

Development of Oleanane-Type Triterpenes as a New Class of HCV Entry Inhibitors

Fei Yu,^{†,§} Qi Wang,^{†,‡,§} Zhen Zhang,^{†,§} Yiyun Peng,[†] Yunyan Qiu,[†] Yongying Shi,[†] Yongxiang Zheng,[†] Sulong Xiao,^{*,†} Han Wang,[†] Xiaoxi Huang,[†] Linyi Zhu,[†] Kunbo Chen,[†] Chuanke Zhao,[†] Chuanling Zhang,[†] Maorong Yu,[†] Dian Sun,[†] Lihe Zhang,[†] and Demin Zhou^{*,†}

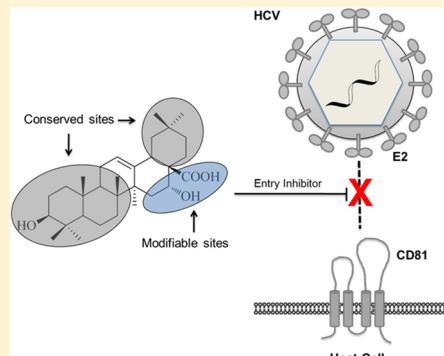
[†]State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China

[‡]Department of Molecular and Cellular Pharmacology, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China

^{*}Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100094, China

Supporting Information

ABSTRACT: Development of hepatitis C virus (HCV) entry inhibitors represents an emerging approach that satisfies a tandem mechanism for use with other inhibitors in a multifaceted cocktail. By screening Chinese herbal extracts, oleanolic acid (OA) was found to display weak potency to inhibit HCV entry with an IC_{50} of 10 μ M. Chemical exploration of this triterpene compound revealed its pharmacophore requirement for blocking HCV entry, rings A, B, and E, are conserved while ring D is tolerant of some modifications. Hydroxylation at C-16 significantly enhanced its potency for inhibiting HCV entry with IC_{50} at 1.4 μ M. Further modification by conjugation of this new lead with a disaccharide at 28-COOH removed the undesired hemolytic effect and, more importantly, increased its potency by ~5-fold (**54a**, IC_{50} 0.3 μ M). Formation of a triterpene dimer via a linker bearing triazole (**70**) dramatically increased its potency with IC_{50} at ~10 nM. Mechanistically, such functional triterpenes interrupt the interaction between HCV envelope protein E2 and its receptor CD81 via binding to E2, thus blocking virus and host cell recognition. This study establishes the importance of triterpene natural products as new leads for the development of potential HCV entry inhibitors.



INTRODUCTION

Hepatitis C virus (HCV) is the leading cause of liver fibrosis and cirrhosis that eventually leads to liver carcinomas.¹ Treatment of HCV infection with ribavirin/interferon has been used for almost 30 years. The recently approved Telaprevir and Boceprevir targeting HCV replication represent the beginning of a new era in the control of HCV infection;² however, resistance to individual antiviral drugs is likely to appear, and a combination of drugs targeting different stages of HCV life cycle is required. Inhibition of virus entry into HCV-permissive cells represents an emerging field for the prevention and reduction of infection. Development of HCV entry inhibitors could satisfy the tandem use with other inhibitors of viral replication, leading to a multifaceted approach to control HCV infection more effectively.

Pentacyclic triterpenes are secondary plant metabolites found in different plant organs, with a few species containing up to 30% of their dry weight.³ These triterpenes have been proposed to possess defense activities due to their capability to prevent various pathogen and herbivore infections⁴ in the host. Betulinic acid, a lupane-type triterpene, has been confirmed by many studies to display significant inhibiting activity against

HIV entry and virus maturation/release. One derivative, bevirimat (PA-457), is already in clinical trial.⁵ Moronic acid and maslinic acid, two oleanane-type triterpenes, also display anti-HIV activity in vitro.⁶ Other diverse and promising biological activities of triterpenes, including anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory, and tonic effects, warrant further pharmaceutical development and even clinical investigation.⁷

Recently, a variety of compounds have been shown to exhibit anti-HCV entry activity with their IC_{50} at the micromolar to even submicromolar level, depending on the sensitivity of the assay in different laboratories.⁸ Here, we report oleanolic acid (OA) and echinocystic acid (EA), two naturally occurring oleanane-type triterpenes (Figure 1), and their derivatives displaying substantial activity to inhibit HCV entry, with one derivative (**70**) showing IC_{50} at ~10 nM. Further studies showed that these compounds represented a group of specific anti-HCVpp agents targeting the viral entry process. The putative mechanism underlying the blocking of HCV entry by

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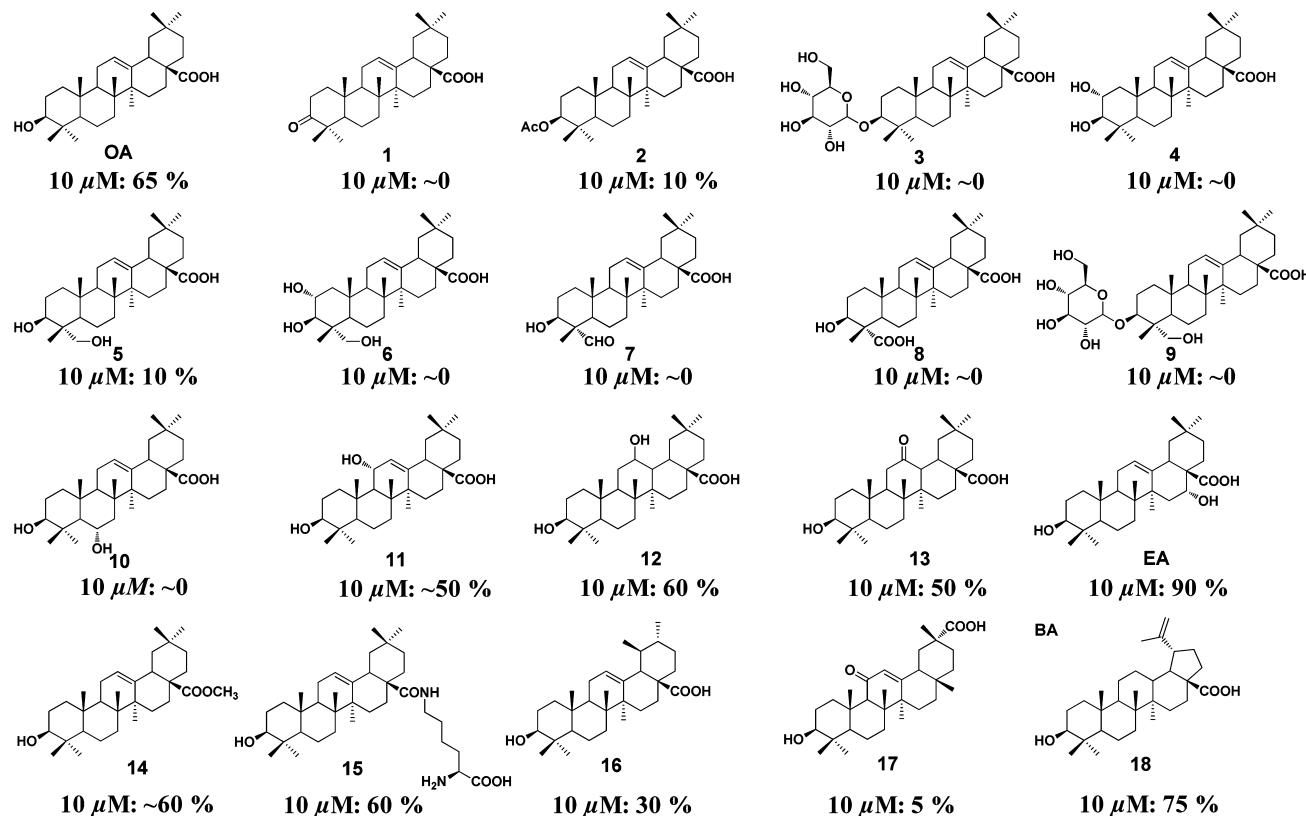


Figure 1. Structures and anti-HCV entry activities of OA and its derivatives (1–15) and analogs (16–18).

EA is its strong binding to E2, one of the envelope proteins of HCV, which blocks the interaction between E2 and CD81, a well-identified receptor for HCV entry. This study establishes the importance of triterpene natural products as leads for the development of potential HCV entry inhibitors.

RESULTS AND DISCUSSION

Discovery of Oleanolic Acid (OA) as a HCV-Entry Inhibitor. A library of Chinese herbal extracts was screened using a HCV pseudo-particle (HCVpp) entry assay.^{8,9} Vesicular stomatitis virus G protein pseudo-particle (VSVpp) was also tested in parallel to determine the specificity and toxicity, as previously reported.^{8,9} The *n*-butanol/H₂O crude extract of *Dipsacus asperoides*, a traditional Chinese herbal medicine, displayed specific anti-HCV entry activity with 50% of viral entry being inhibited at a concentration of 50 $\mu g/mL$ (Supporting Information Figure 1). Subsequent bioactivity-guided phytochemical studies indicated the major active constituents in the extract were saponin due to their strong polarity and weak UV absorption at 254 nm (Supporting Information Figure 2). One major aglycone isolated from the saponin was OA (Figure 1), a relatively nontoxic triterpene natural product exhibiting attractive hepatoprotective, anti-tumor, and antiviral properties.¹⁰ Biological assays indicated that OA displayed substantial activity on blocking HCV entry, with IC_{50} at $\sim 10 \mu M$ (Figure 1), but was far less potent than its parental saponin mixture, which was more potent following an increase in polarity (Supporting Information Figure 2).

Identification of the Pharmacophore of OA. To assess the anti-HCV potential of structurally related triterpene compounds and determine the structure–activity relationship, a series of commercially available triterpenes were acquired

from ChromaDex and Sigma. In addition, a variety of chemical modifications were carried out at the 3-OH and 28-COOH positions of OA to identify the structural requirements. As shown in Figure 1, initial exploration of OA indicated that 3-OH, the chemically modifiable group at ring A, was functionally important since its oxidation (1), acetylation (2), and glucosylation (3) all significantly decreased or even eliminated the activity. Further exploration at ring A indicated that not only 3-OH but also other positions were also less tolerant of some modifications: hydroxylation at C-2 (4), C-23 (5), or both (6) reduced the activity greatly. The potency of 5 was totally lost upon further oxidation of the 23-CH₂OH group into 23-CHO (7) or 23-COOH (8) or glucosidation at 3-OH (9). In addition to ring A, we found ring B was unmodifiable because hydroxylation at C-6 (10) completely abolished the potency of OA. These data suggested the left part of OA, including rings A and B, was conserved, and any modification might significantly decrease its activity.

We subsequently explored the structure–activity relationship of ring C, in particular, the allyl skeleton, and found that a substituent hydroxyl group at either the methylene (11) or vinyl skeleton (12) had almost no or just a marginal effect on the potency of OA. In addition, introduction of a keto at the allyl skeleton (13) also had no obvious effect, suggesting ring C is a modifiable site. Continued exploration of ring D of OA indicated that hydroxylation at C-16 (EA) remarkably enhanced the anti-HCV activity, and the resulting EA showed an increase in the IC_{50} by almost 8-fold (1.4 μM). This result suggested ring D was a modifiable part, and more potent leads might be prepared by modifying ring D. In addition, we synthesized a series of OA derivatives and found ester or amide substitutions at 28-COOH (14, 15) maintained or even

Table 1. Structures and Anti-HCVpp Entry Activities of EA, EA Derivatives and Analogs

Compound	Inhibition of HCVpp entry (%) ^a		Inhibition of VSVpp entry (%) ^a		
	1 μ M	5 μ M	1 μ M	5 μ M	
Positive control 1^b		75.3 ± 1.6	94.4 ± 1.4	7.3 ± 0.7	7.5 ± 0.9
Positive control 2^b		21.2 ± 1.1	34.0 ± 0.9	11.7 ± 2.2	12.8 ± 1.8
EA		27.5 ± 2.1	77.7 ± 3.3	7.2 ± 1.7	9.0 ± 0.8
19		33.4 ± 1.4	55.2 ± 1.8	39.15 ± 2.3	40.35 ± 1.3
20		12.1 ± 0.7	10.5 ± 0.1	23.6 ± 0.7	28.4 ± 0.1
21		10.5 ± 0.7	5.2 ± 0.4	12.6 ± 1.1	25.0 ± 1.5
22		3.5 ± 0.6	64.7 ± 2.9	21.5 ± 1.8	55.4 ± 2.9
23		-14.0 ± 0.9	53.7 ± 2.6	9.9 ± 0.3	30.1 ± 1.2
24		-3.3 ± 0.2	-8.9 ± 0.7	7.6 ± 0.4	9.7 ± 0.7
25		12.3 ± 1.1	16.4 ± 0.7	38.3 ± 2.4	39.3 ± 3.6
26		44.3 ± 2.3	27.8 ± 2.0	27.4 ± 1.6	37.4 ± 3.0
27		14.3 ± 1.1	35.2 ± 2.9	7.9 ± 0.7	11.8 ± 0.9
28		NT	~20 (10 μ M)	NT	NT
29		NT	~0 (10 μ M)	NT	NT

Table 1. continued

Compound	Inhibition of HCVpp entry (%) ^a		Inhibition of VSVpp entry (%) ^a	
	1 μ M	5 μ M	1 μ M	5 μ M
30			NT	\sim 0 (10 μ M)
31			NT	\sim 5 (10 μ M)
32	30.4 \pm 2.6	53.5 \pm 3.4	1.6 \pm 0.1	5.6 \pm 0.6
33	4.9 \pm 0.3	-8.7 \pm 0.7	42.7 \pm 3.5	46.1 \pm 4.1
34	22.6 \pm 1.9	51.0 \pm 0.2	35.8 \pm 1.5	43.5 \pm 0.1
35	34.7 \pm 1.8	87.2 \pm 0.7	20.3 \pm 1.7	21.5 \pm 2.1
36	12.3 \pm 0.8	27.9 \pm 2.7	15.2 \pm 0.2	18.8 \pm 1.4
37	17.5 \pm 2.8	33.7 \pm 3.9	7.3 \pm 1.4	9.9 \pm 3.5
38	4.6 \pm 0.2	5.8 \pm 1.3	1.6 \pm 0.1	-3.9 \pm 0.2
39	1.0 \pm 0.2	-2.7 \pm 0.1	38.5 \pm 2.3	34.7 \pm 1.1
40	48.2 \pm 2.4	70.1 \pm 0.3	-10.0 \pm 0.7	-2.8 \pm 0.2
41	5.5 \pm 0.6	28.2 \pm 1.8	28.8 \pm 1.2	30.6 \pm 2.7
42	1.7 \pm 0.1	7.9 \pm 0.2	8.5 \pm 0.7	14.2 \pm 1.0
43	11.5 \pm 1.1	-3.8 \pm 0.3	45.3 \pm 1.8	40.2 \pm 2.0

Table 1. continued

Compound	Inhibition of HCVpp entry (%) ^a		Inhibition of VSVpp entry (%) ^a	
	1 μ M	5 μ M	1 μ M	5 μ M
44 	13.5 ± 0.5	20.1 ± 1.2	15.8 ± 0.2	24.2 ± 1.8
45 	35.8 ± 2.5	14.0 ± 1.0	20.9 ± 1.5	33.9 ± 2.8
46 	81.7 ± 5.2	94.4 ± 7.1	20.7 ± 1.8	33.2 ± 2.7
47 	16.5 ± 0.5	36.5 ± 3.2	-2.5 ± 0.4	3.6 ± 1.3
48 	NT	~15 (10 μ M)	NT	NT
49 	NT	~90 (10 μ M)	NT	NT
50a 	35.7 ± 0.6	78.3 ± 2.2	3.7 ± 0.3	13.9 ± 1.6
50b 	30.6 ± 2.3	67.5 ± 2.0	12.9 ± 0.2	5.6 ± 0.1
51a 	17.5 ± 1.3	70.7 ± 3.6	20.1 ± 0.1	21.7 ± 2.1
51b 	33.8 ± 2.5	77.7 ± 3.2	36.5 ± 0.6	13.0 ± 1.1
52a 	19.3 ± 1.2	60.6 ± 2.6	17.1 ± 1.0	20.1 ± 0.3
52b 	34.7 ± 2.6	54.6 ± 0.5	15.6 ± 1.1	5.8 ± 0.1
53a 	43.4 ± 2.7	81.6 ± 2.7	29.3 ± 0.5	30.7 ± 1.9
53b 	40.9 ± 3.2	61.6 ± 3.6	8.1 ± .5	14.6 ± 1.2

Table 1. continued

Compound	Inhibition of HCVpp entry (%) ^a		Inhibition of VSVpp entry (%) ^a		
	1 μ M	5 μ M	1 μ M	5 μ M	
54a		80.4 ± 2.8	88.6 ± 0.4	11.2 ± 0.7	14.9 ± 0.3
54b		32.7 ± 1.1	40.1 ± 2.6	22.6 ± 0.5	16.7 ± 1.1
55a		36.5 ± 0.1	50.0 ± 3.2	20.9 ± 1.2	23.4 ± 2.1
55b		36.4 ± 1.9	43.7 ± 2.0	21.6 ± 1.1	21.9 ± 1.9
58a		20.3 ± 0.2	89.7 ± 2.0	-9.1 ± 0.7	12.5 ± 0.3
58b		12.4 ± 0.6	33.1 ± 1.9	-15.2 ± 0.1	19.1 ± 1.6
59a		35.9 ± 2.2	87.3 ± 0.5	39.8 ± 1.6	47.7 ± 0.2
59b		14.8 ± 0.7	27.8 ± 1.4	34.4 ± 0.7	27.0 ± 1.7
60a		26.3 ± 0.7	72.6 ± 3.6	-6.6 ± 0.1	20.9 ± 2.1
60b		18.7 ± 1.8	32.2 ± 2.3	8.4 ± 0.4	25.1 ± 1.4
61a		21.8 ± 0.7	81.5 ± 3.0	-5.3 ± 0.1	14.7 ± 0.8
61b		17.5 ± 0.3	25.0 ± 1.3	17.9 ± 1.5	19.5 ± 1.5
62a		20.4 ± 0.2	66.5 ± 2.6	24.3 ± 2.1	30.6 ± 2.3
62b		16.6 ± 0.3	36.3 ± 0.2	11.5 ± 0.3	25.3 ± 1.5

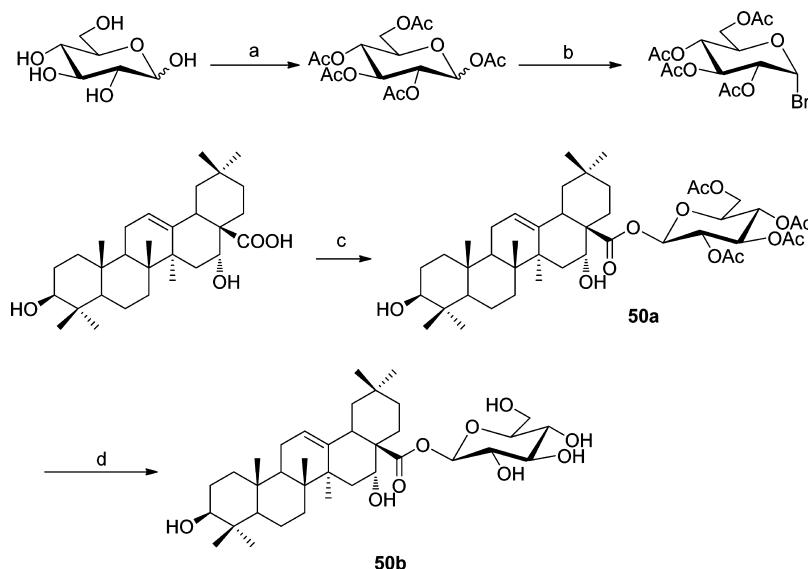
Table 1. continued

Compound	Inhibition of HCVpp entry (%) ^a		Inhibition of VSVpp entry (%) ^a		
	1 μ M	5 μ M	1 μ M	5 μ M	
63a		17.0 ± 1.5	71.6 ± 2.1	32.6 ± 1.5	-10.7 ± 0.8
63b		32.0 ± 2.8	67.0 ± 1.5	7.7 ± 0.8	13.9 ± 0.6
64a		21.8 ± 1.2	65.8 ± 3.0	29.3 ± 1.1	32.2 ± 3.5
64b		20.3 ± 1.5	43.2 ± 3.7	7.0 ± 0.8	35.2 ± 1.7
65a		24.6 ± 1.3	71.8 ± 4.5	39.0 ± 2.8	32.1 ± 0.9
65b		23.1 ± 2.4	14.6 ± 1.5	47.0 ± 2.5	47.3 ± 3.2
66a		56.4 ± 4.6	79.7 ± 1.5	13.2 ± 1.0	28.9 ± 2.3
66b		9.3 ± 0.3	29.7 ± 7.0	25.8 ± 2.3	48.1 ± 1.8
67a		11.9 ± 0.4	64.8 ± 3.4	36.8 ± 3.2	38.8 ± 2.9
67b		15.2 ± 1.2	11.8 ± 0.5	26.6 ± 0.9	50.5 ± 3.5
68a		45.4 ± 1.1	62.9 ± 2.5	17.7 ± 0.2	14.0 ± 0.3
68b		12.8 ± 0.7	39.0 ± 0.3	19.7 ± 1.1	27.4 ± 1.7
69		44.6 ± 7.4	58.2 ± 2.8	18.7 ± 0.9	27.6 ± 1.6
70		86.3 ± 1.8	89.0 ± 0.7	16.5 ± 1.3	11.9 ± 0.2
73		34.2 ± 2.5	76.7 ± 4.6	10.3 ± 1.2	12.0 ± 0.8

^aInhibition (%) = 100% – DMSO negative control (%) of each tested compound; NT, not tested in this assay. ^bPositive control compounds from refs 14b and 8a, respectively.

enhanced the potency, suggesting carboxyl acid is not absolutely required and can be modified.

Shift of C-30-methyl group from C-20 to the adjacent C-19 (16) at ring E decreased the anti-HCV activity by more than

Scheme 1.^a

^aReagents and conditions: (a) Ac_2O , DMAP, pyridine, rt, 12 h; (b) $\text{HBr}\cdot\text{AcOH}$, CH_2Cl_2 , 0 °C to rt, 12 h; (c) acetyl-glucosyl bromide, TBAB, K_2CO_3 , CH_2Cl_2 , N_2 , reflux, overnight; (d) MeONa , MeOH , rt, 1 h.

50%. Switching 28-COOH with 29-methyl group plus oxidation at C-11 (17), a rather tolerant site, also decreased the anti-HCV potency; however, replacement of ring E, a 6-member ring, with a 5-member ring by switching the linker C-21–C-20 to C-21–C-19 (betulinic acid, 18) significantly decreased the report readings in both HCVpp and VSVpp infected cells. This observation might reflect the cellular toxicity rather than potency enhancement by the replacement of oleanolic skeleton with betulinic acid, a relatively cytotoxic triterpene being developed as an anticancer drug.⁵ In this study, the VSVpp entry assay, performed in parallel with HCVpp entry, was originally used to monitor the inhibitory specificity toward different viruses. Later, we found the VSVpp assay is also useful to monitor chemical toxicity because the VSVpp, unlike HCVpp, which infects only liver cells (Huh7), has a broad host range and infects almost all cell lines. When a chemical is cytotoxic, the expression of luciferase reporter, delivered by VSVpp, in host cells will be inhibited. We found the VSVpp assay is even more sensitive to chemical toxicity than the alamarBlue assay. Overall, OA SAR data provide positional requirements and their pharmacophore contributions on the antiviral activity, suggesting part of the OA right side could be modified. Further modification at this region, just like EA, might enhance its anti-HCV potency.

Pharmacophore Confirmation via Chemical Modification of EA. EA is an analog of OA, with a hydroxyl group at C-16 (Figure 1). Such a small variation significantly differentiated EA from OA with an almost 10-fold potency gain for EA. Therefore, EA was chosen as a new lead for SAR exploration, and the same conclusion was obtained. As shown in Table 1, modifications of 3-OH at ring A, including sulfonation (19), amination (20), benzylation (21), oxidation (22), methylation (23), elimination (24), and other modifications (25, 26), made these compounds less potent than EA. Hydroxylation at other sites of ring A, including at C-1 (27) or C-23 (28), also significantly decreased potency. Further oxidation of 23- CH_2OH into 23-CHO (29) and then 23-COOH (30) completely abolished the potency of EA. Similarly, modification of ring B, including hydroxylation at C-6 (31) or C-7 (32) via a

biotransformation approach,¹¹ abolished the antiviral activity of EA. Therefore, the left side of EA, including rings A and B, is functionally conserved and some modification at this side might remarkably attenuate its potency.

Modification of ring C indicated that a keto (33) or a hydroxyl (34) substitute at the vinyl skeleton had almost no effect or only marginal effect on the potency of EA, similar to the case of OA that ring C is tolerant for chemical modifications. Explorations of ring D (35–45) indicated that the potency gained by the introduction of a hydroxyl group at C-16 was lost upon acetylation or elimination of 16-OH; however, oxidation of 16-OH seemed to bring less effect on the potency. Furthermore, 46, a glycoside conjugate at both 3-OH and 28-COOH, also displayed remarkable potency, with >90% HCV entry being inhibited at 10 μM (Table 1). The significant inhibition of HCV entry by glucose substitution at the conserved 3-OH (47) implied that the potency of EA can be further enhanced if the glycoside was kept at 28-COOH, consistent with the initial observation that the more polar the saponin extract, the more potent the virus entry blocking activity. An independent biotransformation experiment indicated that ring E of EA was not a potential modifiable site because hydroxylation at C-21 and C-29 (48, 49) significantly decreased its potency on blocking HCV entry.¹¹ Apparently, intact rings A, B, and E were common structural features that were required for the anti-HCV entry activity.

Development of More Potent HCV Entry Inhibitors. Pentacyclic triterpenes, such as EA and OA, are hydrophobic, which may affect their potential pharmacokinetic properties. To increase their solubility, a variety of carbohydrate moieties, including mono-, di-, and even trisaccharides, were conjugated to EA via 28-COOH, a modifiable site within ring D. As outlined in Scheme 1, direct esterification of EA with 2,3,4,6-tetra-O-acetyl- α -D-glucosyl bromide afforded 50a, which was then hydrolyzed in the presence of CH_3ONa to give 50b.¹² In the same fashion, conjugations of other saccharides with EA (51a–55b, Table 1) were carried out.

The HCV entry assay indicated that the EA–oligosaccharide conjugates, either carrying protective acetyl groups or not,

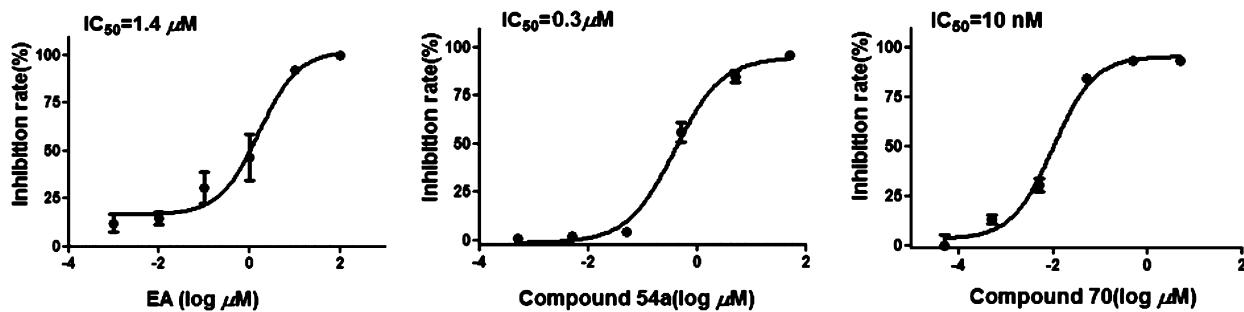
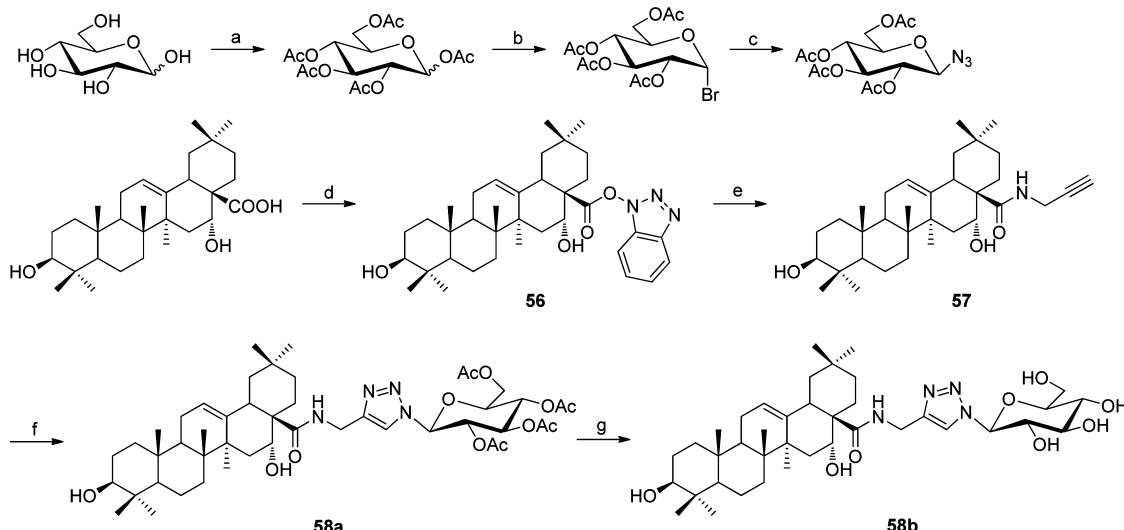


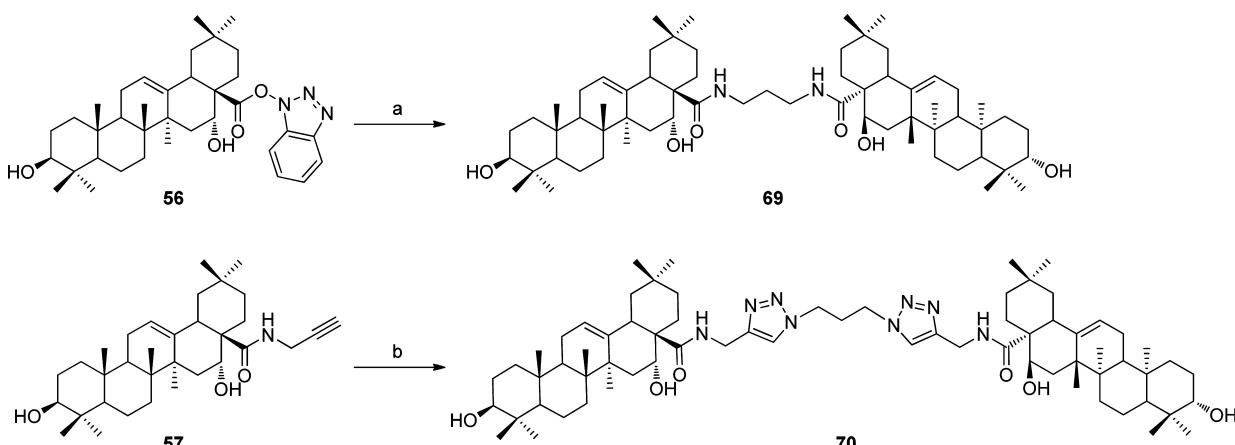
Figure 2. The inhibition curves for compounds EA, 54a, and 70. The concentrations of EA were 1, 10, and 100 nM and 1, 10, and 100 μ M, and the concentrations of 54a and 70 were 0.5, 5, and 50 nM and 0.5, 5, and 50 μ M. Each concentration was tested in triplicate, and the results are expressed as the mean and standard deviation from the triplicate assays.

Scheme 2. ^a



^aReagents and conditions: (a) Ac_2O , DMAP, pyridine, rt, 12 h; (b) $\text{HBr}\text{-AcOH}$, CH_2Cl_2 , 0 °C to rt, 12 h; (c) NaN_3 , DMF, rt, overnight; (d) TBTU, DIEA, THF, rt, overnight; (e) 2-propynylamine, Na_2CO_3 , DMF, rt, 20 min; (f) CuSO_4 , Na-L-ascorbate, azidosaccharides, $\text{CH}_2\text{Cl}_2/\text{H}_2\text{O}$, rt, overnight; (g) MeONa , MeOH , rt, 1 h.

Scheme 3. ^a



^aReagents and conditions: (a) 1,3-propanediamine, Na_2CO_3 , DMF, rt, overnight; (b) CuSO_4 , Na-L-ascorbate, 1,3-diazidopropane, $\text{CH}_2\text{Cl}_2/\text{H}_2\text{O}$, rt, overnight.

exhibited more potent effects than EA (Table 1). Among them, acetylated galactose displayed the highest potency, followed by acetyl protected glucose and ribose. For disaccharide conjugates, the terminally acetylated galactose (54a) showed

the most promising inhibitory activity, with IC_{50} at 0.3 μ M, 5-fold more potent than EA (Figure 2). However, deacetylation of EA glycoconjugates reduced the potency, even though the solubility was significantly increased, especially in the case of

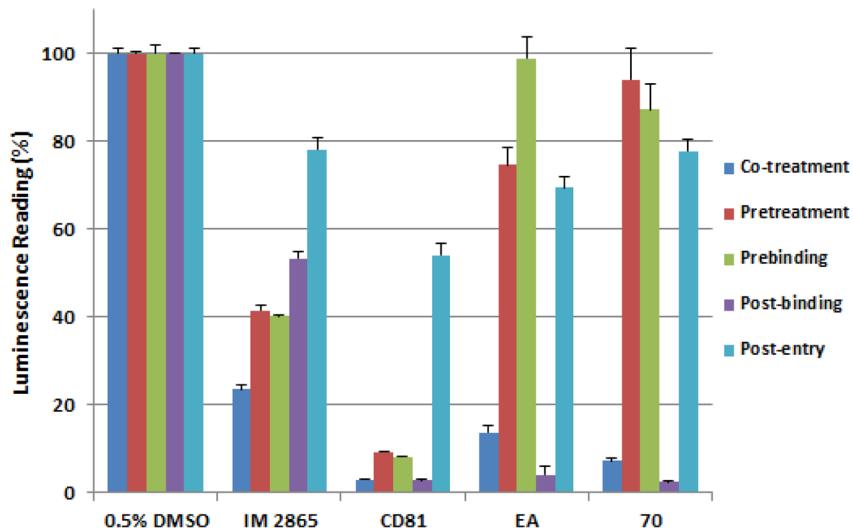


Figure 3. Mechanistic studies of EA-mediated blocking of HCVpp entry by five different assay conditions. CD81 antibody, an entry inhibitor targeting host cell membrane, was utilized as a positive control and IM2865, a nonrelevant compound, was used as negative control. 0.5% DMSO (final concentration) was used for normalization in each condition. The final concentration of all chemicals in the assay was 10 μ M, and the concentration of CD81 antibody was 5 nM.

54a versus **54b**. This result might be due to the reduction of hydrophobicity, which decreased the affinity of EA conjugates to the protein targets that is usually via hydrophobic interactions.

A parallel experiment was carried out by conjugating EA with the same carbohydrate moieties through a triazole linker rather than an ester bond. Such alteration might theoretically enhance the stability of EA glycoconjugates (**58a–68b**, Table 1). The synthesis was based on a Cu(I) azide–alkyne cycloaddition reaction (CuAAC) that linked acetyl-glucosyl azide and **57**, generating 1,4-disubstituted-1,2,3-triazole glucoconjugates in high yields (Scheme 2).¹³ Biological evaluations indicated that almost all such conjugates with acetyl groups in the carbohydrate moieties exhibited a potency that was comparable to their counterparts (Table 1). Deprotection of EA conjugates remarkably decreased their activities, independent of a mono-, di-, or trisaccharide conjugate, just like the cases in the ester bond conjugates (**50a–55b**). In addition, we found that EA glycoconjugates with mono-, di-, or trisaccharide display almost the identical activity, suggesting introduction of a monosaccharide is sufficient to enhance its biological activity.

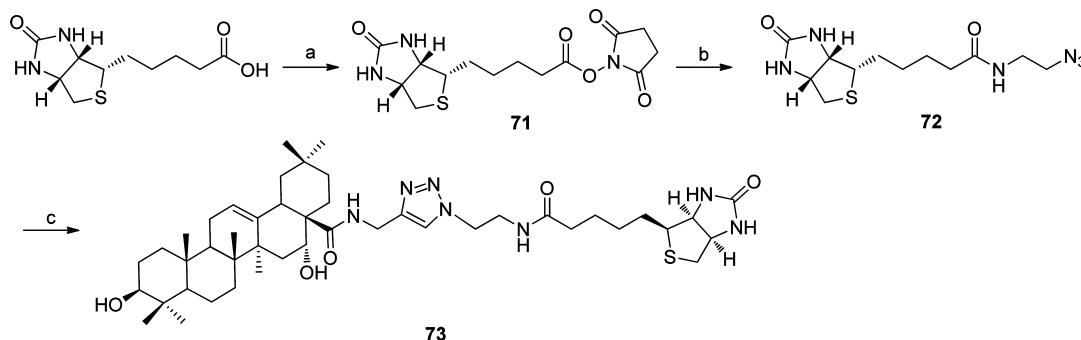
Alternatively, combination of two pharmacophores together is one potential way to further increase the activity of EA. Here, two types of EA dimer, **69** and **70**, were synthesized as shown in Scheme 3. We found that **69**, the EA dimer linked by 1,3-propanediamine, displayed almost the same activity as EA. Unexpectedly, **70**, the EA dimer bearing a triazole linker, exhibited significantly higher potency with IC_{50} at 10.3 nM (Figure 2), almost 2 orders of magnitude more potent than EA (1.4 μ M). To our knowledge, this is the most potent compound based on HCVpp entry assay. Further explorations of this new lead are ongoing to obtain promising HCV entry inhibitors.

Mechanistic Investigation of EA-Mediated HCV Entry Inhibition.

It is clear that intact rings A, B, and E are common structural features among EA and their active derivatives and analogs, which leads us to hypothesize that they are the pharmacophore for blocking HCV entry, via interacting with either HCVpp, host Huh7 cells, or both. To clarify this hypothesis, five different assay conditions were set up as

previously reported.¹⁴ In the first condition, the cotreatment assay, EA was present in the culture medium during the entire viral entry process. Briefly, cells were infected with HCVpp or VSVpp in the presence of 10 μ M EA and incubated for 72 h at 37 °C to allow virus entry. In the second condition, the pretreatment assay, cells were first pretreated with 10 μ M of EA at 37 °C for 3 h, washed to remove unbound compound and then exposed to viruses at 37 °C for 72 h. In the third condition, the prebinding assay, cells were exposed to viruses in the presence of EA at 4 °C for 3 h, washed to remove unbound viruses and compound, and then cultured at 37 °C for 72 h. In the fourth condition, the postbinding and pre-entry assay, cells were first incubated with viruses at 4 °C for 3 h, washed to remove unbound virus, and then treated with EA at 37 °C for 72 h. In the fifth condition, the postentry assay, cells were first treated with viruses at 37 °C for 6 h to allow the virus entry into cells. After washing the unbound viruses, the infected cells were treated with the compound at 37 °C for 72 h.

Under all five conditions, CD81 antibody was utilized as a positive control because it blocks HCV virus entry via binding to CD81 receptors. IM2865 was an unrelated compounds used as another control, and 0.5% DMSO (final concentration) was used for normalization in each condition.¹⁴ As compared with the cotreatment assay, a short pretreatment of the cells with the compound prior to virus infection (pretreatment) or cotreatment of cells with viruses and the compound at 4 °C (prebinding) resulted in very weak, if any, activity (Figure 3), suggesting that EA exerts its inhibitory activity post virus binding. This is remarkably different from the CD81 antibody, which interferes with virus attachment to the target cells by interacting with the surface receptors. In the postbinding or preentry condition, a high activity of EA was noted, suggesting that EA interferes with the subsequent step following virus attachment to target cells, presumably the step of viral envelope–cell membrane fusion. Under the postentry condition, antiviral activity was not observed at all, indicating that EA did not interfere with the multiple processes after viral entry. In conclusion, these data suggested that EA exerts its inhibitory activity at the stage of postvirus binding but before

Scheme 4.^a

^aReagents and conditions: (a) *N*-hydroxysuccinimide, EDC, DMF, rt, overnight; (b) 2-azidoethylamine, TEA, DMF, rt, 12 h; (c) CuSO₄, Na-L-ascorbate, Compound 57, THF/H₂O, 40 °C, overnight.

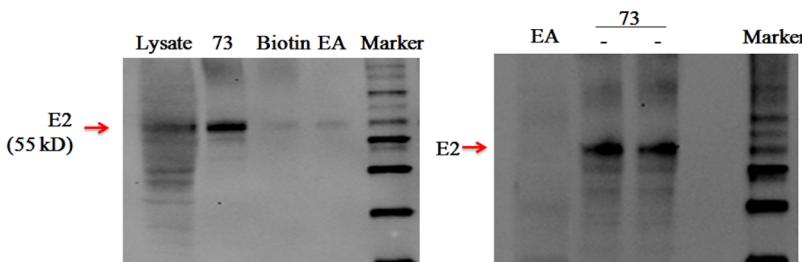


Figure 4. Identification of EA-targeting proteins by pull-down assay. A biotin-labeling EA was utilized as a probe that selectively pulled down HCV envelope protein E2 from HCVpp packaging cell lysate by streptavidin immobilized on agarose.

virus entry, presumably interfering with virus envelope–host cell membrane fusion.

EA-Mediated Disruption of CD81 and E2 Interaction.

To determine which target EA potentially binds to and thereby blocks HCV entry, a biotin-labeled EA conjugate (73) was synthesized (Scheme 4)¹⁵ and then utilized as a probe to pull down the targets in HCVpp packaging cell lysate. Conjugation of the biotin molecule at its 28-COOH through CuAAC had no detectable effect on the potency of EA (Table 1). As shown in Figure 4, HCV envelope protein E2 was selectively pulled down by streptavidin (SA)-immobilized agarose. In contrast, no protein was captured in a parallel pull-down experiment wherein free biotin or EA was utilized, replacing 73 as the probe. Furthermore, competitive experiments indicated that no E2 was pulled down when extra free EA was added into the cell lysate, and such competition was verified by an alternative assay, surface plasmon resonance (SPR) experiments wherein the binding of E2 to the biotin-labeling EA chip was decreased by free EA in a significant dose-dependence manner (Supporting Information Figure 3). Therefore, both supported the direct binding of EA to E2 protein.

To confirm the binding is, indeed, due to the interaction between EA and E2, we then selected another set of experiments in which SA-biotin interaction was used for the immobilization of E2 on the SA sensor tip in BIAcore assay. The sensor was then treated with samples of different concentrations of CD81 and CD81 together with EA. The sensor immobilized E2 on superstreptavidin (SSA) was subjected to treatment with sample EA.¹⁶ A series of BIAcore assays indicated that the K_D between E2 and EA is around 24 μM , about 1000-fold lower than E2 and CD81 ($K_D = 21 \text{ nM}$), and no binding was detected at all for EA to CD81 (Figure 5). Characterization of E2 and CD81 binding in the presence of 10 μM EA indicated that their K_D decreased to 100 nM, ~5-fold

lower than that without EA. On the basis of data from the biological and affinity assays, we proposed a tentative mechanism for EA-mediated blocking of HCV entry (Figure 6): EA binds to HCV envelope protein E2 and thus disrupts its interaction with CD81 receptor. Such disruption does not block virus–host cell attachment, but the followed fusion of the virus envelope and host cell membrane.

Hemolytic and Cytotoxic Studies of EA and Its Derivatives.

A series of studies have demonstrated that the aglycon of triterpenoids possess crucial influence on the hemolytic properties, one of the well-known characteristics for saponins.¹⁷ Compared with OA, which possesses mild hemolytic activity, EA gains a substantial hemolytic side effect with CC₅₀ at 15 μM . Such a hemolytic property may restrict these EA derivatives used as potential anti-HCV entry inhibitors. Here, we found that almost all the EA derivatives depleted the hemolytic activity upon modification of the C-28 carboxyl group (Supporting Information Figure 4). In addition, the cytotoxicity of the most representative compounds in this study (EA, 54a, and 70) toward HepG2 (a liver cancer cell), HeLa (a cervical cancer cell line), and 293T (a derived kidney normal cell) has been tested on the basis of the alamarBlue assay, and no toxicity was detected, even at concentrations as high as 50 μM (Supporting Information Figure 5). Furthermore, data from the VSVpp entry, a useful assay reflecting the cytotoxicity of screen chemicals, also support that most EA derivatives display far less cytotoxicity (Table 1).

CONCLUSION

We identified oleanane-type triterpenes, oleanolic acid (OA) and echinocystic acid (EA), as new scaffolds for blocking HCV entry. Guided by SAR studies, a new class of HCV inhibitors with an IC₅₀ from micromolar to nanomolar was prepared. Mechanistic studies indicated that such functional triterpenes

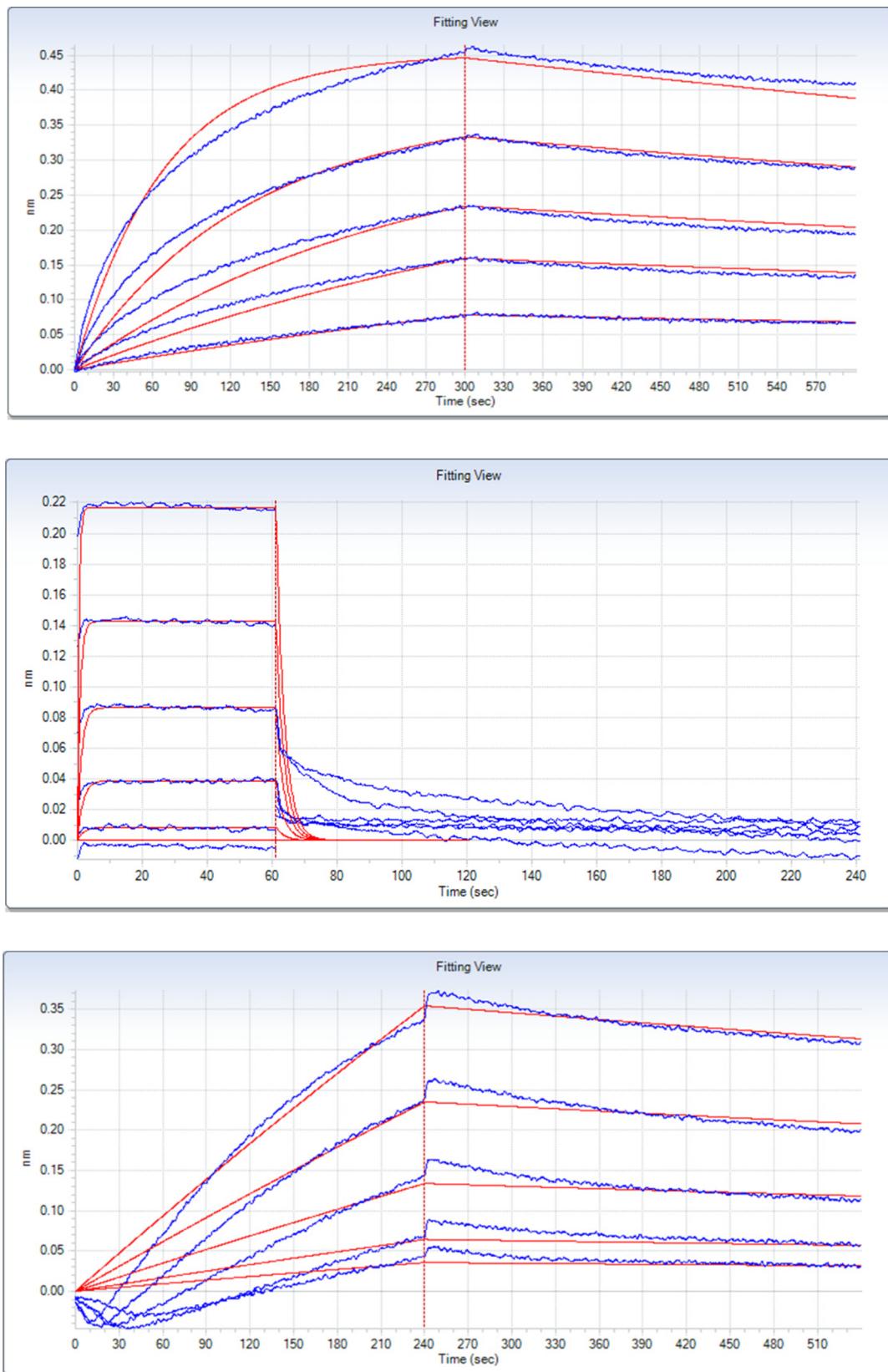


Figure 5. Kinetic assays by Biacore for valuation of E2, CD81, and chemical EA interactions. Blue curves are the experimental trace obtained from biolayer interferometry experiments, and red curves are the best global fits to the data used to calculate the K_D . (A) Binding curves for E2 and CD81 interaction. E2 were loaded onto SA coated biosensors and incubated with varying concentrations of CD81 from 40.9 to 654.5 nM. $K_D = 2.1 \times 10^{-8}$ M ($R^2 = 0.99$). (B) Binding curves for E2 and EA interaction. E2 was loaded onto SSA-coated biosensors and incubated with varying concentrations of EA from 3.125 to 100 μ M. $K_D = 2.4 \times 10^{-5}$ M ($R^2 = 0.94$). (C) Binding curves for E2 and CD81 interaction in the presence of EA. E2 was loaded onto SA-coated biosensors and incubated with varying concentrations of CD81 from 40.9 to 654.5 nM, and then 100 μ M EA was added. $K_D = 1.5 \times 10^{-6}$ M ($R^2 = 0.97$).

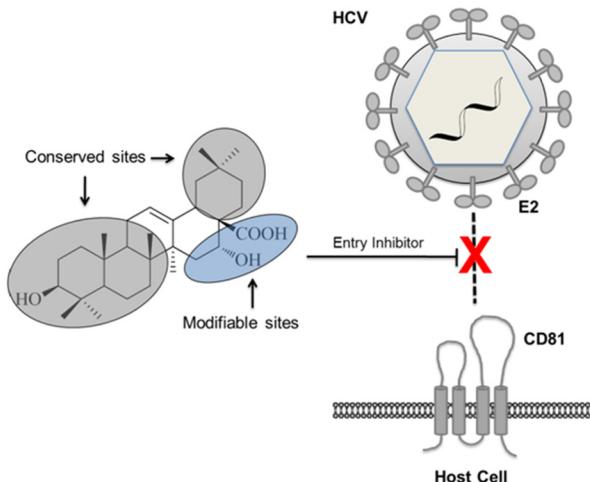


Figure 6. A proposed mechanism for EA-mediated blocking of HCV entry, wherein rings A, B, and E of EA are conserved while ring D is modifiable.

bind to HCV envelope protein E2 and thus interrupt the interaction between E2 and CD81 receptor. Such disruption does not block virus and host cell attachment, but does the following virus–host fusion (Figure 6). This study establishes the importance of triterpene natural products as new leads for the development of potential HCV entry inhibitors.

■ EXPERIMENTAL SECTION

Chemistry. High-resolution mass spectra (HRMS) were obtained with an APEX IV FT_MS (7.0 T) spectrometer (Bruker) in positive ESI mode. NMR spectra were recorded on a Bruker DRX 400 spectrometer at ambient temperature. ¹H NMR chemical shifts were referenced to the internal standard TMS ($\delta_H = 0.00$) or the solvent signal ($\delta_H = 3.31$ for the central line of MeOD). ¹³C NMR chemical shifts are referenced to the solvent signal ($\delta_C = 77.00$ for the central line of CDCl₃, $\delta_C = 49.00$ for the central line of MeOD). Reactions were monitored by thin-layer chromatography (TLC) on a pre-coated silica gel 60 F₂₅₄ plate (layer thickness 0.2 mm; E. Merck, Darmstadt, Germany) and detected by staining with a yellow solution containing Ce(NH₄)₂(NO₃)₆ (0.5 g) and (NH₄)₆Mo₇O₂₄·4H₂O (24.0 g) in 6% H₂SO₄ (500 mL), followed by heating. Flash column chromatography was performed on silica gel 60 (200–300 mesh, Qingdao Haiyang Chemical Co. Ltd.). The method employed in determining the purity of synthesized compounds was HPLC using a Waters e2695 instrument and Waters 2998 photodiode array detector, with chromatography performed on a Diamonsil 250 × 4.6 mm, 5 μm C18(2) column, and eluted with CH₃OH–H₂O (80:20–100:0) at a flow rate of 1.0 mL/min.

General Procedure A for the Synthesis of EA Glycoconjugates (50a–55a). To the glycosyl bromide (0.8 mmol) and EA (189 mg, 0.4 mmol) stirring in CH₂Cl₂/H₂O (10:1, 22 mL) was added K₂CO₃ (138 mg, 1 mmol) and Bu₄NBr (64 mg, 0.2 mmol). The mixture was refluxed under nitrogen atmosphere. After completion (TLC), the reaction mixture was diluted with CH₂Cl₂ (20 mL) and washed with water (10 mL × 3) and brine and dried over Na₂SO₄. The crude product was purified by column chromatography.

General Procedure B for the O-Deacetylation of EA Glycoconjugates (50b–55b, 58b–68b). To the O-pentacylated compound stirring in methanol was added sodium

methoxide (cat.). The mixture was stirred at rt. After completion (TLC), the reaction mixture was neutralized with HCl (1 M). Water was added, and the resulting suspension was filtered. The crude product was purified by column chromatography.

General Procedure C for the CuAAC “Click” Reaction (58a–68a).

To a solution of alkyne (0.16 mmol) and azide (0.19 mmol) in CH₂Cl₂ (3 mL) and H₂O (3 mL) was added CuSO₄ (30 mg, 0.19 mmol) and Na-L-ascorbate (75 mg, 0.38 mmol). The resulting solution was stirred vigorously for 12 h at rt. The reaction mixture was diluted with H₂O (10 mL), then extracted with CH₂Cl₂ (10 mL × 3). The combined organic layer was dried over Na₂SO₄, filtered, and concentrated. The residue was purified by column chromatography.

Compound 50a. Prepared from EA (189 mg, 0.4 mmol) and acetyl-glucosyl bromide (329 mg, 0.8 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/AcOEt, 2/1 v/v) to give **50a** as a white solid (252 mg, 80%). ¹H NMR (400 MHz, CDCl₃): δ 0.70, 0.75, 0.88, 0.89, 0.92, 0.96, 1.32 (7 × CH₃), 1.99, 2.00, 2.00, 2.05 (4 × CH₃CO), 0.72–2.16 (m, other aliphatic ring protons), 2.97 (dd, 1H, J = 4.0, 14.3 Hz), 3.19 (dd, 1H, J = 4.2, 10.6 Hz), 3.74–3.78 (m, 1H), 4.02 (dd, 1H, J = 2.1, 12.4 Hz), 4.25 (dd, 1H, J = 4.4, 12.4 Hz), 4.39 (brs, 1H), 5.08–5.24 (m, 3H), 5.39 (t, 1H, J = 3.2 Hz), 5.54 (d, 1H, J = 8.2 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 15.4, 15.6, 17.0, 18.2, 20.5 (3C), 20.6, 23.2, 24.5, 26.8, 27.1, 28.0, 30.2 (2C), 32.6, 33.0, 35.1, 35.5, 36.9, 38.5, 38.7, 39.5, 40.4, 41.3, 46.0, 46.6, 48.8, 55.2, 61.4, 67.9, 69.9, 72.4, 72.7, 74.2, 78.8, 91.6, 123.2, 141.9, 169.1, 169.4, 170.0, 170.5, 174.7. ESI-HRMS (*m/z*): [M + Na]⁺ calcd for C₄₄H₆₆NaO₁₃, 825.4396; found, 825.4387.

Compound 50b. Prepared from **50a** (50 mg, 0.06 mmol) according to general procedure B. The residue was purified by column chromatography (CH₂Cl₂/MeOH, 10/1 v/v) to afford **50b** as a white solid (12.4 mg, 31%). ¹H NMR (400 MHz, MeOD): δ 0.77, 0.79, 0.89, 0.96, 0.97, 1.37 (7 × CH₃), 0.74–1.96 (m, other aliphatic ring protons), 2.29 (t, 1H, J = 13.3 Hz), 2.99 (dd, 1H, J = 4.0, 14.2 Hz), 3.15 (dd, 1H, J = 5.0, 11.4 Hz), 3.27–3.34 (m, 4H), 3.67 (dd, 1H, J = 4.3, 12.0 Hz), 3.82 (d, 1H, J = 11.1 Hz), 4.53 (brs, 1H), 5.32 (t, 1H, J = 3.4 Hz), 5.35 (d, 1H, J = 8.1 Hz). ¹³C NMR (100 MHz, MeOD): δ 16.1, 16.3, 17.8, 19.5, 24.5, 25.0, 27.3, 27.9, 28.7, 31.3, 31.7, 33.3, 34.2, 36.3, 36.4, 38.2, 39.8, 40.0, 40.8, 42.1, 42.7, 47.8, 48.2, 50.0, 56.9, 62.4, 71.1, 74.0, 74.9, 78.3, 78.7, 79.7, 95.7, 123.6, 144.6, 177.2. ESI-HRMS (*m/z*): [M + Na]⁺ calcd for C₃₆H₅₈NaO₉, 657.3973; found, 657.3987.

Compound 51a. Prepared from EA (189 mg, 0.4 mmol) and acetyl-xylosyl bromide (270 mg, 0.8 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/AcOEt, 2/1 v/v) to give **51a** as a white solid (202 mg, 67%). ¹H NMR (400 MHz, CDCl₃): δ 0.69, 0.74, 0.86, 0.88, 0.91, 0.94, 1.31 (7 × CH₃), 2.01, 2.01, 2.02 (3 × CH₃CO), 0.79–2.15 (m, other aliphatic ring protons), 2.97 (dd, 1H, J = 4.1, 14.1 Hz), 3.17 (dd, 1H, J = 4.2, 10.4 Hz), 3.45 (dd, 1H, J = 8.5, 12.0 Hz), 4.05–4.10 (m, 1H), 4.38 (brs, 1H), 4.89–5.00 (m, 2H), 5.16 (t, 1H, J = 8.2 Hz), 5.37 (t, 1H, J = 3.3 Hz), 5.56 (d, 1H, J = 6.8 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 15.5, 15.6, 17.0, 18.2, 20.6, 20.6, 23.3, 24.6, 26.8, 28.0, 30.2, 32.6, 33.0, 35.1, 35.4, 36.9, 38.5, 38.7, 39.5, 40.4, 41.3, 46.1, 46.6, 48.9, 55.2, 62.5, 68.2, 69.4, 70.9, 74.1, 76.7, 77.0, 77.4, 78.8, 91.9, 123.1, 142.1, 169.2, 169.7, 169.8, 174.7. ESI-HRMS (*m/z*): [M + Na]⁺ calcd for C₄₁H₆₂NaO₁₁; 753.4184; found, 753.4199.

Compound 51b. Prepared from **51a** (90 mg, 0.12 mmol) according to general procedure B. The residue was purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 10/1 v/v) to afford **51b** as a white solid (58.6 mg, 78%). ^1H NMR (400 MHz, MeOD): δ 0.78, 0.89, 0.95, 0.96, 0.97, 1.37 (7 \times CH_3), 0.74–1.93 (m, other aliphatic ring protons), 2.28 (t, 1H, J = 13.3 Hz), 3.02 (dd, 1H, J = 4.0, 14.2 Hz), 3.15 (dd, 1H, J = 5.0, 11.4 Hz), 3.25–3.31 (m, 2H), 3.38 (t, 1H, J = 8.4 Hz), 3.47–3.53 (m, 1H), 3.88 (dd, 1H, J = 5.0, 11.5 Hz), 4.51 (brs, 1H), 5.31–5.33 (m, 2H). ^{13}C NMR (100 MHz, MeOD): δ 16.1, 16.3, 17.8, 19.5, 24.5, 25.1, 27.3, 27.9, 28.8, 31.3, 31.9, 33.4, 34.3, 36.3, 36.4, 38.2, 39.8, 40.0, 40.8, 42.1, 42.7, 47.7, 48.2, 50.1, 56.9, 67.2, 70.8, 73.5, 74.9, 77.3, 79.7, 96.2, 123.7, 144.6, 177.3. ESI-HRMS (m/z) [M + Na]⁺ calcd for $\text{C}_{35}\text{H}_{56}\text{NaO}_8$, 627.3867; found 627.3881.

Compound 52a. Prepared from EA (189 mg, 0.4 mmol) and acetyl-arabinosyl bromide (270 mg, 0.8 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/AcOEt, 2/1 v/v) to give **52a** as a white solid (341 mg, 82%). ^1H NMR (400 MHz, CDCl_3): δ 0.72, 0.75, 0.87, 0.89, 0.92, 0.96, 1.31 (10 \times CH_3), 2.02, 2.04, 2.11 (3 \times CH_3CO), 0.69–2.14 (m, other aliphatic ring protons), 3.00 (dd, 1H, J = 4.0, 14.3 Hz), 3.19 (dd, 1H, J = 4.2, 10.6 Hz), 3.70 (dd, 1H, J = 1.8, 12.9 Hz), 3.97 (dd, 1H, J = 3.8, 13.0 Hz), 4.38 (t, 1H, J = 3.4 Hz), 5.10 (dd, 1H, J = 3.5, 8.9 Hz), 5.19–5.26 (m, 2H), 5.41 (t, 1H, J = 3.2 Hz), 5.51 (d, 1H, J = 6.8 Hz). ^{13}C NMR (100 MHz, CDCl_3): δ 15.5 (2C), 17.0, 18.2, 20.6, 20.7, 20.8, 23.2, 24.8, 26.8, 27.1, 28.0, 29.8, 30.2, 32.6, 33.0, 35.0, 35.5, 36.9, 38.5, 38.7, 39.5, 40.5, 41.4, 46.1, 46.6, 49.0, 55.2, 63.5, 67.0, 68.0, 69.6, 73.9, 78.8, 91.9, 123.1, 141.9, 169.3, 169.8, 170.1, 174.6. ESI-HRMS (m/z) [M + Na]⁺ calcd for $\text{C}_{41}\text{H}_{62}\text{NaO}_{11}$, 753.4184; found, 753.4204.

Compound 52b. Prepared from **52a** (50 mg, 0.07 mmol) according to general procedure B. The residue was purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 10/1 v/v) to afford **52b** as a white solid (38.6 mg, 93%). ^1H NMR (400 MHz, MeOD): δ 0.76, 0.77, 0.88, 0.94, 0.96, 1.36 (7 \times CH_3), 0.73–1.93 (m, other aliphatic ring protons), 2.27 (t, 1H, J = 13.3 Hz), 3.05 (dd, 1H, J = 4.1, 14.3 Hz), 3.14 (dd, 1H, J = 5.0, 11.4 Hz), 3.54 (dd, 1H, J = 9.7 Hz), 3.64–3.65 (m, 2H), 3.86–3.90 (m, 2H), 4.51 (brs, 1H), 5.33 (t, 1H, J = 3.2 Hz), 5.37–5.41 (m, 1H). ^{13}C NMR (100 MHz, MeOD): δ 16.1, 16.3, 17.8, 19.5, 24.5, 25.2, 27.3, 27.9, 28.7, 31.3, 31.8, 33.4, 34.2, 36.2, 36.3, 38.1, 39.8, 39.9, 40.8, 42.0, 42.6, 47.6, 48.2, 50.2, 56.8, 66.1, 68.2, 71.2, 73.5, 74.8, 79.7, 95.7, 123.7, 144.6, 177.2. ESI-HRMS (m/z) [M + Na]⁺ calcd for $\text{C}_{35}\text{H}_{56}\text{NaO}_8$, 627.3867; found, 627.3880.

Compound 53a. Prepared from EA (189 mg, 0.4 mmol) and acetyl-galactosyl bromide (329 mg, 0.8 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/AcOEt, 2/1 v/v) to give **53a** as a white solid (98.5 mg, 31%). ^1H NMR (400 MHz, CDCl_3): δ 0.75, 0.78, 0.91, 0.92, 0.95, 0.99, 1.34 (7 \times CH_3), 1.99, 2.02, 2.04, 2.17 (4 \times CH_3CO), 0.73–2.14 (m, other aliphatic ring protons), 2.99–3.01 (brs, 1H, J = 10.6 Hz), 3.22 (dd, 1H, J = 4.1, 10.4 Hz), 4.00 (t, 1H, J = 6.7 Hz), 4.10–4.15 (m, 2H), 4.39 (brs, 1H), 5.07 (dd, 1H, J = 3.4, 10.4 Hz), 5.31 (t, 1H, J = 10.3 Hz), 5.40–5.44 (m, 2H), 5.54 (d, 1H, J = 8.4 Hz). ^{13}C NMR (100 MHz, CDCl_3): δ 15.5, 15.6, 17.1, 18.2, 20.4, 20.6 (2C), 20.6, 23.3, 24.7, 26.8, 27.1, 28.0, 29.8, 30.2, 32.6, 33.0, 35.1, 35.5, 37.0, 38.5, 38.7, 39.5, 40.5, 41.4, 46.1, 46.6, 49.0, 55.2, 60.7, 66.7, 67.6, 70.7, 71.4, 74.1, 78.8, 92.0, 123.1, 141.9, 169.3, 169.8, 170.1, 170.2, 174.6. ESI-HRMS (m/z)

z) [M + Na]⁺ calcd for $\text{C}_{44}\text{H}_{66}\text{NaO}_{13}$, 825.4396; found, 825.4387; [M + NH₄]⁺ calcd for $\text{C}_{44}\text{H}_{70}\text{NO}_{13}$, 820.4842; found, 820.4839.

Compound 53b. Prepared from **53a** (50 mg, 0.06 mmol) according to general procedure B. The residue was purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 10/1 v/v) to afford **53b** as a white solid (47 mg, 99%). ^1H NMR (400 MHz, MeOD): δ 0.77, 0.78, 0.89, 0.96, 0.97, 1.37 (7 \times CH_3), 0.74–1.96 (m, other aliphatic ring protons), 2.29 (t, 1H, J = 13.3 Hz), 3.00 (dd, 1H, J = 3.8, 14.2 Hz), 3.15 (dd, 1H, J = 4.9, 11.4 Hz), 3.50 (dd, 1H, J = 3.2, 9.7 Hz), 3.58–3.65 (m, 2H), 3.69–3.71 (m, 2H), 3.88 (d, 1H, J = 3.0 Hz), 4.55 (brs, 1H), 5.31–5.33 (m, 2H). ^{13}C NMR (100 MHz, MeOD): δ 16.1, 16.3, 17.8, 19.5, 24.5, 25.0, 27.3, 27.9, 28.7, 31.3, 31.8, 33.4, 34.2, 36.3, 36.4, 38.2, 39.8, 40.0, 40.8, 42.1, 42.6, 47.8, 48.2, 50.0, 56.9, 62.0, 70.0, 71.3, 74.9, 75.2, 77.4, 79.7, 96.2, 123.6, 144.6, 177.3. ESI-HRMS (m/z) [M + NH₄]⁺ calcd for $\text{C}_{36}\text{H}_{62}\text{NO}_9$, 652.4419; found, 652.4415.

Compound 54a. Prepared from EA (189 mg, 0.4 mmol) and acetyl-lactosyl bromide (559 mg, 0.8 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/AcOEt, 2/1 v/v) to give **54a** as a white solid (234 mg, 59%). ^1H NMR (400 MHz, CDCl_3): δ 0.71, 0.78, 0.90, 0.92, 0.94, 0.98, 1.34 (7 \times CH_3), 1.96, 2.02, 2.03, 2.05, 2.07, 2.10, 2.15 (7 \times CH_3CO), 0.74–2.17 (m, other aliphatic ring protons), 2.98 (dd, 1H, J = 3.9, 14.4 Hz), 3.21 (dd, 1H, J = 3.8, 10.6 Hz), 3.69–3.72 (m, 1H), 3.83–3.88 (m, 2H), 4.05–4.15 (m, 3H), 4.36–4.39 (m, 2H), 4.47 (d, 1H, J = 7.9 Hz), 4.95 (dd, 1H, J = 3.4, 10.4 Hz), 5.05–5.12 (m, 2H), 5.23 (t, 1H, J = 9.2 Hz), 5.35 (d, 1H, J = 3.2 Hz), 5.39 (brs, 1H), 5.53 (d, 1H, J = 8.3 Hz). ^{13}C NMR (100 MHz, CDCl_3): δ 15.4, 15.5, 17.0, 18.2, 20.4, 20.5 (2C), 20.6 (2C), 20.7, 20.7, 23.2, 24.5, 26.8, 27.1, 28.0, 30.0, 30.2, 32.6, 33.0, 35.1, 35.4, 36.9, 38.5, 38.7, 39.5, 40.4, 41.3, 46.0, 46.6, 48.8, 55.2, 60.8, 61.6, 66.6, 69.0, 70.3, 70.7, 70.9, 72.6, 73.3, 74.2, 75.7, 78.8, 91.4, 100.9, 123.2, 141.9, 168.9, 169.4, 169.5, 170.0, 170.1, 170.2, 170.3, 174.6. ESI-HRMS (m/z) [M + Na]⁺ calcd for $\text{C}_{56}\text{H}_{82}\text{NaO}_{21}$, 1113.5241; found, 1113.5238; [M + NH₄]⁺ calcd for $\text{C}_{56}\text{H}_{80}\text{NO}_{21}$, 1108.5687; found, 1108.5698.

Compound 54b. Prepared from **54a** (140 mg, 0.13) according to general procedure B. The residue was purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 7/1 v/v) to afford **54b** as a white solid (92.6 mg, 91%). ^1H NMR (400 MHz, pyridine-*d*₅): δ 0.99, 1.03, 1.06, 1.08, 1.17, 1.23, 1.85 (7 \times CH_3), 0.90–2.61 (m, other aliphatic ring protons), 2.83 (t, 1H, J = 13.4 Hz), 3.46–3.56 (m, 2H), 3.98–4.00 (m, 1H), 4.12–4.17 (m, 3H), 4.29–4.57 (m, 7H), 5.12 (d, 1H, J = 7.8 Hz), 5.31 (brs, 1H), 5.65 (brs 1H), 6.27 (d, 1H, J = 8.2 Hz), 6.43 (d, 1H, J = 3.5 Hz). ^{13}C NMR (100 MHz, pyridine-*d*₅): δ 16.3, 17.1, 18.1, 19.4, 24.4, 25.1, 27.8, 28.7, 29.3, 31.4, 32.7, 33.7, 34.1, 36.5, 36.6, 38.0, 39.6, 39.9, 40.7, 41.9, 42.6, 47.7, 47.8, 49.7, 56.5, 62.2, 62.5, 70.6, 73.0, 74.2, 74.9, 75.7, 77.5, 77.7, 77.8, 78.7, 82.0, 95.9, 106.3, 123.3, 144.9, 176.4. ESI-HRMS (m/z) [M + Na]⁺ calcd for $\text{C}_{42}\text{H}_{68}\text{NaO}_{14}$, 819.4501; found, 819.4492.

Compound 55a. Prepared from EA (189 mg, 0.4 mmol) and acetyl-maltosyl bromide (559 mg, 0.8 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/AcOEt, 2/1 v/v) to give **55a** as a white solid (83 mg, 19%). ^1H NMR (400 MHz, CDCl_3): δ 0.72, 0.78, 0.90, 0.92, 0.94, 0.98, 1.34 (7 \times CH_3), 2.00, 2.02, 2.05, 2.10, 2.12 (7 \times CH_3CO), 0.74–2.17 (m, other aliphatic ring protons), 2.98 (dd, 1H, J = 3.9, 14.2 Hz),

3.21 (dd, 1H, $J = 3.9, 10.5$ Hz), 3.76–3.80 (m, 1H), 3.91–3.94 (m, 1H), 4.00–4.05 (m, 2H), 4.20–4.26 (m, 2H), 4.36–4.40 (m, 2H), 4.84 (dd, 1H, $J = 4.0, 10.5$ Hz), 4.98–5.07 (m, 2H), 5.26–5.40 (m, 4H), 5.59 (d, 1H, $J = 8.2$ Hz). ^{13}C NMR (100 MHz, CDCl_3): δ 15.4, 15.5, 17.0, 18.2, 20.5, 20.5 (3C), 20.6, 20.7, 20.8, 23.2, 24.5, 26.8, 27.1, 28.0, 30.1, 30.2, 32.6, 33.0, 35.2, 35.4, 36.9, 38.5, 38.7, 39.5, 40.4, 41.3, 46.1, 46.6, 48.8, 55.2, 61.4, 62.5, 68.0, 68.5, 69.2, 70.0, 70.7, 72.6, 72.8, 74.3, 75.2, 78.8, 91.2, 95.6, 123.2, 141.9, 169.4 (2C), 169.8, 170.0, 170.3, 170.4, 170.5, 174.5. ESI-HRMS (m/z) [M + Na] $^+$ calcd for $\text{C}_{56}\text{H}_{82}\text{NaO}_{21}$, 1113.5241; found, 1113.5247.

Compound 55b. Prepared from **55a** (41 mg, 0.04 mmol) according to general procedure B. The residue was purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 7/1 v/v) to afford **55b** as a white solid (25 mg, 83%). ^1H NMR (400 MHz, MeOD): δ 0.77, 0.79, 0.89, 0.95, 0.97, 1.37 (7 \times CH₃), 0.74–1.96 (m, other aliphatic ring protons), 2.28 (t, 1H, $J = 13.4$ Hz), 3.00 (dd, 1H, $J = 3.9, 14.2$ Hz), 3.15 (dd, 1H, $J = 5.0, 11.3$ Hz), 3.23–3.28 (m, 1H), 3.34–3.38 (m, 1H), 3.43–3.46 (m, 2H), 3.58–3.72 (m, 5H), 3.82–3.84 (m, 3H), 4.53 (brs, 1H), 5.18 (d, 1H, $J = 3.8$ Hz), 5.32(brs, 1H), 5.37 (d, 1H, $J = 8.2$ Hz). ^{13}C NMR (100 MHz, MeOD): δ 16.1, 16.3, 17.8, 19.5, 24.5, 25.1, 27.3, 27.9, 28.7, 31.2, 31.7, 33.3, 34.2, 36.4 (2C), 38.2, 39.8, 40.0, 40.8, 42.1, 42.7, 47.8, 48.2, 50.1, 56.9, 61.8, 62.8, 71.6, 73.6, 74.1, 74.8, 74.9, 75.1, 77.4, 78.1, 79.7, 80.5, 95.6, 102.8, 123.7, 144.6, 177.2. ESI-HRMS (m/z) [M + Na] $^+$ calcd for $\text{C}_{42}\text{H}_{68}\text{NaO}_{14}$, 819.4501; found, 819.4491.

Compound 56. To EA (944 mg, 2 mmol) and TBTU (963 mg, 3 mmol) stirring in 20 mL THF was added DIEA (0.5 mL, 3 mmol). The mixture was stirred at room temperature overnight. After completion (TLC), the reaction mixture was filtered, and the filtrate was concentrated. The crude filtrate was purified by column chromatography (petroleum ether/AcOEt, 4/1 v/v) to give **56** as a white solid (1.01 g, 86%). ^1H NMR (400 MHz, CDCl_3): δ 0.79, 0.83, 0.92, 0.98, 1.01, 1.04, 1.45 (7 \times CH₃), 0.75–2.35 (m, other aliphatic ring protons), 3.16 (dd, 1H, $J = 4.2, 14.2$ Hz), 3.23 (dd, 1H, $J = 3.8, 10.7$ Hz), 4.85 (brs, 1H), 5.49 (t, 1H, $J = 3.2$ Hz), 7.34 (d, 1H, $J = 8.3$ Hz), 7.39–7.55 (m, 2H), 8.05 (d, 1H, $J = 8.4$ Hz). ^{13}C NMR (100 MHz, CDCl_3): δ 15.5, 15.6, 17.3, 18.2, 23.3, 24.4, 26.9, 27.1, 28.1, 30.4, 31.1, 32.6, 33.0, 35.1, 36.0, 37.0, 38.5, 38.7, 39.6, 41.0, 41.5, 46.2, 46.5, 49.3, 55.2, 74.2, 78.9, 108.0, 120.5, 124.2, 124.7, 128.6, 128.6, 141.4, 143.4, 172.7. ESI-HRMS (m/z) [M + H] $^+$ calcd for $\text{C}_{36}\text{H}_{52}\text{N}_3\text{O}_4$, 590.3952; found, 590.3949.

Compound 57. To **56** (589 mg, 1 mmol) and Na_2CO_3 (106 mg, 1 mmol) stirring in 8 mL DMF was added 2-propynylamine (0.1 mL, 1.5 mmol). The mixture was stirred at room temperature for 20 min. After completion (TLC), the solvent was removed under reduced pressure. The mixture was resolved in AcOEt and washed with water and brine twice. The organic layer was dried over MgSO_4 , then filtered and concentrated. The crude residue was purified by column chromatography (petroleum ether/AcOEt, 2/1 v/v) to give **57** as a white solid (473 mg, 93%). ^1H NMR (400 MHz, CDCl_3): δ 0.79, 0.81, 0.91, 0.93, 0.95, 1.00, 1.38 (7 \times CH₃), 0.73–2.30 (m, other aliphatic ring protons), 2.21 (t, 1H, $J = 2.6$ Hz), 2.70 (dd, 1H, $J = 3.8, 13.7$ Hz), 3.22 (dd, 1H, $J = 3.7, 10.8$ Hz), 3.86–4.00 (m, 2H), 4.40 (brs, 1H), 5.54 (t, 1H, $J = 3.4$ Hz), 6.42 (t, 1H, $J = 4.8$ Hz). ^{13}C NMR (100 MHz, CDCl_3): δ 15.6 (2C), 17.1, 18.2, 23.4, 24.9, 26.7, 27.1, 28.0, 29.4, 29.6, 30.2, 32.5, 35.1, 35.2, 36.9, 38.6, 38.7, 39.6, 41.6, 41.8, 46.6, 46.9, 49.0, 55.1, 71.6, 75.3, 78.8, 79.4, 123.4, 144.0, 177.5. ESI-

HRMS (m/z) [M + H] $^+$ calcd for $\text{C}_{33}\text{H}_{52}\text{NO}_3$, 510.3942; found, 510.3941.

Compound 58a. Prepared from **57** (82 mg, 0.16 mmol), acetyl-glucosyl azide (71 mg, 0.19 mmol) according to general procedure C. The residue was purified by column chromatography (petroleum ether/AcOEt, 1/2 v/v) to give **58a** as a white solid (117 mg, 83%). ^1H NMR (400 MHz, CDCl_3): δ 0.71, 0.78, 0.90, 0.91, 0.99, 1.36 (7 \times CH₃), 1.87, 2.03, 2.07, 2.09 (4 \times CH₃CO), 2.76–2.38 (m, other aliphatic ring protons), 2.75–2.76 (m, 1H, $J = 10.9$ Hz), 3.22 (dd, 1H, $J = 2.9, 10.1$ Hz), 4.00–4.03 (m, 1H), 4.15 (d, 1H, $J = 12.6$ Hz), 4.26–4.32 (m, 3H), 4.37 (brs, 1H), 4.55 (dd, 1H, $J = 5.8, 15.2$ Hz), 5.23–5.27 (m, 1H), 5.40–5.47 (m, 2H), 5.55 (brs, 1H), 5.86 (d, 1H, $J = 8.6$ Hz), 6.92 (t, 1H, $J = 4.6$ Hz), 7.79 (s, 1H). ^{13}C NMR (100 MHz, CDCl_3): δ 15.5, 15.6, 16.8, 18.2, 20.1, 20.5 (2C), 20.6, 23.3, 25.2, 26.8, 27.1, 28.0, 29.0, 30.1, 32.4, 32.5, 35.0, 35.1 (2C), 36.9, 38.5, 38.7, 39.6, 41.6, 41.7, 46.7, 46.8, 49.1, 55.1, 61.5, 67.6, 70.3, 72.6, 75.0, 75.1, 78.8, 85.6, 120.9, 123.5, 143.5, 145.2, 168.7, 169.3, 169.9, 170.5, 178.0. ESI-HRMS (m/z) [M + H] $^+$ calcd for $\text{C}_{47}\text{H}_{71}\text{N}_4\text{O}_{12}$, 883.5063; found, 883.5053.

Compound 58b. Prepared from **58a** (80 mg, 0.09 mmol) according to general procedure B. The crude product was washed with water three times without further purification to afford **58b** as a white solid (61 mg, 95%). ^1H NMR (400 MHz, MeOD): δ 0.66, 0.78, 0.89, 0.94, 0.96, 0.97, 1.37 (7 \times CH₃), 0.76–2.00 (m, other aliphatic ring protons), 2.35 (t, 1H, $J = 13.2$ Hz), 2.87 (dd, 1H, $J = 3.2, 13.6$ Hz), 3.15 (dd, 1H, $J = 4.9, 11.4$ Hz), 3.47–3.58 (m, 3H), 3.71 (dd, 1H, $J = 5.3, 12.1$ Hz), 3.83–3.89 (m, 2H), 4.31 (d, 1H, $J = 15.2$ Hz), 4.35 (brs, 1H), 4.43 (d, 1H, $J = 15.2$ Hz), 5.47 (brs, 1H), 5.57 (d, 1H, $J = 9.2$ Hz), 7.98 (s, 1H). ^{13}C NMR (100 MHz, MeOD): δ 16.2, 16.3, 17.7, 19.5, 24.5, 25.4, 27.3, 27.9, 28.7, 31.2, 31.7, 33.3, 33.9, 36.0 (2C), 36.3, 38.1, 39.8, 39.9, 40.8, 42.3, 42.8, 48.0, 48.2, 50.0, 56.8, 62.4, 70.9, 74.0, 75.6, 78.4, 79.7, 81.1, 89.5, 123.5, 124.3, 144.9, 145.9, 180.2. ESI-HRMS (m/z) [M + H] $^+$ calcd for $\text{C}_{39}\text{H}_{63}\text{N}_4\text{O}_8$, 715.4640; found, 715.4647.

Compound 59a. Prepared from **57** (89 mg, 0.17 mmol), acetyl-xylosyl azide (57 mg, 0.19 mmol) according to general procedure C. The residue was purified by column chromatography (petroleum ether/AcOEt, 1/2 v/v) to give **59a** as a white solid (106 mg, 78%). ^1H NMR (400 MHz, CDCl_3): δ 0.67, 0.78, 0.90, 0.91, 0.91, 0.99, 1.36 (7 \times CH₃), 1.88, 2.05, 2.08 (3 \times CH₃CO), 2.63–2.25 (m, other aliphatic ring protons), 2.74 (dd, 1H, $J = 3.3, 13.5$ Hz), 3.22 (dd, 1H, $J = 3.5, 10.4$ Hz), 3.60 (t, 1H, $J = 10.8$ Hz), 4.26–4.31 (m, 2H), 4.38 (brs, 1H), 4.54 (dd, 1H, $J = 5.7, 15.2$ Hz), 5.14–5.18 (m, 1H), 5.40–5.43 (m, 2H), 5.53 (brs, 1H), 5.76 (d, 1H, $J = 8.6$ Hz), 6.90 (t, 1H, $J = 5.1$ Hz), 7.77 (s, 1H). ^{13}C NMR (100 MHz, CDCl_3): δ 15.5 (2C), 16.7, 18.1, 20.1, 20.5 (2C), 23.3, 25.1, 26.7, 27.0, 27.9, 29.2, 30.1, 32.4, 32.5, 35.0 (2C), 35.1, 36.8, 38.5, 38.6, 39.5, 41.5, 41.7, 46.6, 46.7, 48.9, 55.1, 65.3, 68.3, 70.3, 71.9, 75.0, 78.7, 86.1, 120.9, 123.3, 143.5, 145.1, 168.7, 169.6, 169.8, 177.9. ESI-HRMS (m/z) [M + H] $^+$ calcd for $\text{C}_{44}\text{H}_{67}\text{N}_4\text{O}_{10}$, 811.4852; found, 811.4858.

Compound 59b. Prepared from **59a** (55 mg, 0.07 mmol) according to general procedure B. The crude product was washed with water three times without further purification to afford **59b** as a white solid (45 mg, 97%). ^1H NMR (400 MHz, MeOD): δ 0.52, 0.76, 0.88, 0.91, 1.34, 0.95 (7 \times CH₃), 0.70–2.01 (m, other aliphatic ring protons), 2.32 (t, 1H, $J = 13.2$ Hz), 2.85–2.88 (m, 1H), 3.15 (dd, 1H, $J = 4.8, 11.0$ Hz), 3.45–3.55 (m, 2H), 3.66–3.72 (m, 1H), 3.88 (t, 1H, $J = 9.0$), 4.00 (dd, 1H, $J = 5.3, 11.2$ Hz), 4.35 (brs, 1H), 4.39 (brs, 2H), 5.45 (brs,

1H), 5.50 (d, 1H, $J = 9.1$ Hz), 7.97 (s, 1H). ^{13}C NMR (100 MHz, MeOD): δ 16.1, 16.3, 17.6, 19.3, 24.4, 25.3, 27.3, 27.7, 28.7, 31.1, 31.7, 33.2, 33.8, 35.7, 35.8, 36.3, 38.0, 39.7, 39.9, 40.6, 42.2, 42.6, 48.0 (2C), 49.9, 56.7, 69.6, 70.6, 73.7, 75.5, 78.4, 79.7, 89.9, 123.7, 124.2, 144.8, 145.8, 180.1. ESI-HRMS (m/z) [M + H]⁺ calcd for C₃₈H₆₁N₄O₇, 685.4535; found, 685.4526.

Compound 60a. Prepared from 57 (79 mg, 0.15 mmol), acetyl-arabinosyl azide (60 mg, 0.2 mmol) according to general procedure C. The residue was purified by column chromatography (petroleum ether/AcOEt, 1/2 v/v) to give 60a as a white solid (111 mg, 92%). ^1H NMR (400 MHz, CDCl₃): δ 0.69, 0.78, 0.91, 0.99, 1.36 (7 \times CH₃), 1.89, 2.04, 2.23 (3 \times CH₃CO), 0.72–2.30 (m, other aliphatic ring protons), 2.75 (dd, 1H, $J = 2.5$, 13.0 Hz), 3.22 (dd, 1H, $J = 2.0$, 10.0 Hz), 3.95 (d, 1H, $J = 13.3$ Hz), 4.18 (d, 1H, $J = 13.4$ Hz), 4.27 (dd, 1H, $J = 4.4$, 15.0 Hz), 4.36 (brs, 1H), 4.56 (dd, 1H, $J = 5.8$, 15.1 Hz), 5.25 (dd, 1H, $J = 3.3$, 10.0 Hz), 5.43 (brs, 1H), 5.54–5.60 (m, 2H), 5.72 (d, 1H, $J = 9.1$ Hz), 6.90 (t, 1H, $J = 4.7$ Hz), 7.82 (s, 1H). ^{13}C NMR (100 MHz, CDCl₃): δ 15.5 (2C), 16.8, 18.2, 20.2, 20.5, 20.9, 23.3, 25.2, 26.8, 27.1, 28.0, 29.0, 30.1, 32.4, 32.5, 35.0, 35.1 (2C), 36.9, 38.5, 38.7, 39.5, 41.5, 41.7, 46.7, 46.8, 49.0, 55.1, 67.1, 67.6, 68.1, 70.4, 75.1, 78.8, 86.6, 120.9, 123.4, 143.5, 145.0, 168.9, 169.8, 170.1, 177.9. ESI-HRMS (m/z) [M + H]⁺ calcd for C₄₄H₆₇N₄O₁₀, 811.4852; found, 811.4859.

Compound 60b. Prepared from 60a (77 mg, 0.9 mmol) according to general procedure B. The crude product was washed with water three times without further purification to afford 60b as a white solid (64 mg, 99%). ^1H NMR (400 MHz, MeOD): δ 0.59, 0.77, 0.89, 0.92, 0.96, 0.97, 1.36 (7 \times CH₃), 0.72–1.99 (m, other aliphatic ring protons), 2.36 (t, 1H, $J = 13.2$ Hz), 2.87 (dd, 1H, $J = 3.2$, 13.5 Hz), 3.15 (dd, 1H, $J = 4.8$, 11.2 Hz), 3.71 (dd, 1H, $J = 3.3$, 9.4 Hz), 3.85 (d, 1H, $J = 12.4$ Hz), 3.95 (brs, 1H), 4.01 (dd, 1H, $J = 1.6$, 12.7 Hz), 4.14 (t, 1H, $J = 9.3$ Hz), 4.32–4.45 (m, 3H), 5.46 (brs, 1H), 5.46 (d, 1H, $J = 9.0$ Hz), 7.59 (t, 1H, $J = 5.3$ Hz), 8.00 (s, 1H). ^{13}C NMR (100 MHz, MeOD): δ 16.1, 16.4, 17.6, 19.4, 24.4, 25.4, 27.3, 27.9, 28.7, 31.2, 31.9, 33.3, 33.9, 35.9, 36.1, 36.3, 38.0, 39.8, 39.9, 40.7, 42.3, 42.7, 48.0, 48.1, 49.9, 56.7, 70.2, 70.7, 71.3, 74.8, 75.6, 79.6, 90.4, 123.0, 124.3, 144.9, 146.0, 180. ESI-HRMS (m/z) [M + H]⁺ calcd for C₃₈H₆₁N₄O₇, 685.4535; found, 685.4540.

Compound 61a. Prepared from 57 (80 mg, 0.16 mmol), acetyl-mannosyl azide (71 mg, 0.19 mmol) according to general procedure C. The residue was purified by column chromatography (petroleum ether/AcOEt, 1/2 v/v) to give 61a as a white solid (112 mg, 80%). ^1H NMR (400 MHz, CDCl₃): δ 0.51, 0.79, 0.90, 0.90, 0.92, 0.98, 1.35 (7 \times CH₃), 2.00, 2.10, 2.10, 2.15 (4 \times CH₃CO), 0.70–2.27 (m, other aliphatic ring protons), 2.72 (dd, 1H, $J = 3.2$, 13.4 Hz), 3.21 (dd, 1H, $J = 3.5$, 10.3 Hz), 3.93–3.97 (m, 1H), 4.20 (dd, 1H, $J = 1.8$, 12.4 Hz), 4.28–4.46 (m, 4H), 5.25–5.39 (m, 2H), 5.54 (brs, 1H), 5.66 (d, 1H, $J = 2.2$ Hz), 6.14 (brs, 1H), 6.90 (t, 1H, $J = 5.3$ Hz), 7.76 (s, 1H). ^{13}C NMR (100 MHz, CDCl₃): δ 15.5, 15.6, 16.6, 18.1, 20.4, 20.6 (2C), 20.7, 23.3, 24.9, 26.8, 27.1, 28.0, 29.7, 30.2, 32.4, 32.5, 35.0, 35.1, 35.3, 36.9, 38.5, 38.7, 39.5, 41.5, 41.7, 46.6, 46.9, 48.9, 55.2, 62.1, 64.8, 68.7, 70.8, 75.5, 75.6, 78.9, 84.6, 121.5, 123.7, 143.5, 144.6, 169.2, 169.5, 169.8, 170.5, 178.0. ESI-HRMS (m/z) [M + H]⁺ calcd for C₄₇H₇₁N₄O₁₂, 883.5063; found, 883.5065.

Compound 61b. Prepared from 61a (70 mg, 0.08 mmol) according to general procedure B. The crude product was

washed with water three times without further purification to afford 61b as a white solid (53 mg, 93%). ^1H NMR (400 MHz, MeOD): δ 0.62, 0.78, 0.89, 0.94, 0.96, 0.97, 1.37 (7 \times CH₃), 0.73–1.97 (m, other aliphatic ring protons), 2.35 (t, 1H, $J = 13.1$ Hz), 2.87 (dd, 1H, $J = 3.0$, 13.5 Hz), 3.15 (dd, 1H, $J = 4.9$, 11.3 Hz), 3.52–3.54 (m, 1H), 3.73–3.80 (m, 3H), 3.92 (dd, 1H, $J = 1.8$, 12.1 Hz), 4.09 (brs, 1H), 4.31 (d, 1H, $J = 14.6$ Hz), 4.35 (brs, 1H), 4.42 (d, 1H, $J = 15.1$ Hz), 5.47 (brs, 1H), 5.98 (brs, 1H), 8.10 (s, 1H). ^{13}C NMR (100 MHz, MeOD): δ 16.2, 16.3, 17.6, 19.4, 24.5, 25.4, 27.3, 27.9, 28.7, 31.2, 31.8, 33.3, 33.9, 36.0 (2C), 36.3, 38.1, 39.8, 39.9, 40.7, 42.3, 42.8, 48.0, 48.2, 49.9, 56.8, 62.6, 67.8, 72.3, 75.0, 75.6, 79.6, 81.5, 88.2, 124.1, 124.3, 144.9, 145.3, 180.1. ESI-HRMS (m/z) [M + H]⁺ calcd for C₃₉H₆₃N₄O₈, 715.4640; found, 715.4637.

Compound 62a. Prepared from 57 (95 mg, 0.19 mmol), acetyl-galactosyl azide (82 mg, 0.22 mmol) according to general procedure C. The residue was purified by column chromatography (petroleum ether/AcOEt, 1/2 v/v) to give 62a as a white solid (160 mg, 98%). ^1H NMR (400 MHz, CDCl₃): δ 0.72, 0.78, 0.90, 0.92, 0.99, 1.36 (7 \times CH₃), 1.88, 2.01, 2.05, 2.24 (4 \times CH₃CO), 0.76–2.21 (m, other aliphatic ring protons), 2.74 (dd, 1H, $J = 3.5$, 13.6 Hz), 3.22–3.24 (m, 1H), 4.11–4.30 (m, 4H), 4.36 (brs, 1H), 4.56 (dd, 1H, $J = 6.0$, 15.2 Hz), 5.25 (dd, 1H, $J = 3.3$, 10.2 Hz), 5.51–5.56 (m, 3H), 5.80 (d, 1H, $J = 9.3$ Hz), 6.90 (t, 1H, $J = 5.2$ Hz), 7.83 (s, 1H). ^{13}C NMR (100 MHz, CDCl₃): δ 15.5, 15.6, 16.8, 18.2, 20.2, 20.4, 20.6 (2C), 23.3, 25.2, 26.9, 27.1, 28.0, 28.9, 30.1, 32.4, 32.5, 35.0, 35.1 (2C), 36.9, 38.5, 38.7, 39.6, 41.7, 41.8, 46.7, 46.8, 49.1, 55.1, 61.1, 66.7, 67.9, 70.7, 73.9, 75.2, 78.8, 86.2, 121.0, 123.5, 143.5, 145.1, 168.9, 169.8, 170.0, 170.3, 177.9. ESI-HRMS (m/z) [M + H]⁺ calcd for C₄₇H₇₁N₄O₁₂, 883.5063; found, 883.5062.

Compound 62b. Prepared from 62a (70 mg, 0.08 mmol) according to general procedure B. The crude product was washed with water three times without further purification to afford 62b as a white solid (53 mg, 93%). ^1H NMR (400 MHz, MeOD): δ 0.65, 0.78, 0.89, 0.94, 0.96, 0.97, 1.37 (7 \times CH₃), 0.73–1.98 (m, other aliphatic ring protons), 2.36 (t, 1H, $J = 13.2$ Hz), 2.87 (dd, 1H, $J = 3.3$, 13.4 Hz), 3.15 (dd, 1H, $J = 4.9$, 11.4 Hz), 3.69 (dd, 1H, $J = 3.2$, 9.5 Hz), 3.75 (d, 2H, $J = 6.0$ Hz), 3.81–3.84 (m, 1H), 3.99 (d, 1H, $J = 2.9$ Hz), 4.14 (t, 1H, $J = 9.4$ Hz), 4.32 (dd, 1H, $J = 5.2$, 15.6 Hz), 4.36 (brs, 1H), 4.44 (dd, 1H, $J = 5.3$, 15.2 Hz), 5.48 (brs, 1H), 5.54 (d, 1H, $J = 9.2$ Hz), 7.60 (t, 1H, $J = 5.2$ Hz), 8.02 (s, 1H). ^{13}C NMR (100 MHz, MeOD): δ 16.2, 16.3, 17.6, 19.4, 24.5, 25.4, 27.3, 27.9, 28.7, 31.2, 31.8, 33.3, 33.9, 36.0, 36.1, 36.3, 38.1, 39.8, 39.9, 40.7, 42.3, 42.8, 48.0, 48.2, 50.0, 56.8, 62.2, 70.2, 71.3, 75.3, 75.6, 79.7, 79.8, 90.1, 123.1, 124.3, 144.9, 146.0, 180.3. ESI-HRMS (m/z) [M + H]⁺ calcd for C₃₉H₆₃N₄O₈, 715.4640; found, 715.4646.

Compound 63a. Prepared from 57 (77 mg, 0.15 mmol), acetyl-rhamnosyl azide (60 mg, 0.19 mmol) according to general procedure C. The residue was purified by column chromatography (petroleum ether/AcOEt, 1/2 v/v) to give 63a as a white solid (109 mg, 89%). ^1H NMR (400 MHz, CDCl₃): δ 0.57, 0.78, 0.89, 0.91, 0.99, 1.36 (7 \times CH₃), 1.99, 2.10, 2.12 (3 \times CH₃CO), 1.35 (d, 3H, $J = 6.2$ Hz), 0.71–2.46 (m, other aliphatic ring protons), 2.70 (dd, 1H, $J = 3.3$, 13.5 Hz), 3.21 (dd, 1H, $J = 3.6$, 10.2 Hz), 3.79–3.86 (m, 1H), 4.34 (dd, 1H, $J = 5.4$, 15.0 Hz), 4.42–4.47 (m, 2H), 5.15–5.20 (m, 1H), 5.24 (dd, 1H, $J = 3.1$, 10.2 Hz), 5.51 (brs, 1H), 5.66 (dd, 1H, $J = 1.0$, 2.9 Hz), 6.12 (d, 1H, $J = 0.8$ Hz), 6.90 (t, 1H, $J = 10.5$ Hz), 7.74 (s, 1H). ^{13}C NMR (100 MHz, CDCl₃): δ 15.4, 15.5, 16.6, 17.4, 18.1, 20.4, 20.5, 20.6, 23.2, 24.8, 26.7, 27.0,

28.0, 29.8, 30.1, 32.5 (2C), 34.9, 35.0, 35.2, 36.8, 38.4, 38.6, 39.4, 41.4, 41.6, 46.6, 46.8, 48.7, 55.1, 69.0, 69.5, 70.7, 73.8, 75.2, 78.7, 84.6, 121.4, 123.5, 143.4, 144.4, 169.1, 169.7, 169.8, 177.9. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₄₅H₆₉N₄O₁₀, 825.5008; found, 825.5018.

Compound 63b. Prepared from **63a** (70 mg, 0.08 mmol) according to general procedure B. The crude product was washed with water three times without further purification to afford **63b** as a white solid (56 mg, 95%). ¹H NMR (400 MHz, MeOD): δ 0.51, 0.77, 0.89, 0.91, 0.96 (6 \times CH₃), 1.36–1.38 (2 \times CH₃), 0.72–1.99 (m, other aliphatic ring protons), 2.37 (t, J = 13.1 Hz), 2.84 (dd, 1H, J = 3.0, 13.3 Hz), 3.14 (dd, 1H, J = 4.5, 11.0 Hz), 3.51 (brs, 2H), 3.67 (brs, 1H), 4.06 (brs, 1H), 4.31–4.44 (m, 3H), 5.45 (brs, 1H), 5.93 (brs, 1H), 8.01 (s, 1H). ¹³C NMR (100 MHz, MeOD): δ 16.2, 16.4, 17.6, 18.2, 19.5, 24.4, 25.3, 27.3, 27.9, 28.7, 31.3, 32.0, 33.3, 33.9, 35.9, 36.0, 36.4, 38.1, 39.8, 39.9, 40.7, 42.3, 42.8, 48.1, 48.1, 49.9, 56.7, 72.3, 73.1, 74.7, 75.6, 76.9, 79.6, 88.2, 124.0, 124.3, 144.9, 145.3, 180.1. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₃₉H₆₃N₄O₇, 699.4691; found, 699.4691.

Compound 64a. Prepared from **57** (88 mg, 0.17 mmol), acetyl-lyxosyl azide (57 mg, 0.19 mmol) according to general procedure C. The residue was purified by column chromatography (petroleum ether/AcOEt, 1/2 v/v) to give **64a** as a white solid (87 mg, 64%). ¹H NMR (400 MHz, CDCl₃): δ 0.52, 0.79, 0.90, 0.92, 0.99, 1.35 (7 \times CH₃), 2.02, 2.09, 2.13 (3 \times CH₃), 0.70–2.27 (m, other aliphatic ring protons), 2.71 (dd, 1H, J = 3.6, 13.6 Hz), 3.21 (dd, 1H, J = 3.9, 10.4 Hz), 3.54 (dd, 1H, J = 10.0, 11.1 Hz), 4.29–4.46 (m, 4H), 5.25–5.36 (m, 2H), 5.53 (brs, 1H), 5.67 (dd, 1H, J = 1.1, 2.8 Hz), 6.05 (d, 1H, J = 0.84 Hz), 6.88 (t, 1H, J = 5.4 Hz), 7.72 (s, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 15.5 (2C), 16.6, 18.1, 20.5 (2C), 20.7, 23.3, 24.9, 26.8, 27.1, 28.0, 29.8, 30.2, 32.4, 32.5, 35.0, 35.1, 35.3, 36.9, 38.5, 38.7, 39.5, 41.5, 41.7, 46.6, 46.9, 48.9, 55.2, 65.4, 65.8, 68.8, 70.3, 75.4, 78.9, 85.1, 121.4, 123.6, 143.5, 144.5, 169.1, 169.8, 169.9, 177.9. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₄₄H₆₇N₄O₁₀, 811.4852; found, 811.4862.

Compound 64b. Prepared from **64a** (40 mg, 0.05 mmol) according to general procedure B. The crude product was washed with water three times without further purification to afford **64b** as a white solid (32 mg, 95%). ¹H NMR (400 MHz, MeOD): δ 0.56, 0.77, 0.88, 0.93, 0.96, 0.97, 1.36 (7 \times CH₃), 0.72–1.97 (m, other aliphatic ring protons), 2.35 (t, 1H, J = 13.2 Hz), 2.88 (dd, 1H, J = 3.4, 13.6 Hz), 3.15 (dd, 1H, J = 4.9, 11.4 Hz), 3.39 (t, 1H, J = 10.7 Hz), 3.66 (dd, 1H, J = 2.9, 9.2 Hz), 3.92–3.98 (m, 1H), 4.03–4.07 (m, 2H), 4.32–4.43 (m, 3H), 5.45 (brs, 1H), 5.89 (brs, 1H), 8.03 (s, 1H). ¹³C NMR (100 MHz, MeOD): δ 16.1, 16.3, 17.6, 19.4, 24.4, 25.4, 27.3, 27.9, 28.7, 31.2, 31.9, 33.3, 33.9, 36.0 (2C), 36.3, 38.1, 39.8, 39.9, 40.7, 42.3, 42.8, 48.0, 48.2, 49.9, 56.8, 67.3, 69.8, 72.0, 75.0, 75.6, 79.7, 88.8, 124.0, 124.2, 144.9, 145.5, 180.0. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₃₈H₆₁N₄O₇, 685.4535; found, 685.4527.

Compound 65a. Prepared from **57** (60 mg, 0.12 mmol), acetyl-lactosyl azide (126 mg, 0.19 mmol) according to general procedure C. The residue was purified by column chromatography (petroleum ether/AcOEt, 1/2 v/v) to give **65a** as a white solid (120 mg, 86%). ¹H NMR (400 MHz, CDCl₃): δ 0.69, 0.78, 0.91, 0.99, 1.36 (7 \times CH₃), 1.86, 2.00, 2.02, 2.05, 2.11, 2.12, (7 \times CH₃CO), 0.72–2.38 (m, other aliphatic ring protons), 2.71–2.74 (m, 1H), 3.21–3.23 (m, 1H), 3.70–3.71 (m, 1H), 3.93–3.97 (m, 2H), 4.06–4.15 (m, 2H), 4.27 (dd, 1H, J = 4.6, 15.2 Hz), 4.35 (brs, 1H), 4.40 (dd, 1H, J = 4.2, 12.5

Hz), 4.50–4.58 (m, 3H), 4.93–4.97 (m, 1H), 5.06–5.20 (m, 2H), 5.38–5.40 (m, 2H), 5.54 (brs, 1H), 5.75–5.82 (m, 1H), 6.93 (t, 1H, J = 5.1 Hz), 7.73 (s, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 15.5 (2C), 16.7, 18.1, 20.1, 20.3, 20.4 (3C), 20.6, 20.7, 23.3, 25.1, 26.8, 27.0, 27.9, 28.9, 30.1, 32.3, 32.5, 35.0, 35.1 (2C), 36.8, 38.5, 38.6, 39.5, 41.6, 41.7, 46.6, 46.7, 49.0, 55.0, 61.4, 61.5, 67.6, 70.4, 71.4, 72.0, 72.2, 72.7, 75.1, 75.7 (2C), 78.7, 85.4, 100.7, 121.0, 123.4, 143.5, 145.0, 168.9, 169.0, 169.2, 169.5, 170.0, 170.1, 170.4, 178.0. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₅₉H₈₇N₄O₂₀, 1171.5908; found, 1171.5877.

Compound 65b. Prepared from **65a** (80 mg, 0.07 mmol) according to general procedure B. The crude product was washed with water three times without further purification to afford **65b** as a white solid (52 mg, 91%). ¹H NMR (400 MHz, MeOD): δ 0.62, 0.77, 0.88, 0.93, 0.96, 0.97, 1.36 (7 \times CH₃), 0.72–1.97 (m, other aliphatic ring protons), 2.35 (t, 1H, J = 13.3 Hz), 2.89 (dd, 1H, J = 3.3, 13.4 Hz), 3.15 (dd, 1H, J = 5.0, 11.2 Hz), 3.52–3.96 (m, 12H), 4.36–4.47 (m, 4H), 5.46 (brs, 1H), 5.70 (d, 1H, J = 9.2 Hz), 8.19 (s, 1H). ¹³C NMR (100 MHz, MeOD): δ 16.2, 16.4, 17.7, 19.4, 24.4, 25.4, 27.3, 27.8, 28.7, 31.2, 31.8, 33.3, 33.9, 35.5, 36.0, 36.3, 38.1, 39.8, 39.9, 40.7, 42.1, 42.7, 48.0, 48.1, 49.9, 56.7, 61.4, 62.5, 70.3, 72.4, 73.7, 74.7, 75.5, 76.5, 77.1, 79.6 (2C), 89.7, 105.0, 124.2, 124.6, 144.8, 145.2, 180.3. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₄₅H₇₃N₄O₁₃, 877.5169; found, 877.5169.

Compound 66a. Prepared from **57** (75 mg, 0.15 mmol), acetyl-maltosyl azide (126 mg, 0.19 mmol) according to general procedure C. The residue was purified by column chromatography (petroleum ether/AcOEt, 1/2 v/v) to give **66a** as a white solid (163 mg, 93%). ¹H NMR (400 MHz, CDCl₃): δ 0.69, 0.78, 0.91, 0.99, 1.36 (7 \times CH₃), 1.85, 2.02, 2.04, 2.07, 2.11, 2.14, (7 \times CH₃CO), 0.72–2.25 (m, other aliphatic ring protons), 2.71–2.74 (m, 1H), 3.21–3.23 (m, 1H), 3.98 (d, 2H, J = 9.6 Hz), 4.07 (d, 1H, J = 11.8 Hz), 4.14 (t, 1H, J = 9.2 Hz), 4.24–4.28 (m, 3H), 4.36 (brs, 1H), 4.48–4.56 (m, 2H), 4.88 (dd, 1H, J = 3.8, 10.5 Hz), 5.08 (t, 1H, J = 9.8 Hz), 5.30–5.55 (m, 4H), 5.54 (brs, 1H), 5.85 (d, 1H, J = 9.2 Hz), 6.90 (t, 1H, J = 4.8 Hz), 7.71 (s, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 15.5, 15.6, 16.8, 18.2, 20.1, 20.5 (3C), 20.6, 20.7, 20.8, 23.3, 25.1, 26.8, 27.1, 28.0, 29.0, 30.1, 32.4, 32.5, 35.0, 35.1 (2C), 36.8, 38.5, 38.7, 39.5, 41.6, 41.7, 46.7, 46.8, 49.0, 55.1, 61.4, 62.4, 67.8, 68.7, 69.1, 69.9, 70.9, 72.3, 75.0, 75.2, 75.7, 78.7, 85.2, 95.8, 120.9, 123.5, 143.5, 145.1, 169.0, 169.3, 169.9 (2C), 170.2, 170.4, 170.5, 178.0. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₅₉H₈₇N₄O₂₀, 1171.5908; found, 1171.5910.

Compound 66b. Prepared from **66a** (100 mg, 0.09 mmol) according to general procedure B. The crude product was washed with water three times without further purification to afford **66b** as a white solid (64 mg, 86%). ¹H NMR (400 MHz, MeOD): δ 0.63, 0.77, 0.88, 0.94, 0.96, 0.97, 1.36 (7 \times CH₃), 0.72–1.97 (m, other aliphatic ring protons), 2.34 (t, 1H, J = 13.1 Hz), 2.88–2.91 (m, 1H), 3.15 (dd, 1H, J = 4.6, 10.8 Hz), 3.26–3.29 (m, 1H), 3.50 (dd, 1H, J = 3.6, 9.7 Hz), 3.63–3.94 (m, 10H), 4.37–4.48 (m, 3H), 5.26 (d, J = 3.6 Hz), 5.46 (brs, 1H), 5.70 (d, 1H, J = 8.4 Hz), 8.24 (s, 1H). ¹³C NMR (100 MHz, MeOD): δ 16.2, 16.4, 17.7, 19.4, 24.5, 25.4, 27.3, 27.8, 28.7, 31.2, 31.8, 33.3, 33.9, 35.4, 36.0, 36.3, 38.1, 39.8, 39.9, 40.7, 42.1, 42.7, 48.0, 48.1, 50.0, 56.8, 61.6, 62.6, 71.5, 73.7, 74.1, 74.8, 75.0, 75.5, 77.9, 79.6, 80.1, 89.9, 102.9, 124.2, 144.8, 180.3. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₄₅H₇₃N₄O₁₃, 877.5169; found, 877.5174.

Compound 67a. Prepared from **57** (82 mg, 0.16 mmol), acetyl-celllobiosyl azide (126 mg, 0.19 mmol) according to

general procedure C. The residue was purified by column chromatography (petroleum ether/AcOEt, 1/2 v/v) to give **67a** as a white solid (163 mg, 87%). ¹H NMR (400 MHz, CDCl₃): δ 0.69, 0.78, 0.91, 0.99, 1.36 (7 \times CH₃), 1.87, 1.98, 2.06, 2.07, 2.09, 2.11, 2.17 (7 \times CH₃CO), 0.72–2.25 (m, other aliphatic ring protons), 2.71 (dd, 1H, J = 3.0, 13.4 Hz), 3.21–3.23 (m, 1H), 3.92–3.96 (m, 3H), 4.08–4.18 (m, 3H), 4.27 (dd, 1H, J = 4.7, 15.2 Hz), 4.35 (brs, 1H), 4.47–4.54 (m, 3H), 4.98 (dd, 1H, J = 3.4, 10.4 Hz), 5.13 (dd, 1H, J = 8.0, 10.3 Hz), 5.36–5.41 (m, 3H), 5.55 (brs, 1H), 5.79 (dd, 1H, J = 8.7 Hz), 6.90 (t, 1H, J = 5.1 Hz), 7.72 (s, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 15.5, 15.6, 16.8, 18.2, 20.2, 20.4, 20.6 (3C), 20.7 (2C), 23.3, 25.2, 26.8, 27.1, 28.0, 28.9, 30.1, 32.4, 32.5, 35.0, 35.1 (2C), 36.9, 38.5, 38.7, 39.5, 41.7 (2C), 46.7, 46.8, 49.1, 55.1, 60.8, 61.6, 66.5, 69.0, 70.5, 70.8 (2C), 72.5, 75.2, 75.5, 75.8, 78.8, 85.5, 101.0, 121.0, 123.6, 143.5, 145.1, 169.0 (2C), 169.5, 170.0, 170.1 (2C), 170.3, 178.0. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₅₉H₈₇N₄O₂₀, 1171.5908; found, 1171.5901.

Compound 67b. Prepared from **67a** (100 mg, 0.09 mmol) according to general procedure B. The crude product was washed with water three times without further purification to afford **67b** as a white solid (69 mg, 92%). ¹H NMR (400 MHz, MeOD): δ 0.62, 0.78, 0.88, 0.94, 0.96, 0.97, 1.36 (7 \times CH₃), 0.72–1.97 (m, other aliphatic ring protons), 2.34 (t, 1H, J = 13.2 Hz), 2.89 (dd, 1H, J = 2.7, 10.4 Hz), 3.15 (dd, 1H, J = 4.7, 11.0 Hz), 3.26–3.44 (m, 2H), 3.52–3.95 (m, 10H), 4.35–4.50 (m, 4H), 5.46 (brs, 1H), 5.69 (d, 1H, J = 8.4 Hz), 5.70 (d, 1H, J = 8.4 Hz), 8.24 (s, 1H). ¹³C NMR (100 MHz, MeOD): δ 16.2, 16.4, 17.6, 19.4, 24.4, 25.4, 27.3, 27.8, 28.7, 31.2, 31.8, 33.3, 33.9, 35.5, 36.0, 36.3, 38.0, 39.8, 39.9, 40.7, 42.1, 42.7, 48.0, 48.1, 49.9, 56.7, 61.3, 62.3, 71.3, 73.7, 74.8, 75.5, 76.5, 77.7, 78.1, 79.5, 79.6, 79.6, 89.6, 104.5, 124.2, 124.5, 144.8, 145.4, 180.3. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₄₅H₇₃N₄O₁₃, 877.5169; found, 877.5170.

Compound 68a. Prepared from **57** (68 mg, 0.13 mmol), acetyl-maltotriosyl azide (180 mg, 0.19 mmol) according to general procedure C. The residue was purified by column chromatography (petroleum ether/AcOEt, 1/2 v/v) to give **68a** as a white solid (163 mg, 87%). ¹H NMR (400 MHz, CDCl₃): δ 0.69, 0.78, 0.91, 0.99, 1.36 (7 \times CH₃), 1.85, 2.00, 2.04, 2.04, 2.06, 2.10, 2.02, 2.17 (10 \times CH₃CO), 0.72–2.25 (m, other aliphatic ring protons), 2.72 (dd, 1H, J = 3.2, 13.5 Hz), 3.21 (dd, 1H, J = 3.2, 10.3 Hz), 3.93–4.35 (m, 12H), 4.47–4.56 (m, 3H), 4.77 (dd, 1H, J = 4.0, 10.3 Hz), 4.86 (dd, 1H, J = 4.0, 10.5 Hz), 5.08 (t, 1H, J = 9.9 Hz), 5.29–5.49 (m, 5H), 5.54 (brs, 1H), 5.85 (d, 1H, J = 9.1 Hz), 6.88 (t, 1H, J = 5.1 Hz), 7.70 (s, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 15.5, 15.6, 16.8, 18.2, 20.1, 20.5 (4C), 20.6, 20.7 (3C), 20.8, 23.3, 25.1, 26.8, 27.1, 28.0, 29.1, 30.1, 32.5, 35.1, 35.2 (2C), 36.9, 38.5, 38.7, 39.6, 41.7, 41.8, 46.7, 46.8, 49.1, 53.4, 55.1, 61.3, 62.2, 62.6, 67.9, 68.5, 69.2, 69.3, 70.0, 70.4, 71.0, 71.5, 72.5, 73.4, 74.9, 75.2, 75.3, 78.8, 85.2, 95.6, 96.1, 120.9, 123.6, 143.5, 145.1, 169.1, 169.4, 169.6, 169.8 (2C), 170.3 (2C), 170.5 (3C), 178.0. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₇₁H₁₀₃N₄O₂₈, 1459.6753; found, 1459.6784.

Compound 68b. Prepared from **68a** (110 mg, 0.07 mmol) according to general procedure B. The crude product was washed with water three times without further purification to afford **68b** as a white solid (54 mg, 73%). ¹H NMR (400 MHz, MeOD): δ 0.65, 0.78, 0.89, 0.94, 0.95, 0.97, 1.37 (7 \times CH₃), 0.72–1.98 (m, other aliphatic ring protons), 2.35 (t, 1H, J = 7.9 Hz), 2.88 (dd, 1H, J = 3.0, 13.4 Hz), 3.15 (dd, 1H, J = 4.9, 11.3 Hz), 3.26–3.29 (m, 1H), 3.45–3.95 (m, 16H), 4.32 (d, 1H, J =

15.4 Hz), 4.35 (brs, 1H), 4.43 (d, 1H, J = 15.2 Hz), 5.17 (d, 1H, J = 3.6 Hz), 5.26 (d, 1H, J = 3.6 Hz), 5.47 (brs, 1H), 5.62 (d, 1H, J = 8.9 Hz), 7.99 (s, 1H). ¹³C NMR (100 MHz, MeOD): δ 16.2, 16.4, 17.7, 19.5, 24.5, 25.4, 27.3, 27.9, 28.7, 31.2, 31.7, 33.3, 33.9, 36.0, 36.0, 36.3, 38.1, 39.8, 39.9, 40.8, 42.2, 42.8, 48.0, 48.1, 50.0, 56.8, 61.8, 62.1, 62.7, 71.5, 73.4, 73.6, 73.7, 74.2, 74.7, 74.9, 75.0, 75.6, 78.1, 79.5, 79.6, 80.3, 81.3, 89.3, 102.7, 102.9, 123.5, 124.2, 144.9, 146.0, 180.2. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₅₁H₈₃N₄O₁₈, 1039.5697; found, 1039.5707.

Compound 69. To **56** (75 mg, 0.13 mmol) and K₂CO₃ (68 mg, 1 mmol) stirring in 3 mL DMF was added 1,3-propanediamine (5.5 μ L, 0.06 mmol). The mixture was stirred at room temperature overnight. After completion (TLC), the solvent was removed under reduced pressure. The mixture was resolved in CH₂Cl₂ and washed with water and brine twice. The organic layer was dried over MgSO₄, then filtered and concentrated. The crude residue was purified by column chromatography (CH₂Cl₂/CH₃OH, 30/1 v/v) to give the product as a white solid (35 mg, 55%). ¹H NMR (400 MHz, MeOD): δ 0.76, 0.88, 0.92, 0.95, 0.96, 1.35 (14 \times CH₃), 0.71–2.01 (m, other aliphatic ring protons), 2.27 (t, 2H, J = 13.3 Hz), 2.86–2.89 (m, 2H), 3.03–3.17 (m, 6H), 4.30 (brs, 2H), 5.51 (brs, 2H), 7.09 (brs, 2H). ¹³C NMR (100 MHz, MeOD): δ 16.1, 17.7, 19.0, 24.1, 25.6, 27.1, 27.3, 28.5, 30.0, 30.8, 31.0, 33.0, 33.4, 35.6, 35.8, 37.3, 37.6, 39.4, 39.4, 40.3, 41.8, 42.4, 47.5, 47.5, 56.1, 75.0, 79.2, 123.6, 144.4, 179.8. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₆₃H₁₀₃N₂O₆, 983.7811; found, 983.7804.

Compound 70. Prepared from **57** (112 mg, 0.22 mmol), 1,3-diazidopropane (12.6 mg, 0.1 mmol) according to general procedure C. The residue was purified by column chromatography (CH₂Cl₂/CH₃OH, 25/1 v/v) to give the product as a white solid (100 mg, 87%). ¹H NMR (400 MHz, MeOD): δ 0.54, 0.71, 0.76, 0.88, 0.96, 0.96, 0.96, 1.37 (s, 14 \times CH₃), 0.71–2.01 (m, other aliphatic ring protons), 2.35 (t, 2H, J = 13.2 Hz), 2.46–2.53 (m, 2H), 2.87 (dd, 2H, J = 3.1, 9.9 Hz), 3.14 (dd, 2H, J = 5.0, 10.9 Hz), 4.36–4.44 (m, 10H), 5.47 (brs, 2H), 7.82 (s, 2H). ¹³C NMR (100 MHz, MeOD): δ 16.2, 16.4, 17.7, 19.4, 24.5, 25.4, 27.3, 27.9, 28.7, 31.3, 31.8, 32.0, 33.3, 33.9, 36.0, 36.4, 38.1, 39.8, 39.9, 40.7, 42.3, 42.8, 48.1, 48.2, 50.0, 56.7, 75.6, 79.6, 124.2, 124.7, 145.0, 146.1, 180.1. ESI-HRMS (*m/z*) [M + H]⁺ calcd for C₆₉H₁₀₉N₈O₆, 1145.8465; found, 1145.8438.

Compound 71. To D-biotin (488 mg, 2 mmol) stirring in 8 mL DMF, N-hydroxysuccinimide (344 mg, 3 mmol) and EDC (576 mg, 3 mmol) were added. The mixture was stirred at rt overnight. The reaction mixture was then poured into ice (400 mL), and the precipitate was filtered. The precipitate was washed twice and dried under reduced pressure to afford a white solid (580 mg, 85%). The analytical data was in accordance with the reported characterization data.¹⁸

Compound 72. To **71** (96 mg, 0.28 mmol) stirring in 3 mL DMF, TEA (80 μ L, 0.70 mmol) and 2-azidoethylamine (48 mg, 0.56 mmol) were added. The reaction was stirred for 12 h at rt. The contents were evaporated under reduced pressure. The residue was purified by column chromatography (CH₂Cl₂/CH₃OH/AcOH, 100/10/1 v/v/v) to give **72** as a white solid (46.4 mg, 53%). The analytical data was in accordance with the reported characterization data.¹⁸

Compound 73. To a solution of **57** (51 mg, 0.1 mmol) and **72** (37.4 mg, 0.12 mmol) in THF (3 mL) and H₂O (1 mL), CuSO₄ (19.2 mg, 0.12 mmol) and Na-L-ascorbate (47.5 mg,

0.24 mmol) were added. The resulting solution was stirred for 12 h at 40 °C. After completion (TLC), the contents were evaporated under reduced pressure. The residue was purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$, 10/1 v/v) to give 73 as a white solid (58.4 mg, 71%). ^1H NMR (400 MHz, pyridine- d_5): δ 0.88, 0.97, 1.11, 1.04, 1.07, 1.23, 1.75 (7 \times CH_3), 0.90–2.10 (m, other aliphatic ring protons), 2.29–2.37 (m, 3H), 2.44–2.48 (m, 1H), 2.78 (t, 1H, J = 13.1 Hz), 2.88 (d, 1H, J = 12.4 Hz), 2.96 (dd, 1H, J = 4.9, 12.6 Hz), 3.21–3.26 (m, 1H), 3.38 (dd, 1H, J = 3.3, 13.5 Hz), 3.44–3.48 (m, 1H), 3.87–3.92 (m, 2H), 4.38–4.41 (m, 1H), 4.54–4.57 (m, 1H), 4.67–4.75 (m, 3H), 4.85 (dd, 1H, J = 5.5, 14.9 Hz), 5.10 (brs, 1H), 5.66 (brs, 1H), 7.47 (brs, 1H), 8.12 (s, 1H), 8.19 (t, 1H, J = 5.2 Hz), 8.83 (t, 1H, J = 5.7 Hz). ^{13}C NMR (100 MHz, pyridine- d_5): δ 16.2, 16.9, 17.6, 19.1, 24.2, 25.6, 26.3 (2C), 27.6, 28.5, 29.1, 29.3 (2C), 31.1, 32.1, 33.5, 33.6, 36.2, 36.4 (2C), 37.7, 39.5, 39.7, 40.4, 40.4, 41.4, 42.0, 42.6, 47.6, 47.8, 49.6, 50.1, 56.2, 56.6, 61.0, 62.7, 75.2, 78.4, 123.4, 123.9, 145.0, 146.2, 164.9, 173.9, 178.3. ESI-HRMS (m/z) [M + H]⁺ calcd for $C_{45}\text{H}_{72}\text{N}_7\text{O}_5\text{S}$, 822.5310; found, 822.5318.

HCV and VSV Pseudovirus Entry Assay. All compounds were tested using the HCVpp and VSVpp entry assay as described previously. Briefly, pseudotyped viruses were produced by cotransfecting 293T cells with plasmid encoding HCV E1, E2, or vesicular stromatis G protein (VSVG), and the envelope and Vpr-deficient HIV vector carrying a luciferase reporter gene inserted into the Nef position 72 h after transfection. HCVpp or VSVpp was harvested from the supernatant of the transfected cells. For compound library screening, infections were performed in 96-well plates by adding diluted HCVpp or VSVpp into 5×10^3 Huh-7 cells/well in the presence or absence of test compounds, followed by incubation for 72 h at 37 °C. Luciferase activity, reflecting the degree of the pseudoparticles into host cells, was measured 3 days after infection using the Bright-Glo Reagent (Promega). Test compounds were serially diluted to give a final concentration of 1 and 5 μM in 1% dimethyl sulfoxide (DMSO). Maximum activity (100% of control) and background were derived from control wells containing DMSO alone or from uninfected wells, respectively. The individual signals in each of the compound test wells were then divided by the averaged control values (wells lacking inhibitor) after background subtraction and multiplied by 100% to determine percent activity. The corresponding inhibition values were then calculated by subtracting this value from 100. The specificity of the compounds for inhibiting HCV was determined by evaluating inhibition of VSVpp infection in parallel. Each sample was done in duplicate, and experiments were repeated at least three times.

Hemolytic Assay. Hemolytic activity was measured as following:¹⁹ 2% rabbit red blood cells in erythrocyte buffer (130 mM NaCl, 20 mM Tris–HCl, pH 7.4) were incubated with serially diluted compounds. After incubating for 60 min at 37 °C, hemolysis was monitored by measuring absorption at 540 nm with a microplate reader. Percentage of hemolysis was then calculated as the routine method.

ASSOCIATED CONTENT

Supporting Information

Further chemical and biological experimental details, including HPLC data and NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

(D.Z.) Phone: +86-10-82805857. Fax: +86-10-82805857. E-mail: deminzhou@bjmu.edu.cn. (S.X.) Phone: +86-10-82805646. E-mail: slxiao@bjmu.edu.cn.

Author Contributions

[§]F. Yu, Q. Wang, and Z. Zhang contributed equally to this work.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

OA, oleanolic acid; EA, echinocystic acid; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; DMAP, 4-(*N,N*-dimethylamino)pyridine; TBTU, O-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; DIEA, *N,N*-diisopropylethylamine; THF, tetrahydrofuran; TEA, triethylamine; EDC, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; TBAB, tetrabutylammonium bromide

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