7368 J. Med. Chem. 2009, 52, 7368–7371
DOI: 10.1021/jm900275m

Journal of Medicinal Chemistry Letter

Discovery of the First Series of Small Molecule H5N1 Entry Inhibitors

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Received March 4, 2009

Abstract: The occurrence of highly pathogenic avian influenza virus H5N1 highlights the urgent need for new classes of antiviral drugs. Inhibition of H5N1 entry into cells may be an effective strategy. We report the first three small molecule inhibitors saponins with 3-*O*-*β*-chacotriosyl residue, which showed potent inhibitory activity with IC_{50} of 7.22−9.25 μM. The subsequent SAR studies showed the 3-*O*-*β*-chacotriosyl residue was essential for the activity, and the aglycone structure also affected the activity.

Highly pathogenic avian influenza (HPAI) continues to cause outbreaks in poultry and migratory birds in Asia, Europe, and Africa. Recently, humans became infected by this virus with both increasing number of infected individuals and high mortality rates,[1−3] suggesting persisting threat of human pandemic. In the wake of such a pandemic, the World Health Organization (WHO) and many individual nations have developed plans to limit its hazardous consequences. Tremendous efforts are made already to fully understand the pandemic of the virus and to develop effective therapies to control the spread of the virus. To date, only two classes of anti-influenza drugs have been approved that may be effective against H5N1 viruses. The first class is the inhibitors of M2 ion channel such as amantadine and rimantadine. But use of these inhibitors rapidly leads to the emergence of resistant variants and thus they are not recommended for a general and uncontrolled use.[4] The second class is the neuraminidase inhibitors such as oseltamivir, zanamivir, and peramivir. However, resistance of H5N1 to oseltamivir has also been observed recently.[5] Therefore, there is an urgent need for new classes of agents to combat avian H5N1 variants that are resistant to treatment by targeting at other potential viral factors.

The first step of influenza virus infection is the attachment of viral particles to the host cell. This is mediated by viral envelope glycoprotein, hemagglutinin (HA), which binds to its receptor on host cell, sialic acid, leading to viral endocytosis. Therefore, it is important to identify and develop potent entry inhibitors against H5N1 virus as potential antiviral treatments.

Because of the safety concerns in studying highly pathogenic viral pathogens such as Ebola and Marburg viruses, SARS-CoV, pseudotyping systems have been established that alleviate the safety concerns of working with the "live" viruses and also allow easy manipulation of viral glycoproteins in receptor binding and viral entry. Pseudotype reporter viruses have been widely used as a quantitative high-throughput method for assessing viral entry inhibitors and antibody neutralization for highly pathogenic enveloped viruses such as SARS and Ebola viruses.[6,7] Influenza A virus surface glycoprotein HA is the sole surface protein essential for viral entry. Lentiviral vector pseudotyped with high pathogenic H5N1 subtypes influenza A viruses hemagglutintins has been established by us.[8] Others have used the same system to identify antiflu neutralizating antibodies.[9] In this report, we used this efficient HIV-based pseudotyping system to evaluate potential anti-H5N1 entry inhibitors, while the VSV-G/HIV pseudovirions[7] were used as specificity controls. Using these pseudotyping systems, we have screened a saponin library generated from semisynthesis. We found that chlorogenin 3-*O*-*β*-chacotrioside (**1**)[10] and chlorogenin 6-α-*O*-acetyl-3-*O*-*β*-chacotrioside (**2**)[10] displayed strong inhibitory activity against H5N1 entry with IC_{50} of 7.22 and 9.25 μM, respectively. Notably, the two saponins **1** and **2** beared the same *β*-chacotriosyl (α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranosyl) residue, which was a typical sugar chain of natural spirostan saponins with the most cytotoxic activities when compared to the other spirostan saponin members in nature.[11] To our knowledge, this is the first report that these small molecule compounds were discovered to effectively block H5N1 viral entry (Figure 1).

To develop additional small molecule inhibitors with enhanced activity, compound **1** was chosen as the lead compound to design and synthesize a series of analogues **3**−**9** to explore the preliminary structure−activity relations of these designed molecules around the aglycone and sugar chain. We first investigated the effect of aglycone residue on the inhibitory activity. Dihydrochlorogenin, dehydroisoandrosterone, methyl oleanolate, methyl ursolate, and stigmasterine[12] were selected as the aglycone moieties, and the resulting five saponins **3**−**7** with the same 3-*O*-*β*-chacotriosyl residue were derived (Figure 2). Saponins **8** and **9**, simplifing the *β*-chacotriosyl moiety of **1** into α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl and α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl moiety, were also synthesized in order to understand the effect of sugar chain on the activity. Here we report the determination of the saponin inhibitors for H5N1 viral entry and preliminary structure−activity relations (SAR[a]) of these designed molecules **3**−**9**.

The preparation of known saponin **3** was performed according to our previous procedure.[12] The synthesis of target saponins **4**−**6** was done by the similar route as that for compounds **1** and **2** reported previously by us.[10,12] As shown in Scheme 1, glycosylation of dehydroisoandrosterone, methyl oleanolate,

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[a] Abbreviations: Lev, levulinoyl; LevOH, acetylpropionic acid; TMSOTf, trimethylsilyl trifluoromethanesulfonate; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; NIS, *N*-iodobutanimide; AgOTf, silver trifluoromethanesulfonate; SAR, structure−activity relations.

 Published on Web 06/18/2009

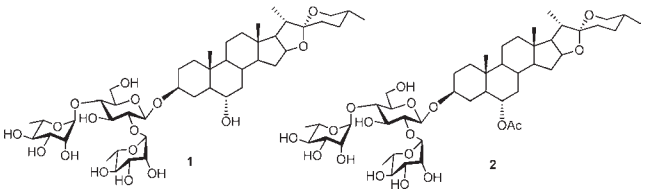


Figure 1. Saponin inhibitors for H5N1 viral entry.

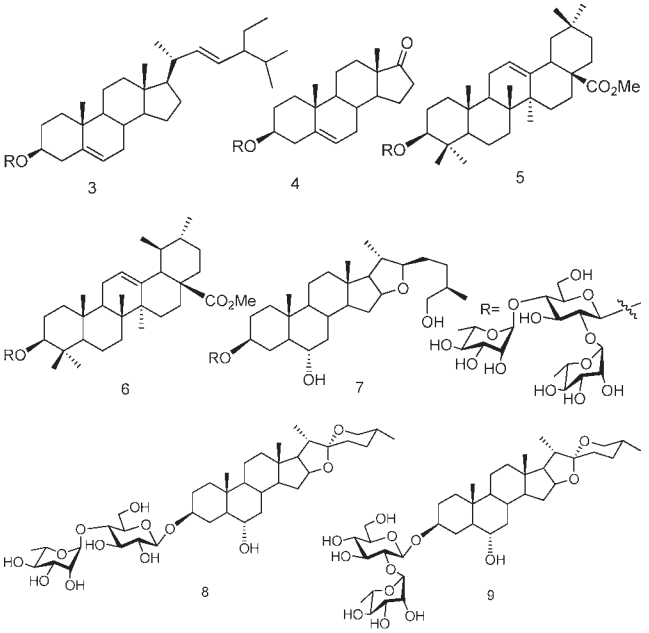


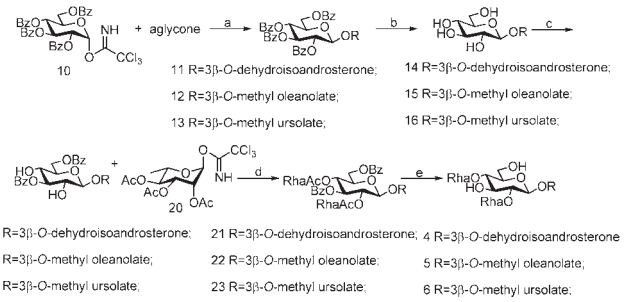
Figure 2. Saponins designed for SAR studies.

and methyl ursolate with 2,3,4,6-tetra-*O*-benzoyl-D-glucopyranosyl trichloroacetimidate (**10**), respectively, under the action of TMSOTf afforded the 3-*O*-*β*-glucopyranosides **11**−**13**. Removal of the benzoyl groups with NaOMe in MeOH gave **14**−**16**, which were subjected to 1-BBTZ [1-(benzoyloxy)-benzotriazole] in the presence of Et_3N to protect selectively the 3,6-OHs of the *β*-glucopyranosyl residues, leading to intermediates **17**−**19**. Subsequent glycosylation of the 2,4-OHs in **17**−**19** with 2,3,4-tri-*O*-acetyl-L-rhamnopyranosyl trichloroacetimidate (**20**) under the "inverse addition conditions" with $BF_3 \cdot Et_2O$ as the promoter led to the desired compounds **21**−**23**. Final treatment with NaOMe in MeOH afforded target saponins **4**−**6** smoothly.

The synthetic route toward **7** is depicted in Scheme 2. Glycosyl acceptor **27** was first prepared starting from 3-*O*-benzyl-chlorogenin **24**.[10] Reductive opening of the spiroketal with $LiAlH_4/AlCl_3$ and followed by protection of **6**, 26-OHs in **25** with acetyl group afforded **26**, in which the 3-*O*-benzyl group was removed by hydrogenolysis to provide the important intermediate **27**.

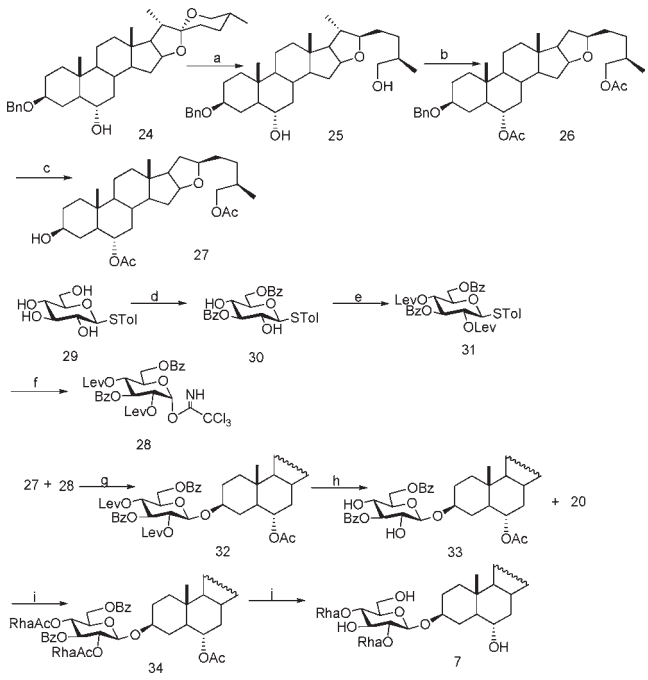
The next step was to elaborate glucopyranosyl donor **28**. Levulinoyl group was chosen to protect the C_2-OH and C_4-OH in **28**, which was helpful to construct *β*-glycosidic linkage as the neighboring ester group and could be selectively removed without affecting the benzoyl group in **28** and acetyl group in acceptor **27**. Thus treatment of thioglycoside **29** with 1-BBTZ in the presence of Et_3N protected selectively the 3,6-OHs to afford compound **30**, which was then masked the following 2,4-OHs with levulinoyl group to furnish **31**. The latter was hydrolyzed with *N*-bromosuccinimide (NBS) in acetone−H_2O, followed by treatment with CCl_3CN and DBU to give

Scheme 1. Synthesis of Target Compounds **4**−**6**[a]



[a]Reagents and conditions: (a) TMSOTf, CH_2Cl_2; (b) MeONa; (c) 1-BBTZ, Et_3N, CH_2Cl_2; (d) $BF_3 \cdot Et_2O$, CH_2Cl_2; (e) MeONa.

Scheme 2. Synthesis of Target Compound **7**[a]



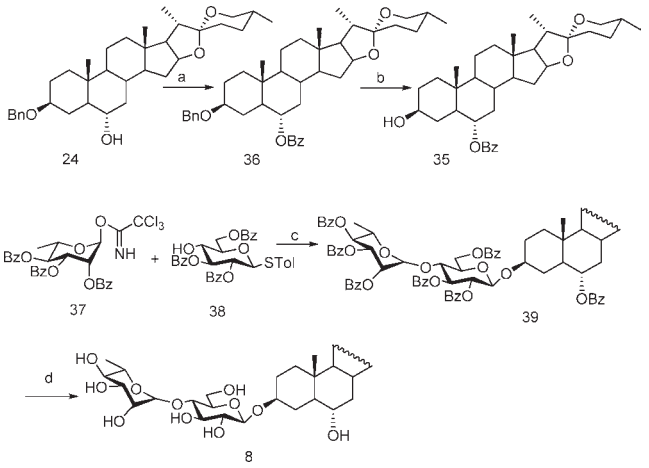
[a]Reagents and conditions: (a) $LiAlH_4$, $AlCl_3$; (b) Ac_2O, pyridine; (c) Pd/C; (d) 1-BBTZ, Et_3N, CH_2Cl_2; (e)LevOH, EDC·HCl, DMAP; (f) i: NBS, acetone-H_2O; ii: DBU, $CNCCl_3$, CH_2Cl_2; (g) TMSOTf, CH_2Cl_2; (h) AcOH-NH_2NH_2; (i) $BF_3 \cdot Et_2O$, CH_2Cl_2; (j) MeONa.

corresponding trichloroacetimidate **28**. With the acceptor **27** and the donor **28** in hand, their coupling reaction was then performed under the promotion of TMSOTf to provide the desired compound **32**, and the following deprotection of the Lev group with hydrazine acetate gave **33**. The target saponin **7** was finally obtained from first glycosylation of **33** with **20** under the promotion of $BF_3 \cdot Et_2O$ and then deprotection of the acyl groups with MeONa.

Target chlorogenin **8** containing α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl moiety was synthesized as shown in Scheme 3. Treatment of the compound **24** with BzCl in the presence of DMAP afforded compound **36**, in which the 3-*O*-benzyl group was removed to give the important intermediate **35**. Target compound **8** could be obtained by taking advantage of a "one-pot" procedure. In this method, trichloroacetamidate glycosyl **37** and thioglycoside **38** were used as sequential glycosyl donors for two glycosidic linkages.

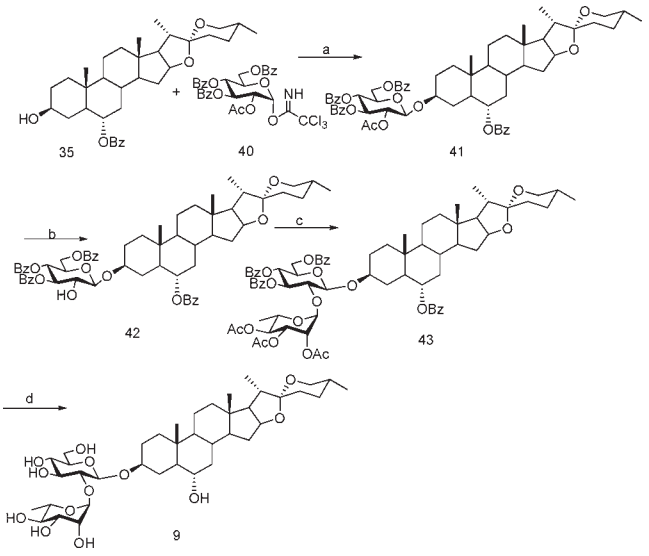
Chlorogenin **9** containing α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl residue was prepared as shown in Scheme 4.

Scheme 3. Synthesis of Target Compound **8**[a]



[a]Reagents and conditions: (a) BzCl, DMAP; (b) Pd/C; (c) i: TMSOTf, CH_2Cl_2; ii: NIS, AgOTf, CH_2Cl_2; (d) MeONa.

Scheme 4. Synthesis of Target Compound **9**[a]



[a]Reagents and conditions: (a) TMSOTf, CH_2Cl_2; (b) AcCl, CH_2Cl_2, MeOH; (c) TMSOTf, CH_2Cl_2; (d) MeONa.

With the acceptor **35** and donor **40** in hand, their coupling reaction was then carried out under the promotion of TMSOTf to provide the compound **41**, in which the 2-*O*-acetyl group was removed from AcCl in MeOH to afford intermediate **42**. The later was glycosylated with **20** promoted by TMSOTf to provide **43**, and final deprotection of all the acyl groups with MeONa gave the target compound **9**.

An HIV-based pseudotyping system was used to screen our semisynthesized saponin library for the entry inhibitors against high pathogenic H5N1 (A/Viet Nam/1203/2004) infection, while VSVG/HIV was applied to evaluate the specificity of H5N1 inhibitors. Potentially effective compounds were further tested by HA (QH)/HIV model in which the HA gene was derived from another highly pathogenic H5N1 virus in migratory birds in Qinghai Lake (Goose/Qinghai/59/05). The Vietnam strain we used was isolated from a fatally infected human patient. Sequence alignments shows these two HAs shares 97% residues similarity. Compounds **1** and **2** showed specific inhibitory effects on both HAs mediated viral entry (Table 1). Similarly, the antiviral activity of

Table 1. Effect of Compounds on HA Mediated Viral Entry

compd	HA(Viet)[a]/ HIV IC_{50} (μM)	HA(QH)[b]/ HIV IC_{50} (μM)	VSVG[c]/HIV conc [10 μM]
1	7.84; 36.3% * (10 μM)	7.22; 42.6% (10 μM)	111%
2	9.25; 46.6% (10 μM)	7.58; 50.4% (10 μM)	101%
3	64.6% (10 μM)	73.6% (10 μM)	76.2%
4	106.1% (10 μM)	108.1% (10 μM)	100.2%
5	17.5% (10 μM)	35.3% (10 μM)	48.4%
6	8.54; 43.3% (10 μM)	6.00; 37.7% (10 μM)	86%
7	102.3% (10 μM)	97.9% (10 μM)	111.1%
8	87.4% (10 μM)	98.2% (10 μM)	116.8%
9	57.9% (10 μM)	61.0% (10 μM)	121.5%
AZT	0.011	0.012	0.015

[a]HA (Viet): HA, H5N1(A/Viet Nam/1203/2004).[13] [b]HA(QH): HA, H5N1(Goose/Qinghai/59/05).[14] [c]VSV-G: vesicular stomatitis virus G protein. *The percentages in this table indicated the infectivity compared to the same amount solvent as 100%.

saponins **3**−**9** was evaluated by these assays. Among the saponins **3**−**7** with the same 3-*O*-β-chacotriose residue, ursolate saponin **6** showed the similar inhibitory activity as **1** and **2**, which exhibited much higher inhibitory activity than its analogue oleanolate saponin **5**, suggesting that the subtle modification of aglycone had the important effect on the antiviral activity. Compound **6** showed slightly different inhibitory activity against HA of the two H5N1 virus like **1** and **2** with IC_{50} of 8.54 and 6.00 μM, respectively, because HA of each H5N1 virus has its own special chemical structure. The reductive ring-opening of spirostan saponin **1**, forming furostan saponin **7**, led to the disappearance of inhibitory activity. Two other steroid saponins **3** and **4** did not show inhibitory activity. These results suggest that the aglycone with more fused rings is favored for the inhibitory activity. Compounds **8** and **9**, two analogues of **1** with reduced sugar chain, showed no activity. This further demonstrated that the 3-*O*-β-chacotriosyl residue plays a very important role in antiviral activity, suggesting that attachment of such trisaccharide chain to some steroids and triterpenes can contribute to enhance inhibitory activity against H5N1 entry.

The potential pandemic of bird flu H5N1 is a global public health concern because there are no effective vaccines available for human. Further, the emergence of drug-resistant H5N1 strains in human patients should serve as a wakeup call for developing new anti-H5N1 (and other flu) therapeutics. Our strategy for developing new anti-influenza therapeutics is to target HA, which is involved in multiple steps during viral entry, including receptor binding, internalization, and fusion. Theoretically, each of these steps can be a target of antiviral therapeutics. However, up to date, no entry inhibitor drug is available for H5N1 or any other influenza viruses. Therefore, it is imperative to develop potent entry inhibitors against H5N1 and other influenza viruses. Here, we first discovered novel small molecule inhibitors against HA mediated viral entry saponins with 3-*O*-β-chacotriosyl residue. They can be used as lead compounds for both prophylactic and therapeutic treatments against bird flu H5N1 and other potential pandemic influenza viruses. Further structure−activity relationship studies on these saponins were carried out for additional small molecule inhibitors with enhanced activity. Such efforts are of vital importance for the further development of novel and effective small molecule inhibitors.

Acknowledgment. This project was supported by the national Natural Basic Research Program of China (no. 2003CB716400) and NSFC Grant no. 30701035.

Supporting Information Available: Procedures for synthesis and characterization of compounds **3**−**43**, details of the in vitro biological protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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