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Synthesis and structure–activity relationships of the halovirs, antiviral natural products from a marine-derived fungus

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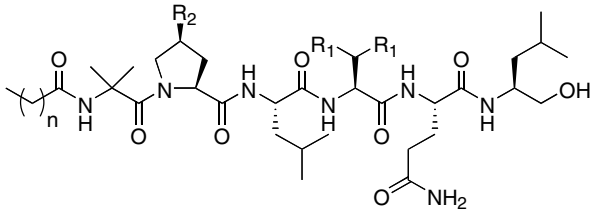
Abstract—The halovirs are linear, lipophilic peptides produced by a marine-derived fungus of the genus *Scytalidium*. We recently reported that these molecules possess potent in vitro activity against the herpes simplex viruses 1 and 2. Here we present structure–activity relationships defining key structural elements for optimal viral inhibition. Results demonstrate that an N^{α}-acyl chain of at least 14 carbons and an Aib-Pro dipeptide are critical for maintaining the antiviral activity.

1. Introduction

Herpes simplex viruses are well known for their ability to remain latent in their host for life and to reactivate and cause lesions near the initial site of infection.[1] The incidence of HSV-2, the serotype most commonly correlated with genital infection, has risen by 30% in the United States since the late 1970s, and infects one in five Americans above the age of twelve.[2] The frequency of HSV-1 associated genital herpes is increasing worldwide,[3,4] and it is becoming clear that herpes lesions facilitate the sexual transmission of other diseases such as HIV. An aspect of concern in current HSV chemotherapy is the emergence of drug resistant strains.[5,6] During viral replication in immunocompromised patients undergoing long-term antiviral chemotherapy, strains have evolved that are impervious to current drugs. Some of these strains have caused progressive disease in immunocompromised patients.[7] Clearly, the discovery of new HSV antivirals with novel modes of action is an important goal.

Recently, we reported a series of linear, lipophilic, neutrally charged peptides possessing antiviral activity from the laboratory cultivation of a marine-derived *Scytalidium* sp.[8] These peptides, designated halovirs A–E (Fig. 1), are potent in vitro inhibitors of the herpes simplex viruses 1 and 2. When HSV-1 is exposed to halovir A prior to cell infection, the peptide inactivates the virus in a time and concentration dependent manner, thereby demonstrating a virucidal mechanism of action. Thus, the halovirs are potential lead molecules in the development of a topical microbicide to ameliorate HSV infections and prevent viral transmission.

Here we report the results of a preliminary synthetic program undertaken to explore structural features critical for the anti-HSV activity of the halovir peptides. Our previous studies of the five natural halovirs hinted that both the length of the fatty acyl chain and the amino



halovir **A** R_1 = Me, R_2 = OH, n = 12
halovir **B** R_1 = H, R_2 = OH, n = 12
halovir **C** R_1 = Me, R_2 = H, n = 12
halovir **D** R_1 = Me, R_2 = OH, n = 10
halovir **E** R_1 = Me, R_2 = H, n = 10

Figure 1. Structures of the naturally occurring halovirs.

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acid sequence influence the antiviral potency and cytotoxic properties. These observations imply that strategic manipulation of the halovir molecular scaffold may lead to more potent and selective inhibitors with increased clinical potential.

2. Results

2.1. Synthesis of halovir analogs

The halovir A core peptide sequence (**1**) was constructed by solution-phase synthesis. Carbodiimide coupling of N^{α}-Boc-Gln-OH with HCl·H-Leu-OMe in MeCN was accomplished using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) in the presence of diisopropylethylamine (DIEA). The N^{α}-*t*-butyloxycarbonyl (Boc) protecting group was then cleaved from the dipeptide product with ethanolic HCl at −20 °C. Subsequent rounds of coupling and deprotection with the appropriate Boc-protected amino acids resulted in the synthesis of the key hexapeptide intermediate **1**.

The total synthesis of halovir A was completed as shown (Fig. 2). The coupling of the ammonium trifluoroacetate salt of **1** with myristic acid proceeded in 89% yield. The

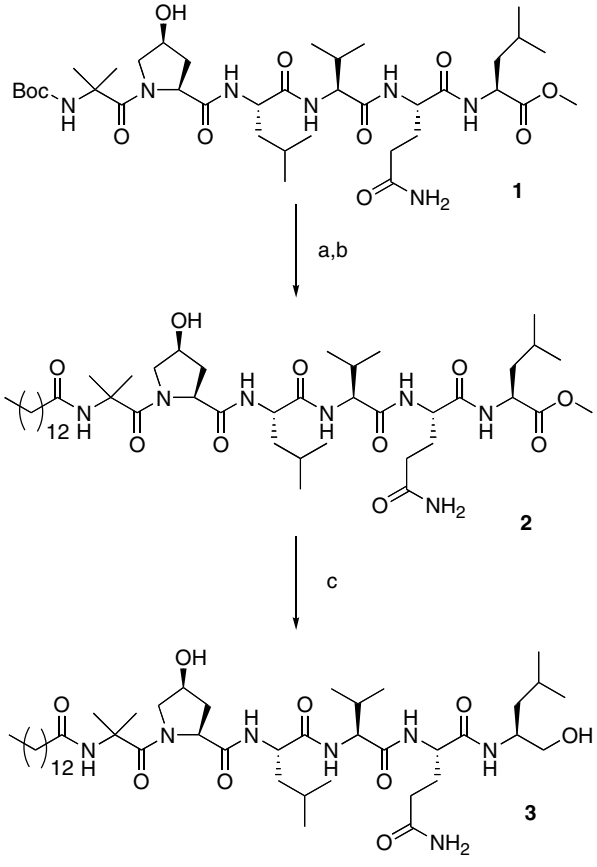

Figure 2. Synthesis of halovir A. Reagents and conditions: (a) TFA, room temperature, ∼quant.; (b) myristic acid, EDC, HOBt, DIEA, DMF, 89%; (c) $LiBH_4$, THF, 82%.

Table 1. Synthetic halovir analogs R_1-R_2-R_3-Leu-Val-Gln-R_4

Analog	R_1	R_2	R_3	R_4
2	Myristoyl	Aib	Hyp	Leu-OMe
4	Acetyl	Aib	Hyp	Lol
5	Hexanoyl	Aib	Hyp	Leu-OMe
6	Decanoyl	Aib	Hyp	Leu-OMe
7	Palmitoyl	Aib	Hyp	Leu-OMe
8	Stearoyl	Aib	Hyp	Leu-OMe
9	Myristoleoyl	Aib	Hyp	Leu-OMe
10	Linoleoyl	Aib	Hyp	Leu-OMe
11	Linolenoyl	Aib	Hyp	Leu-OMe
12	Juniperoyl	Aib	Hyp	Leu-OMe
13	Myristoyl	Ala	Hyp	Leu-OMe
14	Myristoyl	D-Ala	Hyp	Leu-OMe
15	Myristoyl	Aib	Sar	Leu-OMe
16	Myristoyl	Aib	Hyp	Leu-OEt

C-terminal methyl ester of compound **2** was then efficiently reduced with lithium borohydride to yield the desired target **3**. Synthetic halovir A was both chemically and biologically identical to the natural substrate. N^{α}-acyl analogs of halovir A were prepared using the identical methodology (Table 1). Most N^{α}-acyl analogs were tested as their C-terminal methyl esters due to the improved biological properties (see below). Halovir peptide congeners containing amino acid substitutions were prepared by similar synthetic strategies.

2.2. Biological activities of halovir analogs

Antiviral ED_{50} values of each analog were measured in an infectious virus, whole cell assay. Briefly, Vero cells were exposed to HSV-1 for 1 h, the infected cells were then treated with compound or control, and the virus-induced cytopathic effects were measured after five days. ED_{50} values were determined by averaging the results from at least 10 replicate assays.

None of the short sequences synthesized during the construction of the halovir A core peptide (**1**) were active against HSV-1. However, compound **2**, the C-terminal methyl ester derivative of the natural substrate, demonstrated nearly equivalent anti-HSV-1 activity ($ED_{50} = 2.3\,\mu M$), but slightly less cytotoxicity than halovir A (Table 2). The ethyl ester analog of **2** (**16**) was slightly more potent ($ED_{50} = 1.9\,\mu M$), and displayed a further increase in therapeutic index (TI = 14). Due to these improvements in TI, subsequent N^{α}-acyl analogs of halovir A were prepared and tested as their methyl esters.

Figure 3 summarizes the antiviral activities of several targets designed to determine the optimal length of the N^{α}-acyl chain. Biological evaluation of these derivatives clearly established that saturated lipophilic chains shorter than 14 carbons have reduced antiviral potencies. Acetyl (**4**) and hexanoyl (**5**) analogs were completely inactive, while the decanoyl derivative (**6**) was 3.5-fold less potent than compound **2**. The N^{α}-myristoyl (C14, **2**), N^{α}-palmitoyl (C16, **7**), and N^{α}-stearoyl (C18, **8**) analogs all demonstrated similar HSV-1 inhibition, indicating that increases in N^{α}-acyl chain length

Table 2. Biological activities of synthetic halovir analogs

Derivative	Antiviral ED_{50} (μM)	Vero cell IC_{50} (μM)[a]	TI[b]
2 (Methyl ester)	2.3	20	8.7
3 (Halovir A)	1.1	4.0	3.6
4 (Acetyl)	NSA[c]	NSA	
5 (Hexanoyl)	NSA	NSA	
6 (Decanoyl)	7.0	59	8.4
7 (Palmitoyl)	1.7	21	12
8 (Stearoyl)	1.6	25	15
9 (Myristoleoyl)	2.2	20	9.1
10 (Linoleoyl)	3.4	28	8.4
11 (Linolenoyl)	8.5	>42	>4.9
12 (Juniperoyl)	2.3	>43	>19
13 (L-Ala→Aib)	NSA	36	
14 (D-Ala→Aib)	9.3	48	5.1
15 (Sar→Hyp)	NSA	NSA	
16 (Ethyl ester)	1.9	28	14

[a] Cytotoxicity of compound against Vero cells.
[b] Therapeutic index = antiviral ED_{50}/Vero cell cytotoxicity IC_{50}.
[c] No significant activity.

beyond 14 carbons do not significantly enhance viral inhibition.

The effects of unsaturation in the lipophilic acyl chain were next investigated. Myristic and lauric acids weakly inhibit HSV-1 at 16 and 10 mM, respectively, whereas unsaturated linolenic (18:2) acid is more potent at 3.6 mM.[9] Therefore, we hypothesized that introducing unsaturation into the lipophilic chain of halovir A might lead to more potent HSV inhibitors. Myristoleic (**9**), linoleic (**10**), and linolenic (**11**) acyl derivatives were synthesized since their corresponding acids were commercially available. Myristoleic acid is a C14 fatty acid incorporating a single *cis*-double bond, while linoleic and linolenic acids are C18 fatty acids with two and three *cis*-double bonds, respectively. Figure 4 shows that increasing degrees of unsaturation in the N^{α}-acyl chain result in a loss of potency against HSV-1. These findings suggest a possible requirement for flexibility in the lipophilic chain. The inclusion of a primary hydroxyl

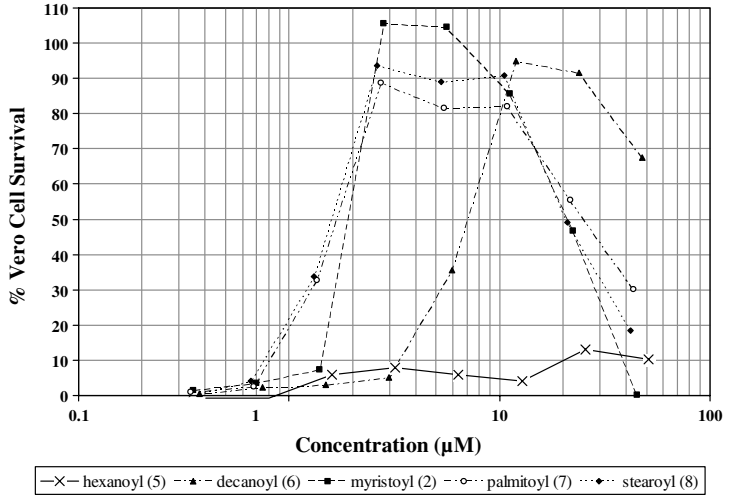

Figure 3. The effects of different lipophilic chain lengths on the anti-HSV-1 activities of halovir A methyl ester analogs.

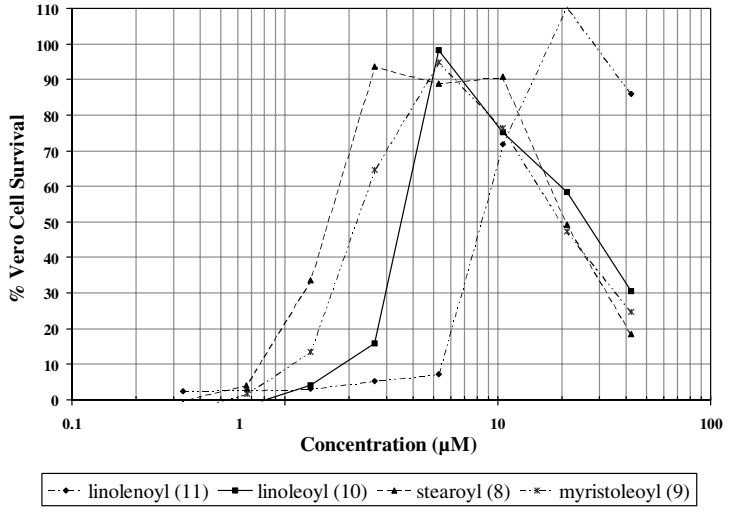

Figure 4. The effects of unsaturation in the lipophilic chain on the anti-HSV-1 activities of halovir A methyl ester analogs.

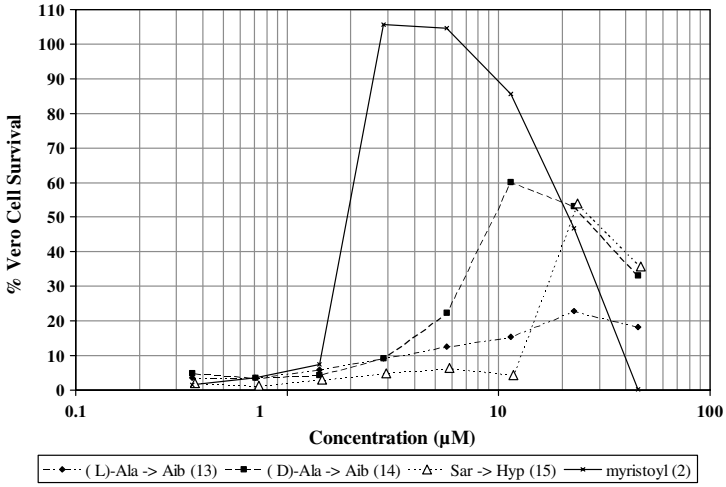

Figure 5. The effect of amino acid substitutions on the anti-HSV-1 activities of halovir A methyl ester analogs.

group on the acyl chain of the juniperoyl (16-hydroxyhexadecanoyl) analog (**12**) had little effect on antiviral potency (Table 2).

Other synthetic targets were designed to probe the importance of the Aib-Hyp dipeptide segment to the antiviral properties (Fig. 5). In DMSO, the halovir peptides adopt a 3_{10}-helical secondary structure leading to an overall amphipathic character.[8] The presence of an Aib residue likely contributes to the helical nature of the halovirs since the additional α-methyl group of Aib restricts the allowed regions of conformational space to those favorable for helices.[10,11] Analogs were synthesized that substituted L- and D-alanine for the N-terminal Aib unit of halovir A. Both L- and D-alanine derivatives were synthesized to determine if either methyl of the Aib residue was critical to the anti-HSV properties. A third target that substitutes sarcosine (Sar, also called *N*-methylglycine) for the hydroxyproline residue of halovir A was also synthesized. Sarcosine was selected as a replacement for Hyp because it induces flexibility into the peptide chain while maintaining the tertiary amide of the parent compound. Figure 5 illustrates the detrimental effects of these three amino acid substitutions on the anti-HSV-1 activity of the halovir peptides. The L-Ala (**13**) and Sar (**15**) halovir congeners were essentially inactive, while the D-alanine analog (**14**) was significantly less potent against HSV-1 than **2**.

Table 2 summarizes the antiviral and cytotoxic activities of various N^{α}-acyl, Leu ester analogs of halovir A.

3. Discussion

Analogs of the halovir peptides were synthesized and evaluated in vitro against HSV-1 in order to establish structure–activity relationships relevant to their antiviral properties. This study has shown that the N^{α}-myristoyl and N^{α}-lauroyl chains of the naturally occurring halovirs play a major role in the anti-HSV activity of these molecules. Specifically, an N^{α}-acyl chain of at least 14 carbons is required to maintain maximum potency. Shorter saturated chains of six or fewer carbons lost all observable antiviral and cytotoxic activities. Also, N^{α}-acyl chain length modulated the cytotoxic properties analogously to the measured differences in the antiviral properties, suggesting that similar mechanisms might be involved. These results suggest that it is unlikely that the antiviral mechanism of action involves the hexapeptide portion engaged in a receptor-binding phenomenon. Nonspecific interaction of the lipopeptides with the lipid envelope of the virus and cellular membrane remains a plausible explanation. Indeed, Toniolo et al. found a similar trend while investigating the significance of the N-terminal fatty acyl chain length of trichogin A IV peptide analogs on membrane destabilization.[12]

Congeners of halovir A incorporating unsaturated lipid chains were found to possess decreased anti-HSV-1 activity. The addition of one point of unsaturation, as demonstrated with the myristoleoyl derivative, only slightly affected HSV inhibition. However, the linoleoyl and linolenoyl targets were two and fivefold less active, respectively, than the saturated stearoyl compound. Thus, increases in unsaturation incrementally decreased the antiviral activity. Lipid chain flexibility may therefore be an important factor in HSV-1 inhibition. These results are in contrast to the antiviral activities of fatty acids themselves, where it was found that unsaturated linoleic (18:2) and arachidonic (20:4) fatty acids were more active against HSV-1 than saturated fatty acids.[9]

The modifications to the Aib-Hyp dipeptide segment in this study nearly abolished the antiviral activity. Substitution of the Aib residue with L-alanine resulted in a completely inactive compound (**13**). Interestingly, the D-alanine isomer (**14**) inhibited some HSV induced cell death, although a maximum of only 60% cell survival was achieved (11 μM). Elimination of the cyclic Hyp residue by substitution with a sarcosine (**15**) also resulted in an inactive peptide. A common effect of these amino

acid substitutions is reduced steric hindrance to rotation between the Aib and Hyp residues. The Aib-Hyp dipeptide has restricted rotation, and therefore may help stabilize the 3_{10}-helical conformation of the short halovir peptide.[13] The amphipathic nature of the halovir 3_{10}-helix should help to increase binding interaction with lipid membranes. Alternatively, Aib-Pro dipeptide segments have been proposed to act as molecular hinges in amphiphilic, membrane modifying peptides such as alamethicin.[13] The Aib-Pro kink may have a dynamic role in facilitating insertion of the peptides into lipid membranes by allowing movement between the N- and C-terminal helical segments.[13] However, it is not apparent what role an Aib-Pro hinge would play in these molecules since there is inherent flexibility in the long hydrocarbon chain.

In summary, several insights into the structure–activity relationships of these antiviral peptides have been established. The indispensability of a sufficiently long N^{α}-acyl chain has been established, and modification of the C-terminal group has been shown to modulate the toxicity of these compounds. Particularly intriguing is the requirement for the Aib-Pro dipeptide segment. Further conformational studies, especially of the natural substrate in the presence of a lipid bilayer, should provide further insight into why this dipeptide segment is crucial to the biological activity.

4. Experimental

Amino acids derivatives were purchased from Calbiochem–Novabiochem Corporation. All materials and reagents were of reagent grade and used without further purification. Reaction solvents (*N*,*N*-dimethylformamide (DMF), acetonitrile (MeCN), tetrahydrofuran (THF), and CH_2Cl_2) were anhydrous as purchased. Purifications were accomplished on a gradient HPLC system comprising a Waters Prep LC 4000 System equipped with a Knauer variable wavelength UV detector set to 210 nm. The column consisted of a Waters PrepLC 25 mm Module fitted sequentially with two 25×100 mm Prep Nova-Pak® C18 6 μm 60 Å cartridges. Flow rates were typically 12 mL/min. Normal-phase silica gel chromatography was performed using Merck Silica gel, 230–400 mesh, 60 Å. Electrospray mass spectrometry was accomplished on a HP 1100 MSD. The ^{1}H NMR data were recorded on either a Varian Inova 300 MHz or Gemini 400 MHz instrument.

4.1. Tetradecanoyl-(α-methyl)alanyl-(*trans*-4-hydroxy)-prolyl-leucyl-valyl-glutaminyl-leucine methyl ester(myristoyl-Aib-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Leu-OMe) (2)

Compound **1** (500 mg, 0.638 mmol) was treated with 10 mL of TFA at ambient temperature for 20 min, and then concentrated under reduced pressure. The resulting ammonium salt was dissolved in 5 mL DMF and treated with diisopropylethylamine (445 μL, 2.55 mmol, 4 equiv). In a separate flask, myristic acid (218 mg, 0.957 mmol, 1.5 equiv), EDC (183.5 mg, 0.957 mmol, 1.5 equiv), and HOBt (129 mg, 0.957 mmol, 1.5 equiv) were dissolved in 5 mL DMF and stirred under N_2 at ambient temperature for 10 min. The two solutions were then combined and stirred under N_2 overnight. The homogeneous reaction was purified directly by C18 HPLC using a 0–100% gradient of MeOH in H_2O, and the desired product **2** was obtained as a white foam (509 mg, 89% yield). ESI-MS $[M+H]^+ = 894.6$, $[M+Na]^+ = 916.6$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.85 (m, 21H), 1.22 (m, 20H), 1.34 (s, 3H), 1.35 (s, 3H), 1.45–1.75 (m, 10H), 1.89 (m, 1H), 2.09 (m, 3H), 2.18 (m, 3H), 3.20 (dd, $J = 12$, 2.9 Hz, 1H), 3.60 (s, 3H), 3.70 (d, $J = 12$ Hz, 1H), 4.06 (m, 2H), 4.25 (m, 3H), 4.38 (t, $J = 8.8$ Hz, 1H), 5.13 (br s, 1H), 6.75 (s, 1H), 7.22 (s, 1H), 7.24 (d, $J = 8.7$ Hz, 1H), 7.59 (d, $J = 7.8$ Hz, 1H), 7.88 (d, $J = 7.8$ Hz, 1H), 7.98 (d, $J = 7.8$ Hz, 1H), 8.66 (s, 1H).

4.2. Tetradecanoyl-(α-methyl)alanyl-(*trans*-4-hydroxy)-prolyl-leucyl-valyl-glutaminyl-leucinol(myristoyl-Aib-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Lol) (3)

Compound **2** (96.4 mg, 0.106 mmol) was dissolved in 4 mL anhydrous THF and treated with 250 μL $LiBH_4$ (2.0 M in THF). After 90 min of vigorous stirring under N_2, the TLC (CH_2Cl_2 9:1 MeOH, $KMnO_4$) showed no starting material remained. The reaction was quenched with 2 mL of MeOH and then concentrated in vacuo. The residue was taken up in MeOH, filtered, and purified by C18 HPLC (75–100% MeOH in H_2O, 15 min). Pure synthetic halovir A (75 mg) was obtained as the desired product. Yield 82%; $[\alpha]_D$ −10 (*c* 0.62, MeOH); MALDI FTMS $[M+Na]^+$ obsd *m*/*z* 888.6175; calcd 888.6144 for $C_{45}H_{83}N_7O_9Na$ (Δ 3.5 ppm).

4.3. Acetyl-(α-methyl)alanyl-(*trans*-4-hydroxy)prolyl-leucyl-valyl-glutaminyl-leucinol(acetyl-Aib-(*trans*-4-hydroxy)-Pro-Leu-Val-Gln-Lol) (4)

Intermediate **1** (40 mg, 0.050 mmol) was treated with saturated HCl in EtOH (−20 °C). After warming to ambient temperature, the reaction was concentrated in vacuo to yellow oil, and 1 mL of anhydrous pyridine, 1 mL of acetic anhydride, and a catalytic amount of 4-dimethylaminopyridine were added. The reaction was stirred at room temperature overnight, was concentrated under reduced pressure, and then subjected to reversed-phase HPLC (5–100% MeOH in H_2O, 25 min). The desired intermediate was obtained as a colorless oil (26.5 mg, 68%). TLC $R_f = 0.25$ in CH_2Cl_2 9:1 MeOH, $KMnO_4$. ESI-MS $[M+Na]^+ = 804$, $[M-H]^- = 780$. The acetamido derivative (14 mg, 0.018 mmol) was then dissolved in 1 mL anhydrous THF at ambient temperature and treated with 60 μL of $LiBH_4$ (2.0 M in THF). After 3 h, TLC analysis indicated the complete conversion to a single product (CH_2Cl_2 9:1 MeOH, $KMnO_4$, $R_f = 0.05$). C18 reversed-phase HPLC (5–100% MeOH in H_2O, 25 min) afforded the desired product as a colorless oil (68%). ESI-MS $[M+Na]^+ = 720$, $[M-H]^- = 696$; ^{1}H NMR (300 MHz, pyridine-d_6): δ 0.96 (m, 9H), 1.06 (d, $J = 3.9$ Hz, 3H), 1.19 (d, $J = 5.7$ Hz, 3H), 1.28 (d, $J = 5.4$ Hz, 3H), 1.56 (s, 3H), 1.74 (s, 3H), 1.75 (m, 1H), 1.98 (m, H), 2.16 (s, 3H), 2.33 (t, $J = 9.6$ Hz, 1H),

2.7 (m, H), 2.92 (m, 2H), 3.73 (d, J = 11 Hz, 1H), 4.04 (m, 2H), 4.32 (d, J = 11 Hz, 1H), 4.65 (m, 4H), 5.24 (t, J = 9.3 Hz, 1H), 5.91 (t, J = 6.3 Hz, 1H), 7.02 (s, 1H), 7.62 (s, 1H), 7.80 (d, J = 9.6 Hz, 1H), 8.07 (d, J = 7.2 Hz, 1H), 8.14 (s, 1H), 8.15 (d, J = 7.5 Hz, 1H), 8.58 (d, J = 5.7 Hz, 1H), 9.74 (s, 1H).

4.4. Hexanoyl-(α-methyl)alanyl-(*trans*-4-hydroxy)prolyl-leucyl-valyl-glutaminyl-leucine methyl ester(hexanoyl-Aib-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Leu-OMe) (5)

ESI-MS $[M+Na]^+$ = 804.4; ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.83 (m, 21H), 1.24 (m, 4H), 1.34 (s, 3H), 1.36 (s, 3H), 1.45–1.80 (m, 10H), 1.90 (m, 1H), 2.09 (m, 3H), 2.19 (m, 3H), 3.21 (dd, J = 11, 3.3 Hz, 1H), 3.60 (s, 3H), 3.70 (d, J = 11 Hz, 1H), 4.06 (m, 2H), 4.24 (m, 3H), 4.39 (t, J = 9.0 Hz, 1H), 5.13 (br d, 1H), 6.73 (s, 1H), 7.21 (s, 1H), 7.24 (d, J = 9.3 Hz, 1H), 7.58 (d, J = 7.8 Hz, 1H), 7.87 (d, J = 7.8 Hz, 1H), 7.96 (d, J = 7.5 Hz, 1H), 8.65 (s, 1H).

4.5. Decanoyl-(α-methyl)alanyl-(*trans*-4-hydroxy)prolyl-leucyl-valyl-glutaminyl-leucine methyl ester(decanoyl-Aib-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Leu-OMe) (6)

ESI-MS $[M+H]^+$ = 838.5; ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.78 (d, J = 5.6 Hz, 3H), 0.84 (m, 18H), 1.22 (m, 12H), 1.34 (s, 3H), 1.36 (s, 3H), 1.45–1.80 (m, 10H), 1.91 (m, 1H), 2.09 (m, 3H), 2.19 (m, 3H), 3.21 (dd, J = 11, 2.8 Hz, 1H), 3.60 (s, 3H), 3.70 (d, J = 11 Hz, 1H), 4.03 (m, 1H), 4.09 (m, 1H), 4.20 (m, 1H), 4.25 (m, 2H), 4.39 (t, J = 8.8 Hz, 1H), 5.14 (d, J = 3.2 Hz, 1H), 6.74 (s, 1H), 7.22 (s, 1H), 7.24 (d, J = 8.8 Hz, 1H), 7.58 (d, J = 8.4 Hz, 1H), 7.88 (d, J = 7.6 Hz, 1H), 7.97 (d, J = 7.2 Hz, 1H), 8.66 (s, 1H).

4.6. Hexadecanoyl-(α-methyl)alanyl-(*trans*-4-hydroxy)-prolyl-leucyl-valyl-glutaminyl-leucine methyl ester(palmitoyl-Aib-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Leu-OMe) (7)

ESI-MS $[M+H]^+$ = 922.6, $[M+Na]^+$ = 944.6; ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (m, 21H), 1.22 (m, 24H), 1.34 (s, 3H), 1.36 (s, 3H), 1.45–1.80 (m, 10H), 1.91 (m, 1H), 2.11 (m, 3H), 2.18 (m, 3H), 3.21 (d, J = 11 Hz, 1H), 3.60 (s, 3H), 3.71 (d, J = 11 Hz, 1H), 4.03 (m, 1H), 4.09 (m, 1H), 4.22 (m, 3H), 4.39 (t, J = 9.2 Hz, 1H), 5.14 (br s, 1H), 6.74 (s, 1H), 7.23 (s, 1H), 7.24 (d, 1H), 7.58 (d, J = 6.8 Hz, 1H), 7.88 (d, J = 7.6 Hz, 1H), 7.96 (d, J = 6.4 Hz, 1H), 8.66 (s, 1H).

4.7. Octadecanoyl-(α-methyl)alanyl-(*trans*-4-hydroxy)-prolyl-leucyl-valyl-glutaminyl-leucine methyl ester(stearoyl-Aib-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Leu-OMe) (8)

ESI-MS $[M+H]^+$ = 950.7, $[M+Na]^+$=972.7; ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (m, 21H), 1.23 (m, 28H), 1.34 (s, 3H), 1.36 (s, 3H), 1.45–1.80 (m, 10H), 1.91 (m, 1H), 2.10 (m, 3H), 2.18 (m, 3H), 3.21 (dd, J = 11 Hz, 1H), 3.60 (s, 3H), 3.71 (d, J = 11 Hz, 1H), 4.03 (m, 1H), 4.09 (m, 1H), 4.24 (m, 3H), 4.39 (t, J = 8.8 Hz, 1H), 5.13 (br s, 1H), 6.74 (s, 1H), 7.22 (s, 1H), 7.24 (d, J = 9.6 Hz, 1H), 7.57 (d, J = 8.4 Hz, 1H), 7.88 (d, J = 7.2 Hz, 1H), 7.95 (d, J = 6.8 Hz, 1H), 8.66 (s, 1H).

4.8. Myristoleoyl-(α-methyl)alanyl-(*trans*-4-hydroxy)prolyl-leucyl-valyl-glutaminyl-leucine methyl ester(myristoleoyl-Aib-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Leu-OMe) (9)

MALDI FTMS $[M+Na]^+$ obsd *m/z* 914.5906; calcd 914.5937 for $C_{46}H_{81}N_7O_{10}Na$ (Δ 3.4 ppm); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (m, 21H), 1.22 (m, 12H), 1.34 (s, 3H), 1.36 (s, 3H), 1.45–1.80 (m, 10H), 1.97 (m, 5H), 2.11 (m, 3H), 2.18 (m, 3H), 3.21 (d, J = 12 Hz, 1H), 3.60 (s, 3H), 3.71 (d, J = 12 Hz, 1H), 4.03 (m, 1H), 4.07 (m, 1H), 4.23 (m, 3H), 4.39 (t, J = 8.8 Hz, 1H), 5.13 (br s, 1H), 5.31 (m, 2H), 6.74 (s, 1H), 7.23 (s, 1H), 7.24 (d, 1H), 7.58 (d, J = 8.0 Hz, 1H), 7.88 (d, J = 7.2 Hz, 1H), 7.96 (d, J = 7.2 Hz, 1H), 8.66 (s, 1H).

4.9. Linoleoyl-(α-methyl)alanyl-(*trans*-4-hydroxy)prolyl-leucyl-valyl-glutaminyl-leucine methyl ester(linoleoyl-Aib-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Leu-OMe) (10)

ESI-MS $[M+H]^+$ = 946.5, $[M+Na]^+$ = 968.6.

4.10. Linolenoyl-(α-methyl)alanyl-(*trans*-4-hydroxy)prolyl-leucyl-valyl-glutaminyl-leucine methyl ester(linolenoyl-Aib-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Leu-OMe) (11)

MALDI FTMS $[M+Na]^+$ obsd *m/z* 966.6291; calcd 966.6249 for $C_{50}H_{85}N_7O_{10}Na$ (Δ 4.2 ppm).

4.11. 16-Hydroxyhexadecanoyl-(α-methyl)alanyl-(*trans*-4-hydroxy)prolyl-leucyl-valyl-glutaminyl-leucine methyl ester(juniperoyl-Aib-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Leu-OMe) (12)

ESI-MS $[M+H]^+$ = 938.6, $[M+Na]^+$ = 960.6; ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (m, 18H), 1.23 (m, 24H), 1.34 (s, 3H), 1.36 (s, 3H), 1.45–1.80 (m, 10H), 1.90 (m, 1H), 2.10 (m, 3H), 2.18 (m, 3H), 3.21 (dd, J = 11 Hz, 1H), 3.36 (t, J = 6.8 Hz, 2H), 3.60 (s, 3H), 3.70 (d, J = 11 Hz, 1H), 4.03 (m, 1H), 4.09 (m, 1H), 4.22 (m, 3H), 4.39 (t, J = 8.8 Hz, 1H), 5.1 (br s, 1H), 6.74 (s, 1H), 7.22 (s, 1H), 7.23 (d, J = 9.6 Hz, 1H), 7.58 (d, J = 8.0 Hz, 1H), 7.88 (d, J = 7.6 Hz, 1H), 7.97 (d, J = 7.6 Hz, 1H), 8.66 (s, 1H).

4.12. Tetradecanoyl-alanyl-(*trans*-4-hydroxy)prolyl-leucyl-valyl-glutaminyl-leucine methyl ester(myristoyl-Ala-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Leu-OMe) (13)

MALDI FTMS $[M+Na]^+$ obsd *m/z* 902.5909; calcd 902.5937 for $C_{45}H_{81}N_7O_{10}Na$ (Δ 3.1 ppm); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (m, 21H), 1.14 (d, J = 6.8 Hz, 3H), 1.23 (m, 20H), 1.45–1.75 (m, 9H), 1.85 (m, 2H), 1.95 (m, 2H), 2.08 (m, 4H), 3.47 (d, J = 10 Hz, 1H), 3.57 (d, J = 10 Hz, 1H), 3.61 (s, 3H), 4.15 (m, 1H), 4.26 (m, 4H), 4.36 (t,

$J=8.0$ Hz, 1H), 4.47 (t, $J=7.2$ Hz, 1H), 5.11 (d, $J=3.2$ Hz, 1H), 6.77 (s, 1H), 7.24 (s, 1H), 7.47 (d, $J=8.8$ Hz, 1H), 7.97 (d, $J=7.6$ Hz, 1H), 8.02 (d, $J=7.6$ Hz, 1H), 8.06 (d, $J=7.2$ Hz, 1H), 8.22 (d, $J=8.0$ Hz, 1H).

4.13. Tetradecanoyl-D-alanyl-(*trans*-4-hydroxyl)prolyl-leucyl-valyl-glutaminyl-leucine methyl ester(myristoyl-D-Ala-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Leu-OMe) (14)

ESI-MS $[M+H]^+=880.6$, $[M+Na]^+=902.6$, $[M+K]^+=916.6$; MALDI FTMS $[M+Na]^+$ obsd *m/z* 902.5970; calcd 902.5937 for $C_{45}H_{81}N_7O_{10}Na$ (Δ 3.7 ppm).

4.14. Tetradecanoyl-(α-methyl)alanyl-sarcosyl-leucyl-valyl-glutaminyl-leucine methyl ester(myristoyl-Aib-Sar-Leu-Val-Gln-Leu-OMe) (15)

MALDI FTMS $[M+Na]^+$ obsd *m/z* 874.6001; calcd 874.5988 for $C_{44}H_{81}N_7O_9Na$ (Δ 1.5 ppm); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (m, 21H), 1.22 (m, 20H), 1.34 (s, 3H), 1.35 (s, 3H), 1.45–1.75 (m, 9H), 1.86 (m, 1H), 1.89 (m, 1H), 2.08 (m, 2H), 2.15 (m, 2H), 3.08 (s, 3H), 3.60 (s, 3H), 3.88 (s, 2H), 4.13–4.25 (m, 4H), 6.75 (s, 1H), 7.23 (m, 2H), 7.88 (d, $J=7.6$ Hz, 2H), 8.17 (d, $J=7.6$ Hz, 1H), 8.60 (s, 1H).

4.15. Tetradecanoyl-(α-methyl)alanyl-(*trans*-4-hydroxyl)-prolyl-leucyl-valyl-glutaminyl-leucine ethyl ester(myristoyl-Aib-(*trans*-4-hydroxy)Pro-Leu-Val-Gln-Leu-OEt) (16)

In the course of constructing peptide **1**, partial transesterification of short peptide sequences during deprotections with ethanolic HCl resulted in a mix of methyl and ethyl ester products. Peptide **16** was separated from **2** during HPLC purification and tested in its pure state. ESI-MS $[M+H]^+=908.6$, $[M+Na]^+=930.6$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (m, 21H), 1.16 (t, 3H, $J=6$ Hz), 1.22 (m, 20H), 1.34 (s, 3H), 1.36 (s, 3H), 1.45–1.80 (m, 10H), 1.91 (m, 1H), 2.11 (m, 3H), 2.18 (m, 3H), 3.21 (d, $J=11$ Hz, 1H), 3.71 (d, $J=11$ Hz, 1H), 4.06 (m, 4H), 4.22 (m, 3H), 4.39 (t, $J=8.4$ Hz, 1H), 5.13 (s, 1H), 6.74 (s, 1H), 7.22 (s, 1H), 7.24 (d, 1H), 7.58 (d, $J=8.0$ Hz, 1H), 7.88 (d, $J=7.2$ Hz, 1H), 7.95 (d, $J=7.6$ Hz, 1H), 8.66 (s, 1H).

4.16. HSV-1 in vitro antiviral assay

Vero cells were dispensed into 96-well plates at a concentration of 10,000 cells/well in 100 μL of minimum essential medium (MEM) containing 5% fetal bovine serum (FBS). The cells were incubated overnight at 37 °C under 5% CO_2. The media was removed by aspiration, and 100 μL of phosphate buffered saline (PBS) was added to each well and then aspirated. The wells were treated with 100 μL of MEM containing 50 plaque forming units (pfu) of virus and incubated for 1 h (multiplicity of infection = 0.005). Each well was then overlaid with 100 μL of media containing 2% FBS and serial dilutions of any one of the halovir analogs dissolved in DMSO. The compounds were evaluated using a minimum of 10 replicates. After incubation for five days, each well was treated with 20 μL of a solution comprising 1.9 mg/mL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and 0.04 mg/mL phenazine methosulfate in phosphate buffered saline (PBS). The cells were incubated for 4 h during which time viable cells metabolize the MTS to a soluble blue formazan. The amount of formazan produced is directly proportional to the number of surviving cells. The optical density (OD) of the wells (490 nm) was then assessed using an ELISA plate reader. The plate reader was linked with a computer outfitted with the Softmax© software for data manipulation. A positive growth control (PG), 4 wells/plate, contained noninfected Vero cells grown in the presence of 0.2% DMSO. A viral death control (VD), 4 wells/plate, contained cells infected with HSV-1 and grown in the presence of 0.2% DMSO. Percent survival was calculated as $(PG-OD_{test})/(PG-VD)\times 100\%$. Serially diluted acyclovir was used as a standard.

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