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Synthesis and anti-viral activity of a series of sesquiterpene lactones and analogues in the subgenomic HCV replicon system

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Dedicated to Professor Iwao Ojima, SUNY at Stony Brook, Stony Brook, NY 11794, USA, on the occasion of his 60th birthday.

Abstract—Hepatitis C virus (HCV) infection is a severe liver disease that often leads to liver cirrhosis and hepatocellular carcinoma (HCC). Current therapy is inadequate to conquer this viral disease. In this study, we identified parthenolide (**1**), an active component in feverfew, a popular remedy for fever and migraine, as a lead compound with an EC_{50} value of 2.21 μM against HCV replication in a subgenomic RNA replicon assay system. Parthenolide is able to potentiate the interferon α-exerted anti-HCV effect. Several commercially available sesquiterpene lactones (**2**–**5**) structurally analogous to parthenolide and a series of synthesized Michael-type adducts of parthenolide (**12**–**18**) also exhibit micromolar concentrations for anti-HCV activities. Structure–activity relationship was elucidated to reveal that the spatial arrangement of the terpenoid skeleton fused with an α-methylene-γ-lactone moiety produces maximal anti-HCV activity. In addition, a strong anti-HCV potency indicates a possibility of secondary amino adducts (**12**–**18**) converting back to parthenolide or being replaced by the nucleophilic residues of proteins inside cells. This work shows that screening of natural products is a viable and fast way for identifying novel molecular diversity as potential drug leads.

1. Introduction

Hepatitis C virus (HCV) was first identified in 1989 as the etiologic agent for non-A, non-B hepatitis and is currently recognized as the leading cause of chronic liver disease.[1] It is estimated that more than 170 million individuals worldwide are afflicted by chronic HCV infection, out of which 20–30% of infected individuals may develop cirrhosis and 1–3% may develop liver cancer.[2] Moreover, end-stage liver associated with HCV has been the leading cause of liver transplantation among adults in western countries. Interferon-α (IFN-α) or its pegylated form, in combination with ribavirin, is the only recommended treatment for HCV infection at present. This combination therapy is limited by its partial efficacy and the adverse effects in individuals with underlined diseases. Given the highly unmet therapeutic (medical) needs, development of new anti-HCV agents has commanded significant attention from academia and the pharmaceutical industry.

A number of advances in the HCV anti-viral research have aimed at the specific virus molecular targets utilizing in vitro assays. These targets include: (i) processing of the viral polyprotein by the NS2-NS3 and NS3-NS4A proteases; (ii) viral RNA replication that uses the NS3 helicase and the NS5B RNA-dependent RNA polymerase (RdRp).[3] The in vitro enzyme-based assays, however, are not informative in cell permissibility and metabolic stability. Screening of anti-HCV agents based on the viral replication in cells would be an alternative method to discover novel anti-viral agents. However, such an approach for anti-HCV drug discovery has been hampered by the inability to efficiently propagate HCV in cell culture. This obstacle was overcome, to a degree, by the recent development of bicistronic subgenomic HCV RNA replicon cells by Drs. Bartenschlager's and

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Rice's laboratories.[4,5] Thus, utilizing HCV subgenomic replicon system as a cell-based screening platform has become an alternative strategy to identify potential lead compounds against HCV and is rigorously employed as a surrogate system.[6] To alleviate the cumbersome way of measuring the viral genome level by Northern blot or PCR-based assay, we have developed an engineered reporter cell line that produces the secreted alkaline phosphatase (SEAP) in proportion to the replication efficiency of the resident HCV subgenomic replicon in the cells.[7,8] The reporter-based cell line (Ava5-EG-(Δ4AB)SEAP) for HCV drug screening was derived from HCV replicon cells (Ava5). Ava5 cells were engineered to contain a reporter gene, EG(Δ4AB)SEAP, and the stable cell line is designated as Ava5-EG (Δ4AB)SEAP. In the EG(Δ4AB)SEAP reporter gene, the enhanced green fluorescent protein (EGFP) was fused to secreted alkaline phosphatase (SEAP) through the NS3/4A protease decapeptide recognition sequence, Δ4AB, which spans the HCV NS4A and NS4B junction region. A high-throughput screening format would thus be amenable by simply measuring the SEAP activities in cell culture supernatants. To examine the effect of HCV inhibition, we treated the engineered HCV replicon cells, Ava5-EG(Δ4AB)SEAP, with the test compounds and harvested the cell culture supernatants for analysis of SEAP activity. Hits obtained from the reporter-based assay were confirmed further by Northern blot analysis in the parental Ava5 cells for their anti-HCV activities (Fig. 1).

For centuries, natural products have served as a major source of drugs and many of these, such as morphine, penicillin G, and quinine, still remain the cornerstones of modern pharmaceutical care. The rapid developments in this area after the 1980s were attributed to major advancements in molecular biology where mechanism-based assays were available for measuring very specific and selective activities.[9] In an effort to discover novel anti-HCV agents, we started with the screening of 'Pure Natural Products Library,' a commercially available library from MicroSource Discovery Systems Inc,[10] consisting of a collection of 720 pure natural products and their derivatives, including simple and complex oxygen heterocycles, alkaloids, sequiterpenes, diterpenes, pentercyclic triterpenes, sterols, and other diverse representatives. We herein report the investigation of novel lead compounds from a series of natural sesquiterpene lactones utilizing HCV subgenomic replicon cell-based assays as screening system. One of the few leads, parthenolide, showed less than 50% of replication at 10 μM concentration. The chemical structure of parthenolide is shown in Figure 2a. Parthenolide dose-dependently decreased the SEAP activity in Ava5-EG(Δ4AB)SEAP cells (Fig. 2b). Less than 10% of SEAP activity was shown as compared to untreated cells when Ava5-EG(-Δ4AB)SEAP cells were treated with 5 μM parthenolide. Northern blot analysis was also used to ascertain the effect of parthenolide on the inhibition of HCV replication. Parental HCV replicon Ava5 cells were treated with parthenolide and harvested 72 h after drug treatment. Subsequently, the total cellular RNAs were extracted and analyzed using Northern blot analysis. As shown in Figure 2c, there was a clear dose-dependent decline in HCV subgenomic RNA levels upon parthenolide treatment, while the mRNA level of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), remained unchanged.

Parthenolide (**1**), a sesquiterpene lactone (Fig. 3), is the putative active ingredient of feverfew (*Tanacetum parthenium*), one of the most commonly used medicinal herbs in the United States.[11] It is a remedy advocated for fever, arthritis, migraine, and is sometimes also used for the treatment of skin disorders, urinogenital complaints, as well as for relief from morning sickness.[12,13] Although parthenolide (**1**) has a wide spectrum of biological activities, it has never been reported to possess any anti-viral activity. Taking a lead from parthenolide, a sesquiterpene lactone, several other commercially available sesquiterpene lactones, including costunolide (**2**), dehydrocostus lactone (**3**), helenalin (**4**), alantolactone (**5**),

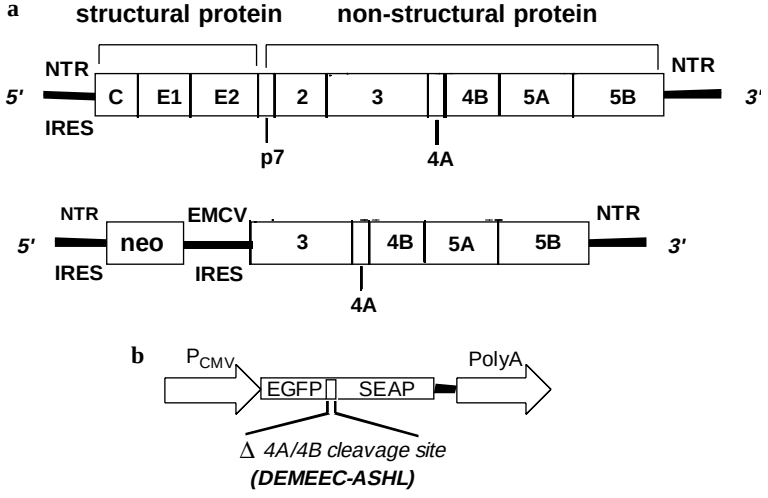


Figure 1. Anti-HCV assay system. (a) Schematic representation of HCV full-length (upper) and subgenomic (lower) genome structures. (b) The construct of EG(Δ4AB)SEAP reporter gene. The Ava5-EG(Δ4AB)SEAP cells were produced by stable integration of the EG(Δ4AB)SEAP reporter gene into Ava5 cells.

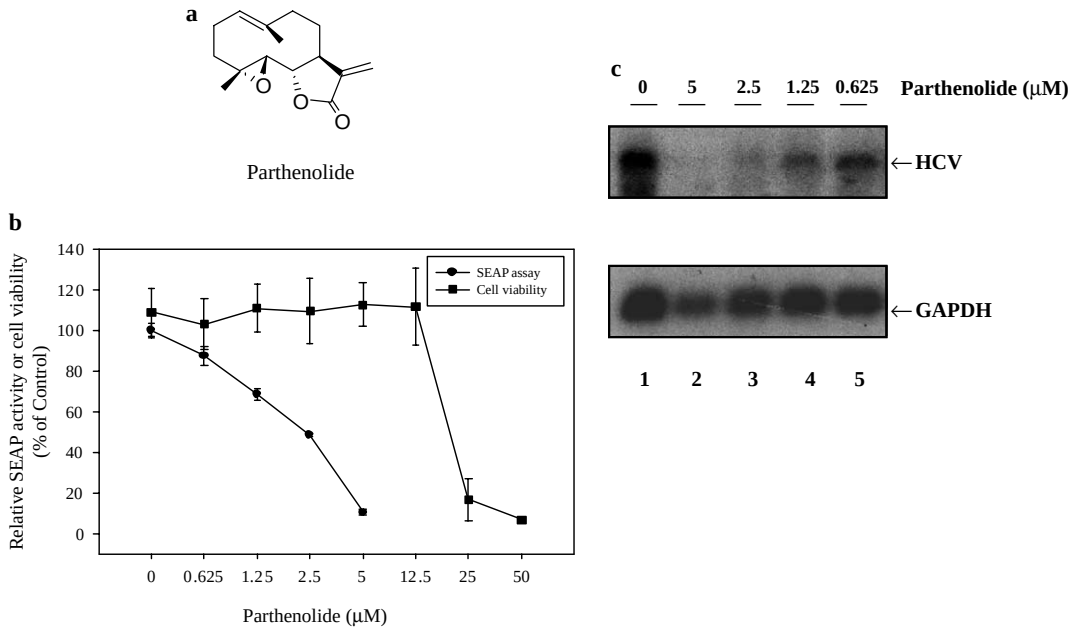


Figure 2. Inhibition of HCV replication by parthenolide (**1**). (a) Chemical structure of parthenolide (**1**). (b) Using Ava5-EGFP(Δ4AB)SEAP cells, parthenolide (**1**) was found to possess potent anti-HCV activity at concentrations that did not cause cellular toxicity. The cellular toxicity was evaluated by MTS assay. Results are expressed as means ± standard derivatives (SD) of three replicate wells. (c) Northern blot analysis of HCV replicon RNAs extracted from Ava5 cells after treatment with 0, 5, 2.5, 1.25, and 0.625 μM parthenolide (**1**). RNAs was extracted 3 days after drug treatment. The amount of GAPDH transcripts was used as a control for mRNA expression in cells treated with parthenolide (**1**). Arrows indicated the HCV replicon RNA and GAPDH RNA.

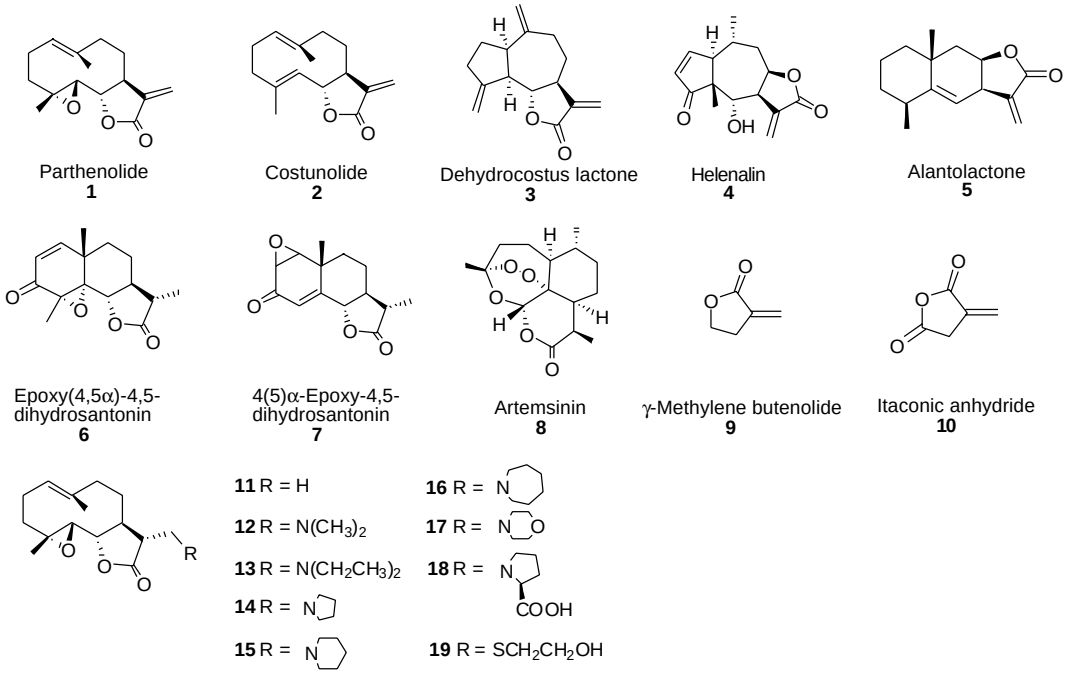


Figure 3. Some sesquiterpene lactones (**1**–**8**), exo-methylene lactones (**9**–**10**), and parthenolide analogues (**11**–**19**).

epoxy(4,5α)-4,5-dihydrosantonin (**6**), 4(5)α-epoxy-4,5-dihydrosantonin (**7**), and artemsinin (**8**) were also examined for their anti-HCV activities.

It is generally perceived that the α-methylene-γ-lactone moiety of sesquiterpene lactones is responsible for their biological effects due to its interaction with biological nucleophiles, such as cysteine sulfhydryl groups of target proteins, by a Michael-type addition.[14–16] Cushman and co-workers in 1995 proposed that several Michael-adducts formed by the reaction with secondary amines could be considered as prodrugs of an anticancer agent,

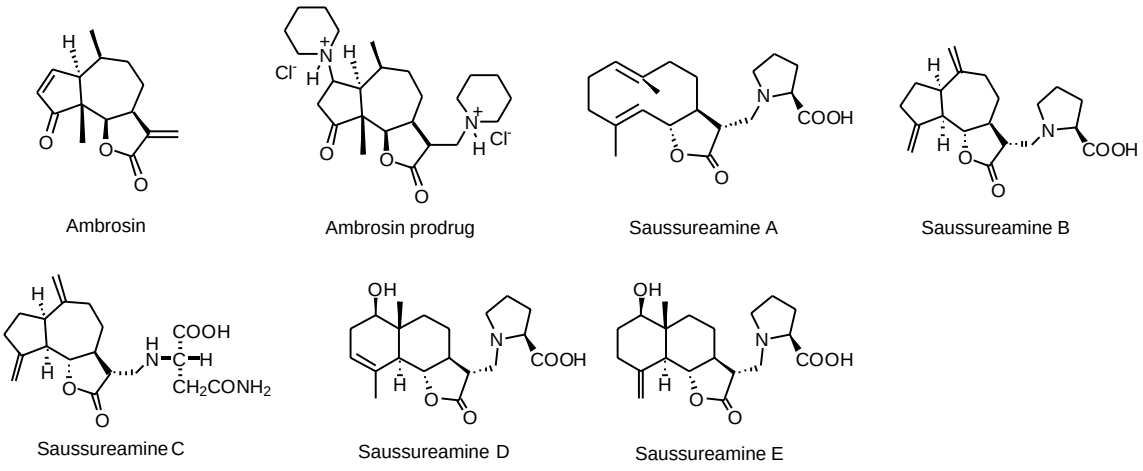


Figure 4. An anticancer agent, ambrosin, and 5 saussureamines isolated from *S. lappa*.

ambrosin.[17] Yoshikawa et al. recently isolated five saussureamines **A**–**E** from Chinese *Sassurea lappa*, which were natural Michael-adducts formed by the reaction of amino acids with the corresponding sesquiterpene lactones.[18] (Fig. 4) These observations prompted us to prepare α-methylene-γ-lactones **10**–**11** and a series of sesquiterpene lactone analogues bearing Michael-adducts, using several secondary amines and 2-mercaptoethanol, to understand the importance of α-methylene-γ-lactone moiety in the anti-HCV activities of various sesquiterpene lactones.

2. Results and discussion

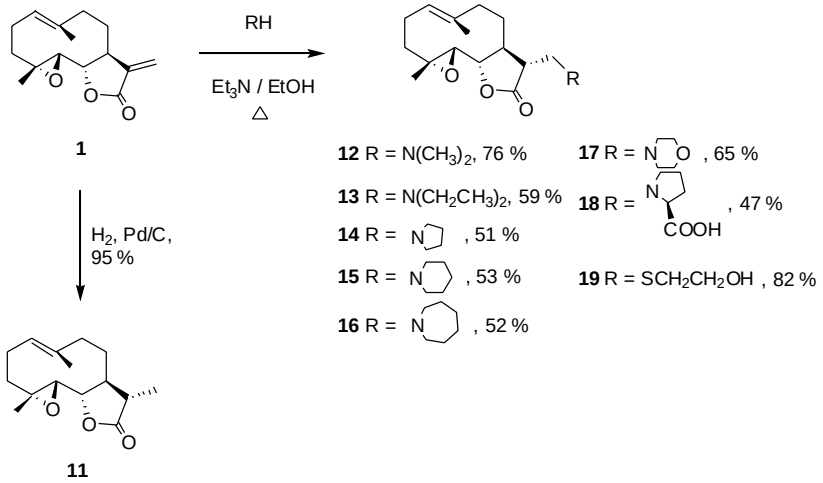
2.1. Chemistry

To understand the importance of the exceptional reactivity of exo-methylene lactone functionality with nucleophilic groups,[19] we first synthesized the reduced parthenolide (**11**),[20] which was obtained in 95% yield by catalytic hydrogenation in the presence of palladium on charcoal. The general synthetic method shown in Scheme 1 was employed for the preparation of the corresponding Michael-adducts **12**–**19**, utilizing several secondary amines including dimethylamine, diethylamine, pyrrolidine, piperidine, 1-aminohomopiperidine, morpholine, and L-proline, as well as one sulfhydryl group from 2-mercaptoethanol, in 47–82% yields.

To unambiguously assign the configuration of the synthesized Michael-adducts, a single crystal X-ray analysis was performed (Fig. 5). Three X-ray crystal structure determinations confirmed that the configuration of the new chiral center at β carbon position in **13**, **17**, and **19** is *S*. None of the other diastereoisomers (with an *R* configuration) was present in the crude reaction mixture. These findings were also consistent with previous observations for the Michael-adducts of alantolactone, costunolide, and dehydrocostus lactone reported in the literature.[21,22] The Michael-type addition was found to occur from the exo-face of the convex-shaped molecule.

2.2. Biology

2.2.1. In vitro subgenomic HCV inhibition assay. Compounds **1**–**10** were evaluated for their anti-HCV activities by analyzing SEAP activities from Ava5-



Scheme 1. Synthesis of parthenolide analogues (**11**–**19**).

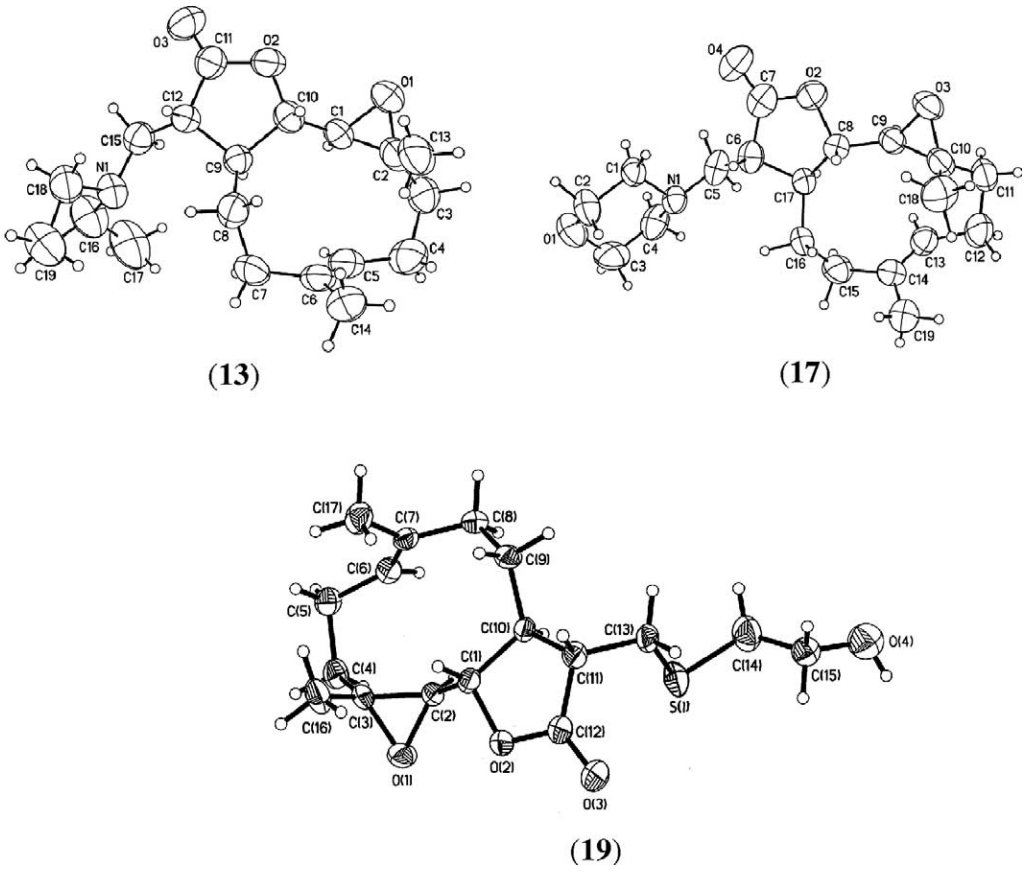


Figure 5. The X-ray crystal structures of compounds **13**, **17**, and **19**.

EG(Δ4AB)SEAP cells. A comparison of EC_{50} values for sesquiterpene lactones **1**–**8**, α-methylene-γ-lactone (**9**), and itaconic anhydride (**10**) is given in Table 1. The EC_{50} values represent the drug concentrations producing a 50% decrease in virus replication, as revealed by a decline in the HCV RNA levels after 3 days of treatment.

Anti-HCV activities of parthenolide (**1**), costunolide (**2**), dehydrocostus lactone (**3**), helenalin (**4**), and alantolactone (**5**) showed a similar potency with EC_{50} values of 1–3 μM, while compounds **6**, **7**, and **8** exhibited EC_{50} values higher than 10 μM, indicating that exo-methylene lactone functionality may be responsible for their anti-HCV activities. Furthermore, α-methylene-γ-lactone (**9**) and itaconic anhydride (**10**) were inactive, suggesting that the spatial arrangement of the terpenoid skeleton fused with α-methylene-γ-lactone moiety enhances the anti-HCV activity. Although the presence of epoxide moiety is of significance in anti-inflammatory activity,[23,24] parthenolide (**1**) and costunolide (**2**) showed similar anti-HCV activity, indicating that the epoxide moiety of parthenolide played a less important role in the anti-HCV activity.

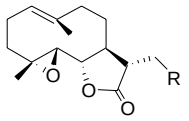
Table 1. Anti-HCV effects of eight sesquiterpene lactones (**1**–**8**) and two exo-methylene lactones (**9**–**10**)

Sesquiterpene lactones	EC_{50} (μM)
Parthenolide (**1**)	2.21 ± 0.15
Costunolide (**2**)	2.69 ± 0.57
Dehydrocostus lactone (**3**)	3.08 ± 0.60
Helenalin (**4**)	1.25 ± 0.35
Alantolactone (**5**)	2.03 ± 0.15
Epoxy(4,5α)-4,5-dihydrosantonin (**6**)	>10
4(5)α-epoxy-4,5-dihydrosantonin (**7**)	>10
Artemsinin (**8**)	>10
α-methylene-γ-lactone (**9**)	>10
Itaconic anhydride (**10**)	>10

The presence of α-methylene-γ-lactone was reported to be essential for the significant cytotoxic activity,[23,25] activation of NF-κB,[26] and the antitumor activity[27,28] of sesquiterpene lactones. To further understand the role of exo-methylene lactone in sesquiterpene lactones in anti-HCV activity, we synthesized a series of parthenolide analogues **11**–**19** starting from parthenolide, focusing on the modification of the exo-methylene moiety, as shown in Table 2.

The loss of anti-HCV activity in a parthenolide analogue **11**, where the methylene group, was reduced to a methyl group was somewhat expected. However, it was rather surprising not to find any significant difference in the EC_{50} values for most of the Michael-adducts **12**–**18**, without the exo-methylene functionality, as compared to the parthenolide (**1**). In an attempt to investigate the transformation of parthenolide analogues in vitro or in cells, the cell lysates from the assays were collected and analyzed by LC/MS/MS for the presence of either parent analogues or parthenolide. Results

Table 2. Anti-HCV effects of parthenolide (**1**) and its analogues (**11**–**19**)



Compounds	EC_{50} (μM)
Parthenolide (**1**)	2.21 ± 0.15
R = H (**11**)	>10
R = $N(CH_3)_2$ (**12**)	2.22 ± 0.97
R = $N(CH_2CH_3)_2$ (**13**)	2.51 ± 1.19
R = pyrrolidine (**14**)	2.82 ± 0.57
R = piperidine (**15**)	1.64 ± 0.01
R = 1-aminohomopiperidine (**16**)	2.05 ± 0.56
R = morpholine (**17**)	5.41 ± 2.1
R = L-proline (**18**)	2.24 ± 0.41
R = 2-mercaptoethanol (**19**)	>10

showed the presence of parthenolide with no signs of residual parent compounds in the cell lysates. The anti-HCV activity of these Michael-adducts therefore may have resulted from either the conversion to parthenolide, in vitro or in cells, or with the direct replacement by cellular proteins. This result is consistent with the results reported by Matsuda et al.,[29] wherein the amino acid sesquiterpene conjugates exhibited the same heat shock responses as the respective unsubstituted sesquiterpene lactones. The 2-mercaptoethanol adduct (**19**) has an activity lower than those of all other amino Michael-adducts, possibly due to the lesser likelihood of its converting back to the parthenolide or it being more difficult to replace by the cellular proteins, since the thio-group is a weaker electrophilic leaving group than a secondary amino group.

2.3. Combination effect with INF-α and parthenolide (1)

A successful anti-viral treatment mandates cocktail therapy. Thus, we examined if parthenolide (**1**) would enhance the anti-HCV activity of IFN-α using an HCV replicon system. The results indicated that there was at least an additive anti-HCV effect when parthenolide (**1**) was used in combination with IFN-α. Figure 6 shows the relative SEAP activity after Ava5 cells were treated with IFN-α alone or in combination with parthenolide (**1**) in different doses for 3 days. In the absence of parthenolide and IFN-α, the relative HCV RNA levels were designated as 100%. The results showed that the efficacy of combination of 5 IU/mL IFN-α and 1.25 μM parthenolide (**1**) in inhibiting HCV replication was similar to that of 10 IU/mL IFN-α alone. Parthenolide (**1**) therefore could possibly enhance the IFN-α-exerted anti-HCV activity in a dose-dependent manner.

3. Conclusion

We have successfully identified parthenolide (**1**), an active component in feverfew, as a lead compound with an EC_{50} value of 2.21 μM against HCV replicon assay from a library of 720 natural products. The natural products library provides a unique set of compounds with a unique and quite an extensive chemical diversity. Based on the structure of the promising lead, parthenolide (**1**), commercially available sesquiterpene lactones (**2**–**8**), and synthesized Michael-adducts (**11**–**19**) of parthenolide, were evaluated to elucidate the structural–activity relationships. X-ray crystal structures confirmed that these Michael-adducts hold *S* configuration at the β chiral carbon position. Structure–activity relationships showed that the spatial arrangement of the terpenoid skeleton fused with an α-methylene-γ-lactone moiety is required for good anti-HCV activity. In addition, a series of secondary amino adducts (**12**–**18**) bearing at β position also showed anti-HCV activities at micromolar concentration, possibly by converting back to parthenolide or by being replaced by the nucleophilic residues of protein in the biological system. These results suggest that an exo-methylene lactone functionality may play an important role in parthenolide's anti-HCV activity.

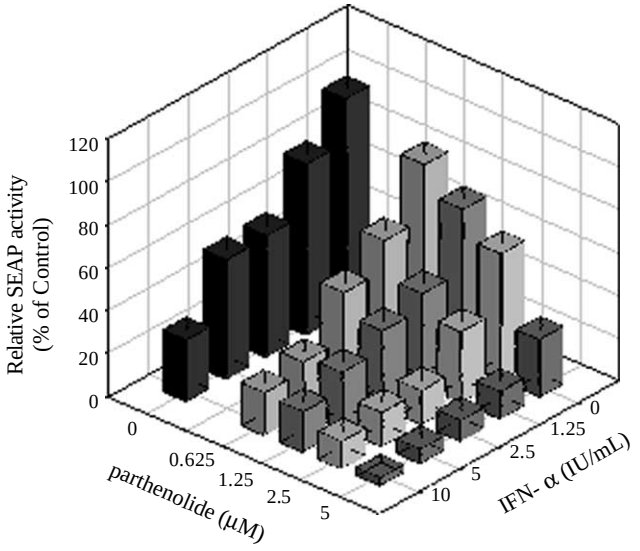


Figure 6. Anti-HCV activities of parthenolide (**1**) and IFN-α in combination. The anti-HCV effects of parthenolide (**1**) and IFN-α in combination were evaluated using the reporter-based cells, Ava5-EG(Δ4AB)SEAP. Ava5-EG(Δ4AB)SEAP cells were treated with these two drugs in combination in a checkerboard titration manner. After drug treatment as described in Section 4, SEAP activities in culture supernatants were measured. Dose–response inhibition of HCV RNA replication was evaluated for varying parthenolide (**1**) concentrations (0, 0.625, 1.25, 2.5, and 5.0 μM) in the presence of various doses of IFN-α (0, 1.25, 2.5, 5, and 10 IU/mL). Results are expressed as means of three replicate wells.

4. Experimental

4.1. Chemistry

High-resolution mass spectra (HRMS) were recorded on a Finnigan (MAT-95XL) spectrometer. Elemental analyses were performed on a Heraeus CHN-O Rapid microanalyzer. ^1H NMR spectra were obtained with a Varian Mercury-300 spectrometer operating at

300 MHz; all values are reported in ppm (δ) downfield from $(CH_3)_4Si$. Flash chromatography was performed on silica gel (Merck Kieselgel 60, No. 9385, 230–400 mesh ASTM). TLC was carried out on silica gel plates (E. Merck 60 F_{254}); zones were detected visually by ultraviolet irradiation (254 nm) or by spraying with phosphomolybdic acid reagent (Aldrich Chemical Co., Milwaukee, WI), followed by heating at 100 °C. All reagents were used as purchased unless otherwise stated. All solvents were dried, according to standard procedures. All reactions were carried out under an atmosphere of dry nitrogen.

Pure Natural Products Library tested for inhibiting HCV replication was obtained from MicroSource Discovery Systems, Inc. (Gaylordsville, CT). The compound library consists of a unique collection of pure natural products and their derivatives. The collection includes simple and complex oxygen heterocycles, alkaloids, sesquiterpenes, diterpenes, pentercyclic triterpenes, sterols, and many other diverse representatives. Parthenolide and helenalin were purchased from Calbiochem (San Diego, CA). Costunolide and dehydrocostus lactone were purchased from Wako Pure Chem. Ltd. (Osaka, Japan). Alantolactone was purchased from Pfaltz-Bauer (New Hyde Park, NY). All other chemicals were purchased from Sigma (St. Louis, MO).

4.1.1. 4,8,12-Trimethyl-3,14-dioxa-tricyclo[9.3.0.0^{2,4}] tetradec-7-en-13-one (11).[20] The title compound was obtained in 95% yield by utilizing catalytic palladium on charcoal and parthenolide (**1**) in the presence of hydrogen gas. ^{1}H NMR (300 MHz, $CDCl_3$): δ1.16–2.46 (m, 19H), 2.71 (d, J = 9.3 Hz, 1H), 3.81 (t, J = 9.0 Hz, 1H), 5.17 (dd, J = 2.1, 12.0 Hz, 1H).

4.1.2. General procedure for the preparation of substituted parthenolide analogues (12–19). The mixture of parthenolide (**1**) (50 mg, 0.20 mmol) and various secondary amines or 2-mercaptoethanol (0.22 mmol) in EtOH (6 mL) was stirred at room temperature overnight. The reaction mixture was quenched by adding water (3 mL) and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to give the residue, which was purified further by silica gel column chromatography to obtain the desired products **12**–**19**.

4.1.2.1. 12-Dimethylaminomethyl-4,8-dimethyl-3,14-dioxa-tricyclo[9.3.0.0^{2,4}]tetradec-7-en-13-one (12). The title compound was obtained by following the above general procedure in the presence of dimethylamine in 76% yield after column chromatography (EtOAc/*n*-hexane = 1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.17–2.46 (m, 23H), 2.68 (ddd, J = 4.6, 13.5, 36.3 Hz, 1H), 2.74 (d, J = 9.3 Hz, 1H), 3.82 (t, J = 9.0 Hz, 1H), 5.19 (dd, J = 2.4, 12.0 Hz, 1H). HRMS (EI, M^+) calcd for $C_{17}H_{27}NO_3$, 293.1991; found, 293.1992. Anal. ($C_{17}H_{27}NO_3 \cdot 0.3C_2H_2O_4$) C, H, N.

4.1.2.2. 12-Diethylaminomethyl-4,8-dimethyl-3,14-dioxa-tricyclo[9.3.0.0^{2,4}]tetradec-7-en-13-one (13). The title compound was obtained by following the above general procedure in the presence of diethylamine in 59% yield after column chromatography (EtOAc/*n*-hexane = 1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.01 (t, J = 7.2 Hz, 6H), 1.17–2.59 (m, 20H), 2.72 (d, J = 9.0 Hz, 1H), 2.80 (ddd, J = 4.9, 14.1, 29.8 Hz, 2H), 3.81 (t, J = 9 Hz, 1H), 5.15 (dd, J = 2.4, 12 Hz, 1H). HRMS (EI, M^+) calcd for $C_{19}H_{31}NO_3$, 321.2304; found, 321.2309. Anal. ($C_{19}H_{31}NO_3 \cdot 0.1H_2O$) C, H, N.

4.1.2.3. 4,8-Dimethyl-12-pyrrolidin-1-ylmethyl-3,14-dioxa-tricyclo[9.3.0.0^{2,4}]tetradec-7-en-13-one (14). The title compound was obtained by following the above general procedure in the presence of pyrrolidine in 51% yield after column chromatography (CH_2Cl_2/EtOAc = 1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.17–1.33 (m, 24H), 2.74 (d, J = 9.0 Hz, 1H), 2.89 (qd, J = 4.6, 12.6 Hz, 2H), 3.82 (t, J = 8.9 Hz, 1H), 5.19 (dd, J = 2.4, 12.0 Hz, 1H). HRMS (EI, M^+) calcd for $C_{19}H_{29}NO_3$, 319.2147; found, 319.2142. Anal. ($C_{19}H_{29}NO_3$) C, H, N.

4.1.2.4. 4,8-Dimethyl-12-piperidin-1-ylmethyl-3,14-dioxa-tricyclo[9.3.0.0^{2,4}] tetradec-7-en-13-one (15). The title compound was obtained by following the above general procedure in the presence of piperidine in 53% yield after column chromatography (EtOAc/*n*-hexane = 1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.18–2.49 (m, 26H), 2.70 (ddd, J = 5.3, 13.4, 43.8 Hz, 2H), 2.71 (d, J = 8.7 Hz, 1H), 3.82 (t, J = 9.0 Hz, 1H), 5.18 (dd, J = 2.2, 11.8 Hz, 1H). HRMS (EI, M^+) calcd for $C_{20}H_{31}NO_3$, 333.2304; found, 333.2305. Anal. ($C_{20}H_{31}NO_3 \cdot 0.1H_2O$) C, H, N.

4.1.2.5. 12-Azepan-1-ylmethyl-4,8-dimethyl-3,14-dioxa-tricyclo[9.3.0.0^{2,4}]tetradec-7-en-13-one (16). The title compound was obtained by following the above general procedure in the presence of 1-aminohomopiperidine in 52% yield after column chromatography (EtOAc/*n*-hexane = 1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.18–2.74 (m, 29H), 2.92 (t, J = 4.0 Hz, 2H), 3.82 (t, J = 8.8 Hz, 1H), 5.19 (dd, J = 2.1, 12.0 Hz, 1H). HRMS (EI, M^+) calcd for $C_{21}H_{33}NO_3$, 347.2460; found, 347.2507. Anal. ($C_{21}H_{33}NO_3 \cdot 0.3H_2O$) C, H, N.

4.1.2.6. 4,8-Dimethyl-12-morpholin-4-ylmethyl-3,14-dioxa-tricyclo[9.3.0.0^{2,4}]tetradec-7-en-13-one (17). The title compound was obtained by following the above general procedure in the presence of morpholine in 65% yield after column chromatography (EtOAc/*n*-hexane = 2:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.18–2.56 (m, 20H), 2.72 (d, J = 9.0 Hz, 1H), 2.75 (ddd, J = 5.4, 13, 26.1 Hz, 2H), 3.60 (t, J = 4.5 Hz, 4H), 3.83 (t, J = 9.0 Hz, 1H), 5.19 (dd, J = 2.4, 12.0 Hz, 1H). HRMS (EI, M^+) calcd for $C_{19}H_{29}NO_4$, 335.2097; found, 335.2098. Anal. ($C_{19}H_{29}NO_4 \cdot 0.1H_2O$) C, H, N.

4.1.2.7. 1-(4,8-Dimethyl-13-oxo-3,14-dioxa-tricyclo[9.3.0.0^{2,4}]tetradec-7-en-12-yl-methyl)-pyrrolidine-2-carboxylic acid (18). The title compound was obtained by following the above general procedure in the presence of L-proline in 47% yield after column chromatography (CH_2Cl_2/MeOH/H_2O = 15:4:1). ^{1}H NMR (300 MHz,

$CDCl_3$): δ 1.21–2.49 (m, 19H), 2.91 (d, J = 9.3 Hz, 1H), 3.07–3.71 (m, 4H), 3.84 (ddd, J = 3.0, 7.5, 10.8 Hz, 1H), 4.00 (dd, J = 4.8, 9.3 Hz, 1H), 4.15 (t, J = 4.8 Hz, 1H), 5.26 (dd, J = 2.1, 12 Hz, 1H). HRMS (EI, M^+-COOH) calcd for $C_{20}H_{29}NO_5$, 318.2069; found, 318.2082. Anal. ($C_{20}H_{29}NO_5$) C, H, N.

4.1.2.8. 12-(2-Hydroxy-ethylsulfanylmethyl)-4,8-dimethyl-3,14-dioxa-tricyclo[9.3.0.0^{2,4}]tetradec-7-en-13-one (19). The title compound was obtained by following the above general procedure in the presence of 2-mercaptoethanol in 82% yield after column chromatography (CH_2Cl_2/MeOH = 15:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.20–2.84 (m, 20H), 3.00 (ddd, J = 4.5, 13.8, 30 Hz, 2H), 3.79 (t, J = 5.4 Hz, 2H), 3.87 (t, J = 9.0 Hz, 1H), 5.21 (dd, J = 2.1, 12.0 Hz, 1H). HRMS (EI, M^+) calcd for $C_{20}H_{29}NO_5$ ($C_{17}H_{26}O_4S$), 326.1552; found, 326.1542. Anal. ($C_{17}H_{26}O_4S$) C, H.

4.2. Biology

Huh-7 cells containing HCV subgenomic replicons (Ava5) were provided by Apath, LLC (St. Louis, MO). The reporter-based HCV subgenomic replicon, Ava5-EG(Δ4AB)SEAP, has previously been described.[7,8] Cell culture reagents were obtained from Life Technologies (Gaithersburg, MD). Cell viability was determined by the MTS assay that was essentially as described.

4.2.1. Subgenomic HCV inhibitory assay. In 96-well plates, Ava5-EG(Δ4AB)SEAP cells were seeded at a density of 7×10^3 cells per well. After incubation at 37 °C for 1 day, cells were treated with various drugs at final 10 μM. Two days later, culture medium was replaced with fresh phenol red-free DMEM/10% FBS containing the same concentration of drugs and cells were incubated for one more day. Culture supernatants were collected from each well and SEAP activities were measured using Phospha-Light assay kit (Tropix, Foster City, CA), according to the manufacturer's instruction.

4.2.2. Northern blot analysis. In each 10 cm culture dish, we seeded around 1×10^6 Ava5 cells and maintained the cells in the culture medium supplemented with 1 mg/mL G418. One day after seeding, we treated the cells with drugs for 3 days and harvested cells for RNA extraction. Three micrograms of total RNA was denatured by treatment with 2 M formaldehyde/50% formamide at 55 °C for 15 min and separated by denaturing agarose gel electrophoresis. The gel was then transferred to a positively charged nylon membrane, BrightStar-Plus™ (Ambion, Austin, TX) by a vacuum blotter (Vacu. GeneXL, Pharmacia, MI). After drying, RNA was then cross-linked to membrane by UV irradiation (Stratagene, La Jolla, CA). The membrane was probed separately with the NS5B gene fragment of HCV and human glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene fragment labeled with [α-^{32}P]dCTP by rediprime™ II random prime labeling system (Pharmacia, MI) in accordance with manufacturer's instructions. Hybridization was carried out with denatured probes in Rapidhyb™ hybridization buffer (Pharmacia) at 65 °C for 2 h. After hybridization, membranes were washed once in 2× SSC–0.2% SDS at 60 °C for 20 min, once in 1× SSC–0.2% SDS at 60 °C for 20 min, and twice in 0.1× SSC–0.2% SDS at 65 °C for 15 min. The blots were visualized by autoradiography.

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References and notes

1. Choo, Q. L.; Kuo, G.; Weiner, A. J.; Overby, L. R.; Bradley, D. W., et al. *Science* **1989**, *244*, 359–362.
2. Lauer, G. M.; Walker, B. D. *N. Engl. J. Med.* **2001**, *345*, 41–52.
3. Locarnini, S. A.; Bartholomeusz, A. *J. Gastroenterol. Hepatol.* **2002**, *17*, 442–447.
4. Lohmann, V.; Korner, F.; Koch, J.; Herian, U.; Theilmann, L., et al. *Science* **1999**, *285*, 110–113.
5. Blight, K. J.; Kolykhalov, A. A.; Rice, C. M. *Science* **2000**, *290*, 1972–1974.
6. Yeh, C. T.; Hwang, D. R.; Lai, H. Y.; Hsu, J. T. *Biochem. Biophys. Res. Commun.* **2003**, *310*, 537–541.
7. Lee, J. C.; Shih, Y. F.; Hsu, S. P.; Chang, T. Y.; Chen, L. H., et al. *Anal. Biochem.* **2003**, *316*, 162–170.
8. Lee, J.-C.; Chang, C.-F.; Chi, Y.-H.; Hwang, D.-R.; Hsu, J. T. A. *J. Virol. Methods* **2004**, *116*, 27–33.
9. Clark, A. M. *Pharm. Res.* **1996**, *13*, 1133–1144.
10. MicroSource MicroSource Discovery Systems Inc; http://www.msdiscovery.com.
11. O'Hara, M.; Kiefer, D.; Farrell, K.; Kemper, K. *Arch. Fam. Med.* **1998**, *7*, 523–536.
12. Knight, D. W. *Nat. Prod. Rep.* **1995**, *12*, 271–276.
13. Groenewegen, W. A.; Knight, D. W.; Heptinstall, S. *Prog. Med. Chem.* **1992**, *29*, 217–238.
14. Baeuerle, P. A.; Baltimore, D. *Cell* **1988**, *53*, 211–217.
15. Ruben, S. M.; Dillon, P. J.; Schreck, R.; Henkel, T.; Chen, C. H., et al. *Science* **1991**, *251*, 1490–1493.
16. Schmitz, M. L.; Baeuerle, P. A. *Embo J.* **1991**, *10*, 3805–3817.
17. Hejchman, E.; Haugwitz, R. D.; Cushman, M. *J. Med. Chem.* **1995**, *38*, 3407–3410.
18. Yoshikawa, M.; Hatakeyama, S.; Inoue, Y.; Yamahara, J. *Chem. Pharm. Bull. (Tokyo)* **1993**, *41*, 214–216.
19. Hausen, B. M.; Schmalle, H. W. *Contact Dermatitis* **1985**, *13*, 329–332.
20. Neukirch, H.; Kaneider, N. C.; Wiedermann, C. J.; Guerriero, A.; D'Ambrosio, M. *Bioorg. Med. Chem.* **2003**, *11*, 1503–1510.
21. Matsuda, H.; Kagerura, T.; Toguchida, I.; Ueda, H.; Morikawa, T., et al. *Life Sci.* **2000**, *66*, 2151–2157.
22. Lawrence, N. J.; McGown, A. T.; Nduka, J.; Hadfield, J. A.; Pritchard, R. G. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 429–431.
23. Kupchan, S. M.; Eakin, M. A.; Thomas, A. M. *J. Med. Chem.* **1971**, *14*, 1147–1152.
24. Lyss, G.; Schmidt, T. J.; Merfort, I.; Pahl, H. L. *Biol. Chem.* **1997**, *378*, 951–961.
25. Rodriguez, E.; Towers, G. H.; Mitchell, J. C. *Phytochemistry* **1976**, *15*, 1573–1580.

26. Hehner, S. P.; Heinrich, M.; Bork, P. M.; Vogt, M.; Ratter, F., et al. *J. Biol. Chem.* **1998**, *273*, 1288–1297.
27. Kupchan, S. M.; Fessler, D. C.; Eakin, M. A.; Giacobbe, T. J. *Science* **1970**, *168*, 376–378.
28. Hanson, R. L.; Lardy, H. A.; Kupchan, S. M. *Science* **1970**, *168*, 378–380.
29. Matsuda, H.; Toguchida, I.; Ninomiya, K.; Kageura, T.; Morikawa, T., et al. *Bioorg. Med. Chem.* **2003**, *11*, 709–715.