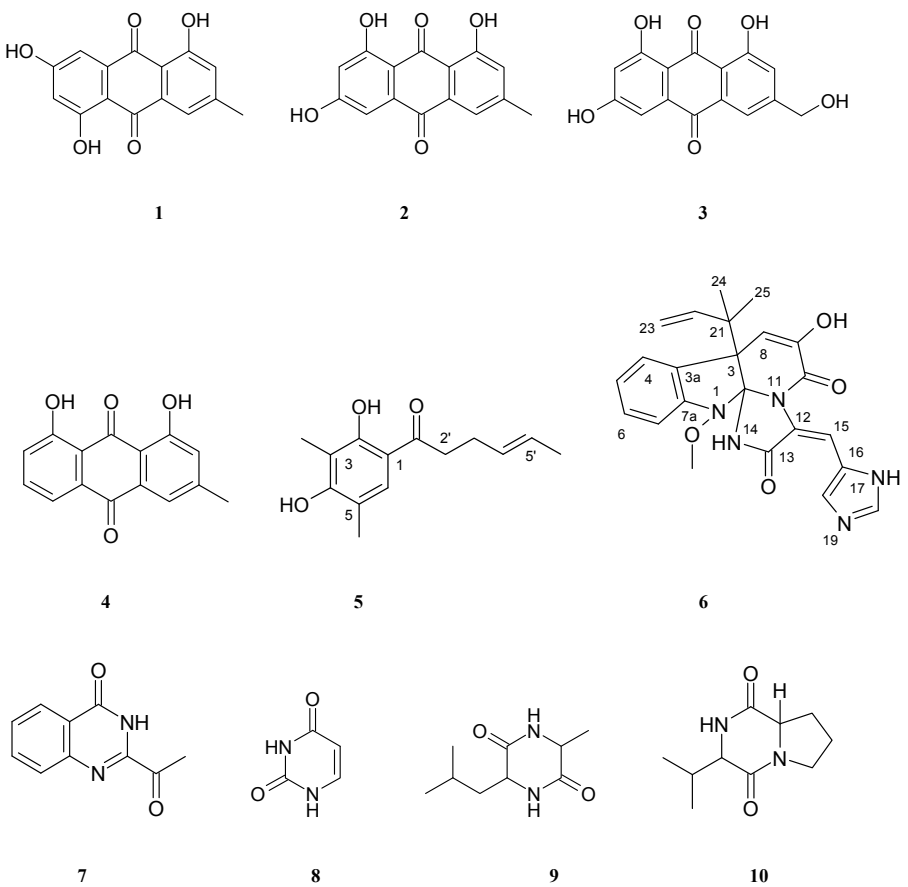

Fig. 1. Compounds isolated from *Penicillium chrysogenum*: alatinone (**1**), emodin (**2**), *ω*-hydroxyemodin (**3**), chrysophanol (**4**), 2',3'-dihydrosorbicillin (**5**), meleagrin (**6**), chrysoginone (**7**), uracil (**8**), cyclo-L-Ala-L-Leu (**9**), and maculosin-5 (**10**).

(TLC), and semi-preparative high-performance liquid chromatography (HPLC) to afford twelve known metabolites, **1**–**12** (Fig. 1). NMR spectroscopy (1D- and 2D-NMR) and mass spectrometry (MS) were extensively used in structural elucidation. The isolated compounds were identified based on the spectral analyses and comparison with literature data.

The presence of *peri*-hydroxyanthraquinones in compounds **1**–**4** was detected by TLC on silica gel plates sprayed with 1.0% vanillin/H_2SO_4 and by their red colour with 5% KOH solution, respectively. The NMR spectra of these compounds revealed tricyclic anthraquinones with chelated hydroxy, methyl, and methoxy groups. These compounds were identified as alatinone (**1**) (Hemlata and Kalidhar, 1993), emodin (**2**) (Cohen and Towers, 1995), ω-hydroxyemodin (**3**) (Benfaremo and Cava, 1985), and chrysophanol (**4**) (Chang *et al*., 1998). However, this is the first report on the isolation of alatinone (**1**) from a microorganism. The phenone compound 2',3'-dihydrosorbicillin (**5**) was obtained by semi-preparative HPLC as a light yellow solid soluble in MeOH (Maskey *et al*., 2005).

Alkaloid metabolites **6**–**10** with substituted nitrogen atoms showed both aromatic (compounds **6**–**8**) and aliphatic (compounds **9** and **10**) proton characters in their ^{1}H NMR spectra. The compounds were characterized as meleagrin (**6**) (Kawai *et al*., 1984), chrysoginone (**7**) (Blight and Grove, 1974), uracil (**8**) (Huang *et al*., 1995), cyclo-L-Ala-L-Leu (**9**) (Caesar *et al*., 1969), and maculosin-5 (**10**) (Lee *et al*., 1994). In addition, two unsaturated fatty acids, oleic acid (**11**) and linoleic acid (**12**), were isolated from the *n*-hexane fraction (Carballeira *et al*., 2000).

Antimicrobial activity

The results of the antimicrobial activity tests of the extract and the isolated pure compounds are shown in Table I. The tested pathogens were found to be resistant to emodin (**2**), ω-hydroxyemodin (**3**), and cyclo-L-Ala-L-Leu (**9**). *Candida albicans* was sensitive to the extract and meleagrin (**6**) with inhibition diameters of 18 and 15 mm, respectively. *Klebsiella pneumoniae* was highly sensitive to the extract and the isolated compounds 2',3'-dihydrosorbicillin (**5**) and meleagrin (**6**). Chrysophanol (**4**), 2',3'-dihydrosorbicillin (**5**), chrysoginone (**7**), and maculosin-5 (**10**) moderately inhibited the growth of *Aspergillus niger*, *Escherichia coli*, *Bacillus subtilis*, and *Bacillus megaterium.*

Anticancer activity

The ethyl acetate extract and the isolated compounds were evaluated for their *in vitro* anticancer activity against cancer and normal cell lines using the disk diffusion assay (Table II). As a result of this bioassay, the extract demonstrated some selectivity against colon 38 cells with a zone

Table I. Antimicrobial potential (inhibition zone in mm) of the extract and nine compounds isolated from *Penicillium chrysogenum* in a radial diffusion assay.

Sample	Gram-positive bacteria[a]				Gram-negative bacteria[a]			Fungi[a]		
	S.a.	*B.m.*	*B.c.*	*B.s.*	*E.c.*	*K.p.*	*Es.c.*	*C.a.*	*A.n.*	*S.c.*
Ethyl acetate extract	–	12 ± 1.1	8	8	–	17 ± 0.5	–	18 ± 0.1	–	–
Alatinone (**1**)	–	11	9	–	–	10	–	9	–	–
Emodin (**2**)	–	–	–	–	9	–	–	–	–	–
ω-Hydroxyemodin (**3**)	10	–	–	–	–	–	–	8	–	–
Chrysophanol (**4**)	–	–	–	10	–	–	13 ± 1.5	–	10	–
2',3'-Dihydrosorbicillin (**5**)	–	14 ± 1.5	–	–	–	18 ± 1.1	–	–	11	–
Meleagrin (**6**)	–	9	–	–	–	19 ± 2.0	–	15 ± 2.5	–	11
Chrysoginone (**7**)	8	12	–	15 ± 1.1	–	–	8	–	–	8
Cyclo-L-Ala-L-Leu (**9**)	–	–	–	–	12	–	–	10	–	–
Maculosin-5 (**10**)	–	–	11	–	–	–	14 ± 1.3	11	–	10
Oxytetracycline (30 μg) (control)	17	20	10	10	22	–	15	–	–	–

[a] *S.a.*, *Staphylococcus aureus*; *B.m.*, *Bacillus megaterium*; *B.c.*, *Bacillus cereus*; *B.s.*, *Bacillus subtilis*, *E.c.*, *Enterobacter cloacae*; *K.p.*, *Klebsiella pneumoniae*; *Es.c.*: *Escherichia coli*, *C.a.*, *Candida albicans*; *A.n.*, *Aspergillus niger*; *S.c.*, *Saccharomyces cerevisiae.*

Table II. Cytotoxicity results presented in zu[a] (200 zu ≙ 6 mm inhibition) of the extract and compounds isolated from the endophytic *Pencillium chrysogenum*.

Sample	Normal cells	Leukemia		Solid tumours			
				Colon cancer		Lung cancer	Liver cancer
	CFU-GM	L1210	CCRF-CEM	HCT-116	C38	H-125	HEP-G2
Ethyl acetate extract	250	300	0	250	400	250	250
Alatinone (**1**)	50	200	0	50	200	50	150
Emodin (**2**)	–	100	–	0	250	0	100
ω-Hydroxyemodin (**3**)	300	400	300	200	350	250	450
Meleagrin (**6**)	650	700	500	450	600	600	600
Oleic acid (**11**)	–	200	–	200	150	150	300
Linoleic acid (**12**)	–	200	–	150	150	200	250

[a] zu, zone units.

Table III. Inhibition of HCV NS3-NS4A protease and human trypsin by the extract and compounds isolated from *Penicillium chrysogenum*.

Sample	IC_{50} [μg/ml]	
	HCV PR inhibitory activity (%)	Trypsin inhibitory activity (%)
Ethyl acetate extract	20.0 ± 4.3	> 1000
Alatinone (**1**)	100.0 ± 3.7 (370 μM)	> 1000
Emodin (**2**)	22.5 ± 1.6 (80 μM)	450.5 ± 4.7 (1.6 mM)
ω-Hydroxyemodin (**3**)	10.6 ± 0.3 (30 μM)	45.2 ± 5.1 (157 μM)
Chrysophanol (**4**)	> 1000	Nt
2',3'-Dihydrosorbicillin (**5**)	> 1000	Nt
Meleagrin (**6**)	> 1000	Nt
Chrysoginone (**7**)	> 1000	Nt
Uracil (**8**)	> 1000	Nt
Cyclo-L-Ala-L-Leu (**9**)	> 1000	Nt
Maculosin-5 (**10**)	> 1000	Nt
HCV-I_2	1.5 ± 0.5 (1.64 μM)	Nt
T-I	Nt	0.01 ± 0.4 (0.5 μM)

HCV-I_2, HCV NS3-NS4A protease inhibitor 2 (positive control for HCV PR); T-I, soybean trypsin-chymotrypsin inhibitor (positive control for trypsin); Nt, not tested.

difference of 150 units compared to the normal cells (CFU-GM) at a concentration of 30 μg/disk. Of the compounds in the extract, only **3** and **6** were shown to have clearly inhibitory activity at 3 μg/disk.

HCV NS3-NS4A protease inhibition

The ethyl acetate extract along with compounds **1**–**10** isolated from this extract were tested for their inhibitory activity against HCV PR using HCV NS3-NS4A protease inhibitor 2 as a positive control (Table III). The ethyl acetate extract exhibited potent activity against HCV NS3-NS4A protease with an IC_{50} value of 20 μg/ml. Compounds **2** and **3** were strongly inhibitory with IC_{50} values of 22.5 and 10.6 μg/ml, respectively, while compound **1** was weakly inhibitory with an IC_{50} value of 100 μg/ml. The selectivity of the ethyl acetate extract and the active compounds for HCV PR was tested by comparison with their ability to inhibit human trypsin. Trypsin is a serine protease similar to HCV PR (Love *et al*., 1996; Wei *et al*., 2009). Compound **2** was 20 times more selective as inhibitor of HCV PR than human trypsin (Table III). Although compound **3** inhibited HCV PR more potently than **2**, it was less selective. It is noteworthy that this is the first report on the inhibition of HCV PR by anthraquinones which warrants further investigation of other members of this widely distributed class of compounds.

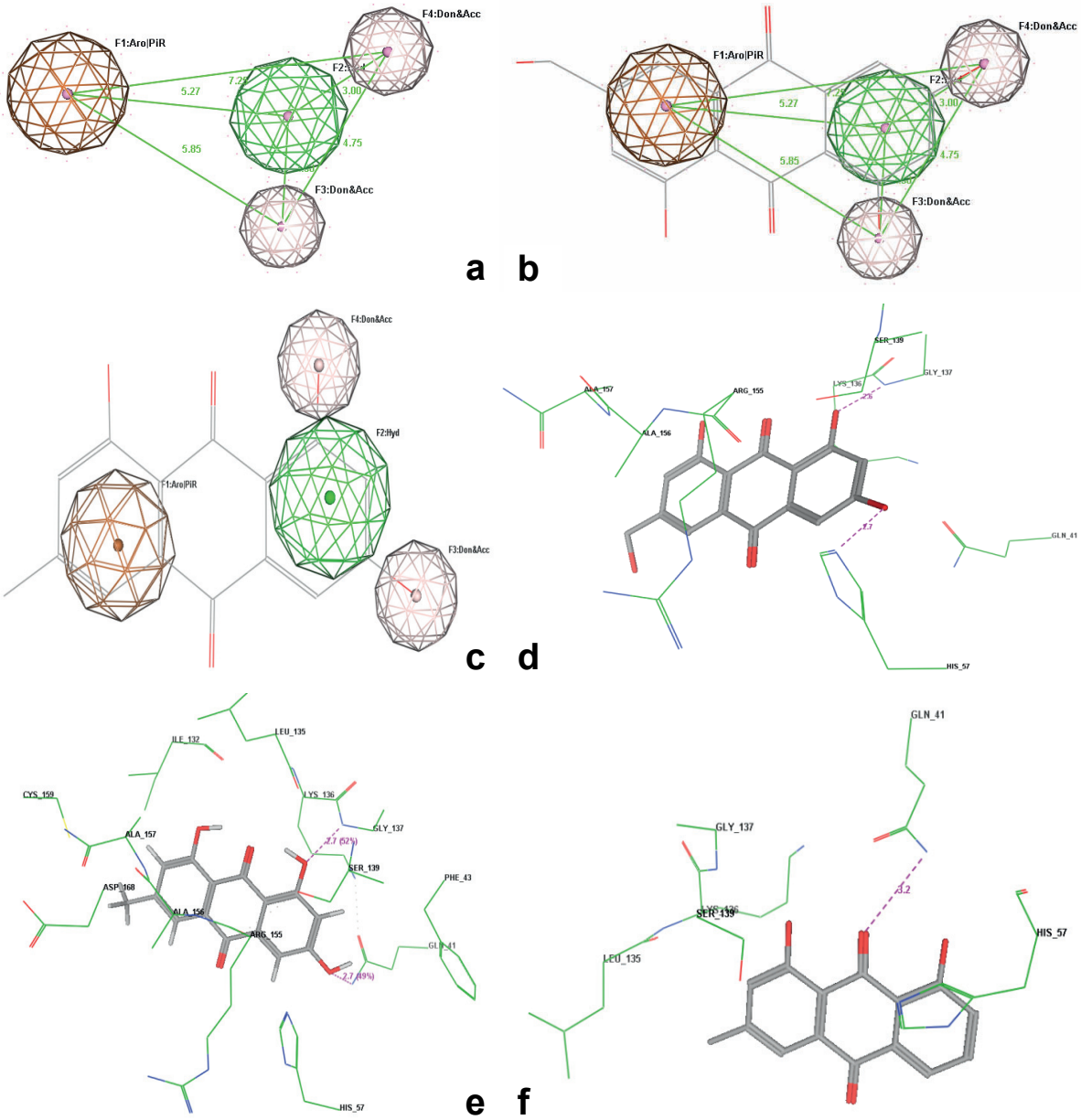

Fig. 2. Pharmacophore model and docking: Requirement of structural features in anthraquinones as HCV protease inhibitors and docking of the isolated compounds to the HCV NS3-NS4A protease active site. H-bonds are represented by dashed lines, H-bonding length is measured in angstrom. (a) Pharmacophore features of anthraquinone, distances between features are given in angstrom. (b) Superimposition of the pharmacophoric features on compound **3**. (c) Superimposition of the pharmacophoric features on compound **2**. (d) Docking of compound **3**. (e) Docking of compound **2**. (f) Docking of compound **4**. Aro, aromatic; Pir, π-ring; Hyd, hydrophobic; Don&Acc, donor and acceptor.

Due to the lack of any information on the structural requirements of anthraquinones as HCV PR inhibitors, the significant pharmacophoric features of these compounds were determined and verified by docking the active compounds to the HCV PR active site.

Pharmacophore elucidation

The generated pharmacophore model consists of four essential structural features (Fig. 2a), where the presence of aromatic and hydrophobic moieties in the anthraquinone nucleus is important for activity. In addition, the presence of *meta*-coupled H-bond donors and/or acceptors is essential for binding to H-bond donors and acceptors in the HCV PR active site (Fig. 2a). Screening of the isolated compounds on this suggested pharmacophore revealed the lack of these features in all compounds except compounds **2** and **3** (Figs. 2b and c).

Docking study

It is well established that interactions between the HCV PR active site and conventional electrophiles such as aldehydes, ketones, α-ketoacids, and α-ketoamides, followed by trapping of the resulting covalently bound intermediate by the active site triad (Ser139, His57, and Asp81), will provide effective inhibition (Njoroge *et al*., 2008). Additionally, it has been reported that the fitting of ligand functional groups to the shallow, solvent-exposed active site of the protease through other forces, such as van der Waals, aromatic, H-bonding, and hydrophobic interactions, plays an important role in the inhibition of HCV PR (Njoroge *et al*., 2008). The co-crystal structure ligand 2A4Q used in this study inhibits HCV PR by forming a reversible covalent bond between the enzyme active site Ser139 hydroxy and the ketone carbonyl group of the inhibitor (code 2A4Q). This compound also forms multiple H-bonds with Gln41, Gly137, and Ala153 in the protease active site through its amide chain (Chen *et al*., 2005). There is little information available on the binding of naturally occurring nonpeptidomimetic compounds to HCV PR and no information at all regarding the binding of anthraquinones to HCV PR (El Dine *et al*., 2011).

Compound **3** is predicted to bind with the *meta*-coupled phenolic hydroxy groups to Gln41 and His57, one of the active triad amino acids in the active site, which could be one reason for its higher activity (Fig. 2 d). Compound **2** is predicted to form a H-bond between the *meta*-coupled phenolic hydroxy groups and Gln41 and Gly137 (Fig. 2e). On the other hand, compound **4** binds only to Gln41 through its carbonyl group, while failing to bind to any other amino acid due to the lack of *meta*-coupled phenolic hydroxy groups (Fig. 2f).

These docking results are in accordance with the proposed pharmacophore model regarding the significance of the hydroxy groups as H-bond donors and/or acceptors (Fig. 2a). Most of the active compounds in this study are predicted to form H-bonds with amino acids other than Ser139, and the distance between the functional group and the hydroxy group of Ser139 was not sufficient to permit the formation of a covalent bond. This may be the reason why these compounds are not as potent as the positive control or the inhibitor used in the docking study (Chen *et al*., 2005).

Conclusion

The ethyl acetate extract from the endophytic *P. chrysogenum* has been established here as a source of a variety of natural compounds with diverse biological activities, such as antimicrobial, anticancer, and anti-HCV PR effects. The main constituents of the extract were anthraquinones and nitrogenous compounds.

Anthraquinones are a group of widely distributed natural constituents with diverse pharmacological activities, such as anticancer (Yang *et al*., 2013), antimicrobial (Rhea *et al.*, 2012), and hepatoprotective effects (Byun *et al.*, 2007). Emodin (**2**), as an example, exhibited anticancer activity through induction of apoptosis (Hsu and Chung 2012; Liu *et al*., 2012), antiviral activity against herpes simplex (Xiong *et al*., 2011), and inhibition of HIV-1 reverse transcriptase (Kharlamova *et al*., 2009). Moreover, emodin was identified as a potent inhibitor of casein kinase (CK2), a target in neurodegenerative diseases and cancer therapy, with an IC_{50} value of 2 μM (Yim *et al*., 1999).

Surprisingly, even though there is little if any similarity between the active sites of HCV PR (serine protease), CK2 (protein kinase), and HIV-1 reverse transcriptase, emodin was a potent inhibitor of all these targets. The possible explanation for this finding, as revealed by our pharmacophore model, could be the diversity of the

functional groups in the emodin molecule. The presence of H-bond donors and acceptors, such as the phenolic hydroxy groups which can bind to the nitrogen atoms of several amino acid residues, *e.g.* histidine, in the active sites of these enzymes could be cited as an example. In addition, the presence of electrophilic moieties, such as the carbonyl group, is considered important for the attack of amino acids with nucleophilic hydroxy groups, as in serine. Moreover, the rigidity of the molecule and its hydrophobic and aromatic moieties are important for aromatic interactions with amino acids such as phenylalanine. The presence of this wide variety of functional groups makes most anthraquinones promising candidates for enzyme inhibition and small ligand-protein interactions.

Finally, the pharmacophore model proposed here can serve as a tool in the ligand-based virtual screening of other anthraquinones for their possible anti-HCV PR effects.

Thus, we introduce emodin (**2**) and ω-hydroxyemodin (**3**) as promising lead HCV PR inhibitors for further semi-synthetic modification aiming at increasing their potency and selectivity.

Material and Methods

General experimental procedures

Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and silica gel (60–120 mesh; Qualigens, Mumbai, India) were used for CC. Culture media of Czapek agar and potato dextrose broth were procured from Lab M (Bury, Lancashire, UK). *n*-Hexane, ethyl acetate, and methanol were used for CC. Flash chromatography was carried out on silica gel (230–400 mesh). TLC was performed on Polygram SIL G/UV_{254} plates (Macherey-Nagel, Düren, Germany). Mixtures of methanol and methylene chloride (3:2 and 1:1, v/v) were used as mobile phase for TLC analysis. Compounds were visualized as intense dark blue and yellow coloured spots on TLC plates under UV light. Most of the coloured spots changed their colour after spraying with vanillin/H_2SO_4 followed by heating at 120 °C. UV/Vis spectra were recorded on a Shimadzu model UV-240 spectrometer (Tokyo, Japan). NMR spectra were measured on a Jeol ECA 300 (Tokyo, Japan) (^{1}H, 300 MHz; ^{13}C, 75.4 MHz) and a Varian Inova 500 (International Equipment Trading Ltd, Vernon Hills, IL, USA) (^{1}H, 500 MHz; ^{13}C, 125.7 MHz) spectrometer, respectively. ESI-mass spectra were recorded on a Finnigan LCQ ion trap mass spectrometer (San Jose, CA, USA).

Enzymes and chemicals

The Sensolyte™ 520 HCV protease assay kit fluorimetric (Lot# AK71145–1020), HCV NS3-NS4A protease, HCV NS3-NS4A protease inhibitor 2 (cat# 25346), and Sensolyte™ Green protease assay kit fluorimetric (Lot# AK71124–1011) were purchased from AnaSpec (San Jose, CA, USA). Soybean trypsin-chymotrypsin inhibitor was purchased from Sigma-Aldrich (St. Louis, MO, USA). Falcon™ Microtest™ 384-well 120 μl black assay plates (nonsterile, no lid) were purchased from Becton Dickinson (Tokyo, Japan).

Fungal isolation and culture conditions

The red alga *Liagora viscida* (Forsskål) C. Agardh was collected from the Egyptian Red Sea at a depth of 5–8 m off the coast of Rass Mohamed (South Sinai, Egypt) in March 2010. The sample was selected solely on the basis of a clean and healthy exterior, and brought to the laboratory in ice. In the laboratory, specimens were washed with sterile water and processed immediately. The sample was identified by the Coral Reef Ecology and Biology Group, National Institute of Oceanography and Fisheries, Suez, Egypt.

After proper washing, samples were cut into 0.5- to 1-cm pieces, surface-sterilized with 70% ethanol for 1 min, and rinsed three times with sterile distilled water. The algal pieces were blotted on sterile blotting paper. Each piece was placed in a Petri dish on the surface of solid potato dextrose agar (PDA) medium containing (g/l) potato (200), glucose (10), and agar (15), at pH 7.5, prepared in 50% sea water supplemented with penicillin benzyl sodium salt (0.02 g/l) to avoid any bacterial growth.

Morphological and molecular identification of the endophytic isolate

After 1 d of incubation in PDA medium, sandy-brown, velvety colonies were observed. The strain was identified as *Penicillium chrysogenum* based on the morphological features of its conidiophores, and a voucher specimen of the fungus was deposited at the Microbiology Department, Assiut University, Assiut, Egypt.

The mycelium was scraped directly from the surface of the PDA culture (1 d old) and weighed. DNA was extracted and purified, using the GenElute™ DNA isolation kit for genomic DNA (Sigma-Aldrich), by the method of Chomczynski (1993). For identification of the fungal species, the ITS regions ITS1 and ITS4 and the intervening 5.8S rDNA region were amplified and sequenced by electrophoretic sequencing on a 3130-genetic analyzer (Fermentas, Glen Burnie, MD, USA; taq polymerase, dntps) using the GenJET™ sequencing kit (Sigma-Aldrich). The DNA fragment of the ITS regions was amplified using the polymerase chain reaction (PCR) with the pair of primers ITS1 [5'-GGAAGGG(G/A)TGTATTATTAG-3'] and ITS4 (5'-GTAAAAGTCCTGGTTCCC-3'). Multiple sequence alignment was performed and the molecular phylogeny established using BioEdit (Hall, 1999). According to sequencing similarities and multiple alignment, the fungus was found to be closely related to *P. chrysogenum* strain CBS 306.48 (ac: GU733359.1) with 91% identity. DNA sequencing was carried out by Sequencer Scientific Bourg El-Arab, Alexanderia, Egypt.

Extraction and isolation of compounds

The isolated fungus *P. chrysogenum* was cultivated on Czapek's peptone liquid medium containing (g/l) glucose (30), yeast (2), peptone (10), $NaNO_3$ (3), KH_2PO_4 (0.5), and KCl (0.5), in 50% sea water at room temperature. Twenty one-day-old fermentation broth (12 l) was separated from the fungal mat by filtration. The fungal mycelia were suspended in distilled water for easy blending by a dispersing tool (Ultra Turrax model 25; IKA, Staufen, Germany) at 8000 min^{-1} and the homogenate was extracted with ethyl acetate. The resultant extract was evaporated to dryness using a rotavapor at 40 °C, followed by de-fatting with *n*-hexane.

The crude extract was chromatographed over a silica gel column using *n*-hexane as starting nonpolar eluent; the polarity was gradually increased with ethyl acetate as polar solvent in the eluent mixture (5%, 10%, until 100% ethyl acetate, v/v), followed by methanol/ethyl acetate (1:4 and 1:1, v/v. The combined fractions were further purified by preparative TLC (CH_3OH/CH_2Cl_2, 5:95) and semi-preparative HPLC using a C18 column, eluted with acetonitrile/water (90:10, isocratic method, 1.5 ml/min), and a Sephadex LH-20 column, eluted with CH_3OH, CH_3OH/CH_2Cl_2 (1:1 and 2:3), and CH_3OH/CH_2Cl_2/*n*-hexane (2:2:1), to yield pure compounds **1**–**12**, which were subjected to analysis by mass and NMR spectroscopy.

Alatinone (1,5,7-trihydroxy-3-methylanthraquinone) (**1**): Orange powder. – Yield: 7 mg. – TLC: R_f = 0.76 (CH_3OH/CH_2Cl_2, 5:95). – ^{1}H NMR (DMSO-d_6, 600 MHz): δ = 12.05 (2H, br, 8-OH), 12.0 (2H, br, 1-OH), 7.66 (1H, br, H-4), 7.53 (1H, d, J = 2.3 Hz, H-8), 7.25 (1H, br, H-2), 7.05 (1H, d, J = 2.3 Hz, H-6), 2.53 (3H, s, 3-CH_3). – ^{13}C NMR (DMSO-d_6, 150 MHz): δ = 189.7 (C-9), 181.2 (C-10), 163.2 (C-7), 161.4 (C-1), 160.8 (C-5), 148.5 (C-3), 134.4 (C-4a), 132.9 (C-10a), 124.0 (C-2), 120.5 (C-4), 113.5 (C-9a), 112.2 (C-8a), 111.3 (C-6), 111.1 (C-8), 21.4 (3-CH_3). – (–)-ESI-MS: m/z = 269 (100%) $[M - H]^-$.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) (**2**): Red powder. – Yield: 26 mg. – TLC: R_f = 0.70 (CH_3OH/CH_2Cl_2, 5:95). – ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 12.14 (2H, br, 1- & 8-OH), 7.39 (1H, d, J = 1.2 Hz, H-4), 7.09 (1H, d, J = 1.2 Hz, H-2), 6.96 (1H, d, J = 2 Hz, H-5), 6.19 (1H, d, J = 2 Hz, H-7), 2.41 (3H, s, 6-CH_3). – ^{13}C NMR (DMSO-d_6, 125.7 MHz): δ = 188.3 (C-9), 181.4 (C-10), 164.7 (C-3), 161.2 (C-1/8), 147.4 (C-3), 134.7 (C-10a), 132.6 (C-4a), 123.7 (C-2), 120.0 (C-4), 113.3 (C-9a), 110.3 (C-8a), 107.7 (C-5), 107.4 (C-7), 21.3 (6-CH_3). – (–)-ESI-MS: m/z = 269 (100%) $[M - H]^-$.

ω-Hydroxyemodin (1,3,8-trihydroxy-6-hydroxymethylanthraquinone) (**3**): Red powder. – Yield: 12 mg. – TLC: R_f = 0.72 (CH_3OH/CH_2Cl_2, 5:95). – ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 12.09 (2H, br, 1- & 8-OH), 7.58 (1H, d, J = 1.3 Hz, H-4), 7.18 (1H, d, J = 1.3 Hz, H-2), 7.05 (1H, d, J = 2.3 Hz, H-5), 6.51 (1H, d, J = 2.3 Hz, H-7), 4.57 (2H, s, 6-CH_2). – ^{13}C NMR (DMSO-d_6, 125.7 MHz): δ = 189.2 (C-9), 181.3 (C-10), 166.5 (C-3), 164.5 (C-1), 161.3 (C-8), 152.5 (C-3), 134.8 (C-10a), 132.7 (C-4a), 120.7 (C-2), 116.9 (C-4), 114.0 (C-9a), 109.2 (C-5), 108.4 (C-8a), 107.8 (C-7), 61.8 (6-CH_2). – (–)-ESI-MS: m/z = 285 (100%) $[M - H]^-$.

Chrysophanol (1,8-dihydroxy-3-methylanthraquinone) (**4**): Orange powder. – Yield: 18 mg. – TLC: R_f = 0.68 (CH_3OH/CH_2Cl_2, 5:95). – ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 12.01 (1H, s, OH-8), 11.99 (1H, s, OH-1), 7.74 (1H, d, J = 7.5 Hz, H-5), 7.61 (1H, d, J = 7.5 Hz, H-6), 7.55

(1H, br, H-4), 7.23 (1H, d, J = 7.5 Hz, H-7), 7.01 (1H, br, H-2), 2.41 (3H, s, 3-CH_3). – ^{13}C NMR (DMSO-d_6, 125.7 MHz): δ = 192.3 (C-9), 181.8 (C-10), 162.5 (C-1), 162.2 (C-8), 149.1 (C-3), 136.8 (C-6), 133.6 (C-10a), 133.2 (C-4a), 124.3 (C-7), 124.2 (C-2), 121.2 (C-4), 119.8 (C-5), 115.7 (C-9a), 113.5 (C-8a), 22.3 (3-CH_3). – (–)-ESI-MS: m/z = 253 (100%) $[M - H]^-$.

2',3'-Dihydrosorbicillin (**5**): Light yellow powder. – Yield: 8 mg. – TLC: R_f = 0.6 (CH_3OH/CH_2Cl_2, 5:95). – ^{1}H NMR (acetone-d_6, 300 MHz): δ = 13.0 (1H, s, 2-OH), 7.49 (1H, s, H-6), 5.47 (2H, m, H-4'/H-5'), 2.94 (2H, t, J = 7.2 Hz, H_2-2'), 2.31 (2H, m, H_2-3'), 2.31 (3H, d, J = 0.8 Hz, 5-CH_3), 2.07 (3H, s, 3-CH_3), 1.59 (3H, m, H_3-6'). – ^{13}C NMR (acetone-d_6, 125.7 MHz): δ = 204.6 (C-1'), 162.2 (C-2), 160.9 (C-4), 130.9 (C-4'), 129.5 (C-6), 126.2 (C-5'), 116.2 (C-5), 113.2 (C-3), 111.5 (C-1), 37.8 (CH_2-2'), 28.1 (CH_2-3'), 18.1 (CH_3-6'), 16.1 (5-CH_3), 8.1 (3-CH_3). – (–)-ESI-MS: m/z = 233 (100%) $[M - H]^-$. – (+)-ESI-MS: m/z = 257 (22%) $[M + Na]^+$, 487 (100%) $[2M + Na]^+$.

Meleagrin (**6**): White powder. – Yield: 17 mg. – TLC: R_f = 0.26 (CH_3OH/CH_2Cl_2, 5:95). – ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 12.6 (1H, s, 19-NH), 8.23 (1H, s, H-18), 7.54 (1H, s, H-8), 7.52 (1H, d, J = 7.6 Hz, H-4), 7.24 (1H, s, H-20), 7.23 (1H, t, J = 7.6 Hz, H-6), 7.23 (1H, s, H-15), 7.05 (1H, t, J = 7.6 Hz, H-5), 6.95 (1H, d, J = 7.6 Hz, H-7), 6.09 (1H, br, H-22), 5.46 (1H, s, 14-NH), 5.02 (2H, d, J = 18 & 13 Hz, H-23), 3.71 (3H, s, 1-OCH_3), 1.31 (3H, s, H_3-24), 1.19 (3H, s, H_3-25). – ^{13}C NMR ($CDCl_3$, 125.7 MHz): δ = 165.7 (C-13), 159.6 (C-9), 159.6 (C-10), 146.9 (C-22), 142.2 (C-7a), 136.7 (C-18), 133.4 (C-3a), 128.6 (C-4/8), 125.9 (C-16), 125.0 (C-6), 123.9 (C-5/20), 113.3 (C-12), 112.0 (C-7), 109.4 (C-23), 108.2 (C-15), 102.6 (C-2), 65.2 (1-OCH_3), 53.8 (C-3), 42.7 (C-21), 24.0 (C-25), 23.7 (C-24). – (–)-ESI-MS: m/z = 432 (100%) $[M - H]^-$. – (+)-ESI-MS: m/z = 434 (100%) $[M + H]^+$, 456 (20%) $[M + Na]^+$, 867 (40%) $[2M + H]^+$, 889 (25%) $[2M + Na]^+$.

Chrysoginone [2-acetylquinazolin-4(3H)-one] (**7**): White powder. – Yield: 14 mg. – TLC: R_f = 0.34 (CH_3OH/CH_2Cl_2, 5:95). – ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 10.01 (1H, s, 3-NH), 8.35 (1H, d, J = 8 Hz, H-8), 7.84 (1H, t, J = 8 Hz, H-5), 7.83 (1H, d, J = 8 Hz, H-7), 7.60 (1H, t, J = 8 Hz, H-6), 2.71 (3H, s, 2-$COCH_3$). – ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ = 193.8 (2-CO), 160.2 (C-4), 147.9 (C-8a), 144.6 (C-3), 134.3 (C-7), 129.3 (C-5), 129.1 (C-8), 126.8 (C-6), 123.4 (C-5a), 23.2 (2-$COCH_3$). – (+)-ESI-MS: m/z = 211 (25%) $[M + Na]^+$, 399 (100%) $[2M + Na]^+$, 587 (100%) $[3M + Na]^+$.

Antimicrobial activity

The antifungal and antibacterial activities of the ethyl acetate extract of *P. chrysogenum* and the isolated compounds were determined using the agar diffusion method with 6 mm paper disks loaded with 100 and 400 μg of the extract and each pure compound, respectively (Cosentino *et al.*, 1999). The metabolites were tested against the following indicator strains: *Bacillus megaterium*, *Bacillus cereus*, *Bacillus subtilis*, and *Staphylococcus aureus* (Gram-positive bacteria) and *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Escherichia coli* (Gram-negative bacteria), as well as *Candida albicans*, *Saccharomyces cerevisiae*, and *Aspergillus niger* (fungi). Oxytetracycline was used as a positive control.

Cancer cell line assays

An *in vitro* cell-based assay, using murine L1210 (leukemia), C38 (colon), and CFU-GM (normal) cells and human HCT-116 (colon), H-125 (lung), CCRF-CEM (leukemia), and HEP-G2 (liver) cells, was employed to assess the general and differential cytotoxicity of the pure compounds (Valeriote *et al.*, 2002). Samples were dissolved in 250 μl of dimethyl sulfoxide (DMSO), and 15-μl aliquots were applied in duplicate to cellulose disks in agar plates containing the respective cells. After a period of incubation, the zone of cell colony inhibition (z) was measured from the edge of each disk to the edge of colony growth, and expressed as zone units (zu), where 200 zu $\triangleq$ 6 mm. General cytotoxic activity for a given sample was defined as an antiproliferation zone of 300 zu or greater. The differential cytotoxicity of a pure compound was expressed by observing a zone differential of 250 units or greater between any solid tumour cell (murine colon C38, human colon HCT-116, human lung H-125, human liver HEP-G2) and either leukemia cells (murine L1210 or human CCRF-CEM) or normal cells (CFU-GM).

Assay for determination of HCV protease inhibitory activity

Samples of 2 μl of each compound dissolved in DMSO were placed in each well of a 384-well

microplate, then 8 μl of recHCV PR (0.5 μg/ml) were added, and the plate was briefly agitated. Finally, 10 μl of the freshly prepared substrate [Ac-Asp-Glu-Dap (QXLTM520)-Glu-Glu-Abu-COO-Ala-Ser-Cys(5-FAMsp)-NH_2] were added with sequential rotational shaking. The reaction mixture was incubated for 30 min at 37 °C. The fluorimetric analyses were performed on an automated TECAN GENios plate reader (Männedorf, Switzerland) with excitation wavelength at 485 nm and emission wavelength at 530 nm. Each compound was tested in triplicate. HCV PR inhibition (%) was calculated using the following equation:

$$\text{inhibition (\%)} = (F_{\text{substrate}} - F_{\text{test}}) \cdot 100/F_{\text{substrate}},$$

where $F_{\text{substrate}}$ is the fluorescence of the substrate and enzyme without test compounds, and F_{test} is the fluorescence of the assay mixture with the added compound.

Green protease assay

Compounds **1**–**10** were dissolved in DMSO (2.5 μl; final content, 10%, w/v) and placed in the wells of a 384-well microplate. Then 17.5 μl of assay buffer and 2.5 μl of trypsin (0.1 U/μl) were added and the plate was briefly agitated. Finally, 2.5 μl of the freshly diluted protease substrate Hi-Lyte Fluor™ 488-labeled casein were added under sequential rotary shaking and the mixture incubated at 37 °C for 30 min. The positive control was the soybean trypsin-chymotrypsin inhibitor. Inhibition was calculated as for HCV.

Pharmacophore elucidation for the HCV PR inhibitors

The pharmacophore model was generated using the pharmacophore elucidation function of MOE software 10/2008 (Chemical Computing Group, Montreal, Canada). Prior to running the pharmacophore elucidation, a database of all isolated molecules and their IC_{50} values was prepared. The 3D conformations of the compounds were generated using the conformation import function of the software with the default MMFF94x forcefield for energy minimization. In the pharmacophore elucidation function the activity field was adjusted to the respective IC_{50}. The features used were Aro/Pir with radius 1.4 Å, Hyd (1.4 Å), Don and Acc (1.0 Å), Cat and Ani (1.0 Å), and O2 (1.4 Å) (for abbreviations see legend to Fig. 2). The query cluster was adjusted to 1.25 and conformation to As-Is.

Docking study

The docking study was carried out using MOE software 10/2008. The crystal structure of HCV NS3-NS4A protease (2A4Q) was downloaded from the protein data bank (www.pdb.org). The 3D structures of the tested compounds were generated using the ligx function of the MOE program followed by energy minimization of the generated structures. The crystal structure of HCV PR was prepared for the docking study using the protonate 3D function adjusting the temperature to 300 K and the pH value to 7. The electrostatic functional form was used for calculation; electrostatic interactions were computed using GB/VI (generalized born/volume integral formalism) between two atoms if their separation distance was smaller than the cutoff value of 10 Å. The electrostatic constants of the solute and solvent were set to 1 and 80, respectively. The van der Waals forces were set to 800R3. The energy was minimized using the MMFF94x force field. The active site was detected using the surface and mapping function of the program. Docking was carried out by setting the placement to triangle matcher, rescoring 1 to London dG and its retain to 10, refinement to force field and rescoring 2 to none, and its retain to 10. The parameters were selected as they gave the best redocking result for the co-crystal ligand with the root mean square deviation (RMSD) equal to 0.5 Å.

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