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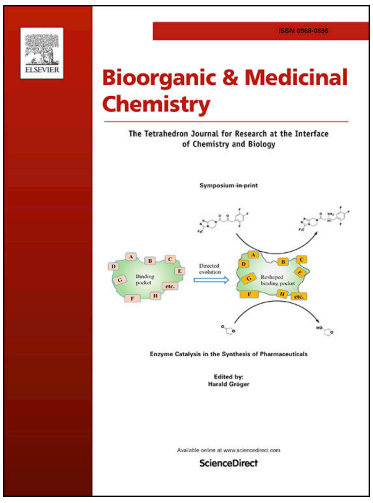

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ABSTRACT

We describe here the design, synthesis, and evaluation of a macrocyclic peptidomimetic as a potent agent targeting enterovirus A71 (EV71). The compound has a 15-membered macrocyclic ring in a defined conformation. Yamaguchi esterification reaction was used to close the 15-membered macrocycle instead of the typical Ru-catalyzed ring-closing olefin metathesis reaction. The crystallographic characterization of the complex between this compound and its target, 3C protease from EV71, validated the design and paved the way for the generation of a new series of anti-EV71 agents.

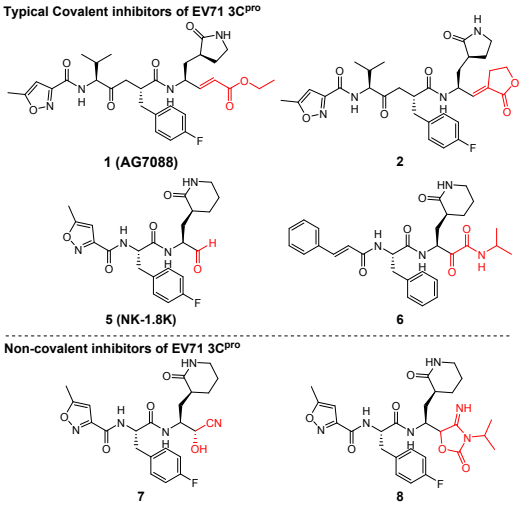
Introduction

The human Enterovirus A71 (EV71) is the major causative agent in outbreaks of the hand, foot and mouth disease (HFMD) in Asia in recent years. In particular, EV71 can cause infections in the central nervous system, leading to high mortality rates in young children.[1,2] Up to date, there is no specific antiviral therapy available to treat HFMD. [3]

EV71 is an icosahedral virus with a single stranded and positive sense RNA genome. After entry to the host cell, its replication starts with the translation of a polyprotein precursor from the monocistronic genome, which is subsequently processed into mature proteins by virally encoded 2A protease and 3C protease ($3C^{pro}$). $3C^{pro}$ also plays crucial roles in evading and suppressing the host defense systems.[4] Because of the essential role in the life cycle of enteroviruses, $3C^{pro}$ has been widely recognized as an attractive therapeutic target. Recent years, several examples of $3C^{pro}$ inhibitors for enteroviruses, including those for Human Rhinoviruses (HRV), have been reported in literatures but no effective anti-enterovirus compounds have entered the market up to date.[5-7] It is of great importance to identify novel series of compounds targeting enteroviruses.

Design Strategy

EV71 $3C^{pro}$ is a cysteine protease, and its enzymatic activity depends on the Cys-His-Glu catalytic triad in the active site. The substrate-binding pocket of $3C^{pro}$ is shallow and a non-covalent chemical binder generally does not afford sufficient binding affinity to inhibit $3C^{pro}$. Consequently typical $3C^{pro}$ inhibitors are with warheads to form covalent bonds. **AG7088** (Figure 1), also known as Rupintrivir, is a peptidomimetic inhibitor containing an α,β-unsaturated ester as the warhead to form a covalent bond with the nucleophilic Cys147 in the catalytic triad of $3C^{pro}$. **AG7088** is of high potency designed to target the $3C^{pro}$ of HRV and was advanced to clinical trials for common cold. However, due to unfavorable stability, bioavailability, and efficacy, it did not pass the Phase II trial.[8]



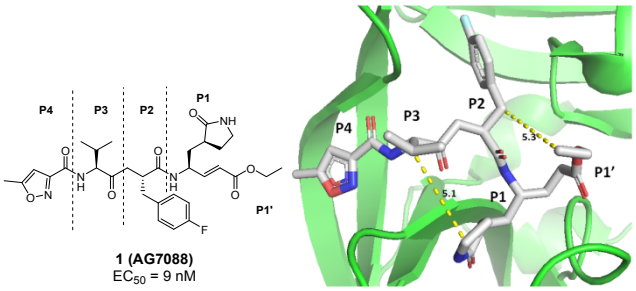
	Warhead type	Cell-based EC50	Mouse plasma stability	Reference
1	α,β-unsaturated ester	1 ~ 9 nM	2.7% left @ 2h	9, 10
2	α,β-unsaturated lactone	28 nM	88% left @ 2h	9
5	Aldehyde	108 nM	-	11, 12
6	α-keto amide	1.12 μM	-	13
7	Cyanohydrin	48 / 88 nM	$t_{1/2}$ = 7 min	14, 15
8	4-Iminooxazolidin-2-one	0.1 μM	$t_{1/2}$ > 2 h	15

Figure 1. Chemical structures and profile comparison of typical inhibitors of EV71 3C^{pro} with various warheads

We previously showed that **AG7088** was a very potent anti-EV71 agent with an EC_{50} value of 9 nM in a cell-based assay. Other typical EV71 3C^{pro} inhibitors such as compound **2**, **5** and **6**, shown in Figure 1, contain various types of covalent warheads, and recent years, some novel inhibitors, like compound **7** and **8**, with non-covalent warheads were also reported.[9-15] Among above mentioned inhibitors, some of them achieved a better balance between the potency and stability. But up to date, except **AG7088**, none of the inhibitors advanced into the clinical trials.

Although **AG7088** is the most advanced compound in development, it was unstable in the mouse plasma with only 2.7% remains after 2 hours of incubation.[9] We found however that the stability of **AG7088** could be improved by increasing the size of the esters and replacing the ethyl ester with lactone. For example, Compound **2** (Figure 1) was of enhanced mouse plasma stability and PK profiles.[9] These data suggested that the increase of steric hindrance, which restricted the conformation, might be an avenue to address the issues of **AG7088** in stability and pharmacokinetics (PK). Limiting the number of conformations would also reduce the entropy loss and facilitate compound interaction with the target.

Macrocyclization has been adopted in medicinal chemistry to generate more rigid products with better stability and potency.[16-18] Our design of a new series of anti-EV71 compounds starting with **AG7088** for macrocyclization. The basic design principle was for the product to maintain, if not enhance, the interaction with the target as for **AG7088** with a suitable macrocyclic linkage.



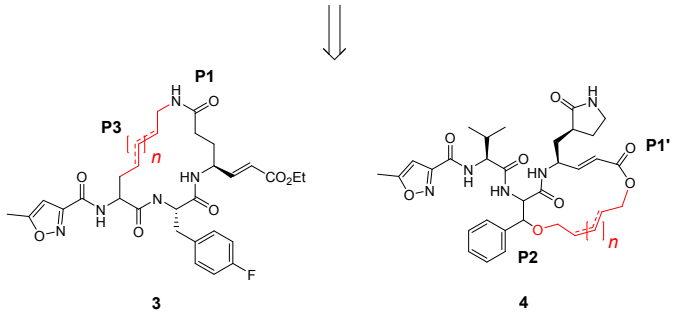


Figure 2. According to the structure of AG7088 and its conformation adopted in EV71 3C^{pro}, it can be found the orientation & distances of P3-P1 (5.1 Å) and P2-P1' (5.3 Å) are quite suitable for macrocylic linkers. And our preliminary design of macrocylic compounds with different type of linkers is shown as general structures 3 and 4.

AG7088 fits snuggly in the substrate-binding pocket of 3C^{pro}, and, based on its complex structure with 3C^{pro}, the possible place that could accommodate extra moieties in a macrocylic compound was either in between P3 and P1 or in between P2 and P1', judging by the orientations to extend the newly attached atoms and the distances to the nearest atoms (5.1 Å and 5.3 Å). Synthesis feasibility were also taken into consideration. The Ring-Closing Metathesis (RCM) reaction was our initial choice for the macrocylic compounds synthesis.[19, 20] The unsaturated olefins in the intermediates of the RCM reaction however were kept to reduce the total number of rotatory bonds for the further enhancement of the rigidity of the molecules.

The search for macrocycles with double bonds was first carried out with the Shape Screening module in the Schrodinger suite. The *in silico* screening resulted in several macrocycles with the desired linkage types that could fit in the space either in between P3 and P1 or in between P2 and P1' (Figure 2). Further modeling indicated that connecting the P1' and P2 of **AG7088** to form the macrocylic compound **4** could maintain most of the interactions. Compound **4** has four chiral isomers. Among these, isomer-4 was the one that fit the substrate-binding pocket of 3C^{pro} in the similar fashion as **AG7088** did (Figure 3).

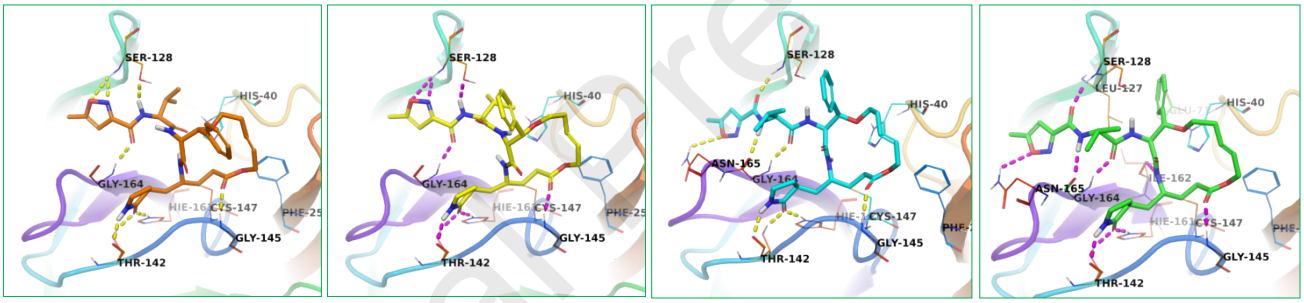


Figure 3. Predicted binding modes of different isomers of compound 4. Isomer-1 was colored brown, isomer-2 was colored yellow, isomer-3 was colored cyan, and isomer-4 was colored green.

Encouraged by the *in silico* screening results, compound **4** was selected for synthesis and evaluation.

Chemistry

Since we did not know which synthetic route would result in the desired isomer in advance, the two chiral centers were made as a mixture. As shown in Figure 4, retrosynthetic analysis of compound **4** indicated that the desired macrocycle could be built in a convergent manner using three building blocks **B1**, **B2**, and **B3** (Figure 3). In the initial attempt, RCM reaction was employed to link the terminal olefin moieties of **B2** and **B3** to make the macrocylic ring.

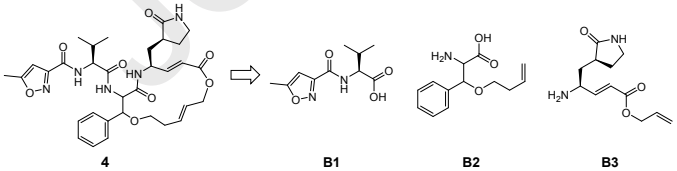
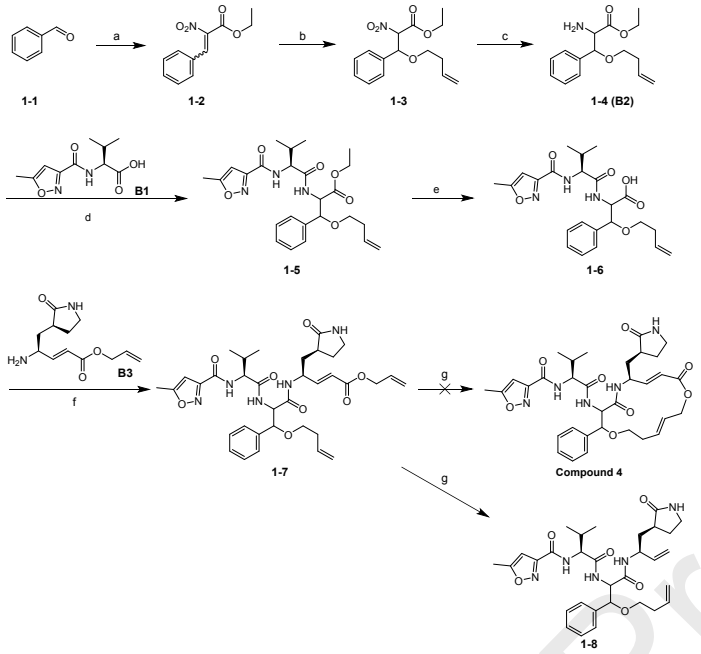


Figure 4. Retrosynthetic analysis of compound 4

Scheme 1 was the first synthetic route for compound **4** based on the retrosynthetic analysis**.** The synthesis of **B1** was illustrated in the supporting information. **B3** was prepared according to the method described previously.[21] Commercially available benzaldehyde **1-1** was converted to **1-2** *via* condensation with ethyl 2-nitroacetate under catalysis of $TiCl_4$. Michael addition of but-3-en-1-ol to **1-2** can provide **1-3** in near quantitative yield. The nitro group of **1-3** was then reduced by zinc dust to the corresponding intermediate amine **1-4** (**B2**), which was coupled with intermediate **B1** to give condensation product **1-5** under T_3P condition**.** The ethyl ester

moiety in **1-5** was hydrolyzed to give acid **1-6**, which was transformed to **1-7** *via* HOBT and EDCI mediated condensation reaction. However, the ring-closing olefin metathesis reaction was not successful in the transformation of **1-7** to compound **4** under several standard ruthenium-catalyzed olefin metathesis conditions including 1st, 2nd generation Grubbs' catalysts and Zhan Catalyst. Interestingly, instead of desired conversion, olefin metathesis reaction occurred between the two double bonds on **B3** moiety, and mass of **1-8** was observed on LCMS as the likely main byproduct (data not shown).

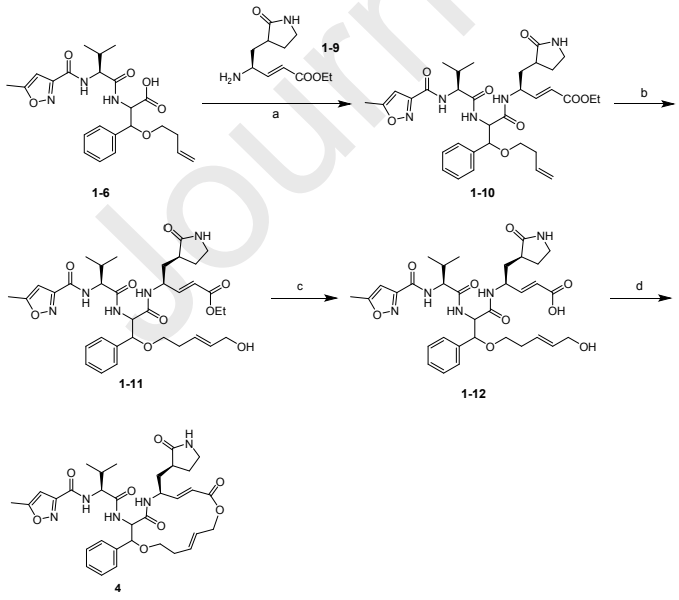
Scheme 1 [a]



[a] Synthetic Method A. Reagents and conditions: (a) ethyl 2-nitroacetate,$TiCl_4$, THF,-10~25°C, 18h; (b) but-3-en-1-ol, LHMDS, 18-C-6, -78 °C; (c) Zn, HCl a.q., EtOH, 0-25 °C, 12 h; (d) T_3P, THF, 0-25 °C, 12h; (e) LiOH, THF/H_2O, 0-20 °C, 12 h; (f) EDCI, HOBT, NMM, CH_2Cl_2, 0-25 °C, 12 h; (g) Grubbs, Catalyst 2_{nd} Generation, DCM, 25 °C, 12h.

Since the olefin metathesis reaction failed to close the large ring in the last step, an alternative route was adopted by using the Yamaguchi esterification reaction,[22] which could use the available **1-6** as the common intermediate.

Scheme 2 [a]



[a] Synthetic Method B. (a) EDCI, HOBT, NMM, CH_2Cl_2, 0-25 °C, 12 h; (b) (E)-but-2-ene-1,4-diol, Grubbs Catalyst 2nd Generation, DCM, 25 °C, 12h (c)

LiOH, THF/H_2O, 25°C, 12 h; (d) 2,4,6-trichlorobenzoyl chloride, Et_3N, THF, 0-25°C, 2 h.

As Shown in Scheme 2, EDCI and HOBT mediated the coupling of intermediate **1-6** and **1-9** (prepared according to established methods [9]) to afford **1-10,** which underwent standard olefin metathesis reaction by the catalysis of 2nd generation Grubbs' catalyst with (E)-but-2-ene-1, 4-diol to give alcohol **1-11** under a very mild reaction condition with a 78% yield. Hydrolysis of α,β-unsaturated ester moiety of **1-11** can give the corresponding acid **1-12** which was cyclized in the presence of 2, 4, 6-trichlorobenzoyl chloride and Et_3N to give the macrocyclic Compound **4** as a mixture of four diastereoisomers with moderate yield. Compound **4** was resolved into three products after preparative HPLC purification. One of the three products was a mixture of two inseparable diastereoisomers. The products were examined for potency and, if it was so identified, the stereochemistry of the active product would be determined.

The Properties of Compound 4

The inhibitory activities of the three isolated products from HPLC purification were separately evaluated in the anti-EV71 CPE assay, and one of the single products exhibited a high anti-EV71 potency with an EC_{50} value of 4.5 μM.

To identify the chiral configuration of the active diastereoisomer in compound **4**, the complex structure between the active diastereoisomer in compound **4** and EV71 $3C^{pro}$ were analyzed by macromolecular X-ray crystallography. The complex was obtained by soaking the compound into the crystalline EV71 $3C^{pro}$ for the structure determination.[23] The stereochemistry of active diastereoisomer of compound **4** was well defined in the X-ray structure, as is shown in Figure 5C. The crystal structure (PDB ID: 6LKA, Table 1) showed that isomer-4 was indeed the active isomer of compound **4** (Figure 5) in agreement with the modeling results (Figure 3) and formed a covalent bond with Cys147 with a bond length of 1.8 Å, which was similar to **AG7088**. Like **AG7088**, compound **4** bound at the S1-S4 and S' sub-pocket and formed hydrogen bonds with Gly164, Thr142, and Gly145. The NH group that replaced the methylene moiety in **AG7088** formed an additional hydrogen bond with the side chain from Ser128. Although the linker of macrocyclic ring did not form any specific interactions with the protein, it fitted well with defined electron density, probably due to its rigid conformation (Figure 5).

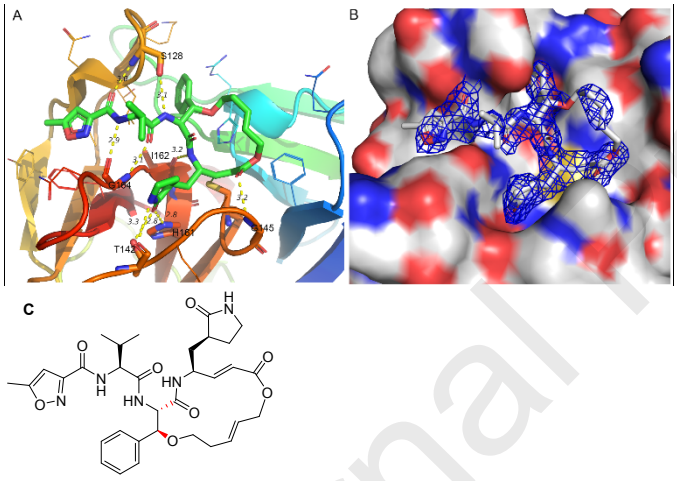


Figure 5. Compound 4 bound at the active site of $3C^{pro}$ (A), its density (B) and the defined stereochemistry of the active diastereoisomer (C), PDB ID: 6LKA.

Further test indicated, however, that the mouse plasma stability for isomer-4 of compound **4** was still unsatisfactory and over 90% (5.6% left@2h) were degraded after an incubation of 2 hours.

Concluding Remarks

Macrocyclic compound **4** was synthesized by a highly convergent route from acyclic precursors *via* the Yamaguchi esterification reaction instead of the standard Ru-catalyzed ring-closing olefin metathesis reaction.

The macrocyclic compound bound in the mode as was designed in the complex with EV71 $3C^{pro}$ and its potency against EV71 was maintained although not to the full extent. With the complex structure in high resolution, a novel insight for $3C^{pro}$ inhibitor development was gained from the interactions of the first macrocyclic inhibitor with the linker between P2 and P1'. It was obvious that the macrocyclic linker was well accommodated in the substrate-binding groove of $3C^{pro}$. However, none of additional interactions was formed, which could be the reason that activity was dampened. Additional interactions between linker atoms and $3C^{pro}$ should be vigorously designed and incorporated in future studies, especially for adopting this macrocycle strategy to other types of warheads.

The stability of compound **4** still needs further improvement. We further compared the stability data of **AG7088**, compound **2**, and other $3C^{pro}$ inhibitors with different esters. It seems that the stability could improve only if the steric hindrance group was placed at the carbon atom adjacent to the carboxyl group, differing our current macrocyclic ester. It is an indication that the introduction of additional steric hindrance would be of benefit to the stability of macrocycles.

In conclusion, we demonstrated the feasibility of a novel macrocyclic strategy for developing inhibitors to $3C^{pro}$ with a novel compound **4** and its complex structure with EV71 $3C^{pro}$. With the improvements in potency and stability, new series of macrocyclic compounds would be a way forward to develop new generations of EV71 $3C^{pro}$ inhibitors.

Methods

Cell-Based Assay

Human embryonic rhabdomyosarcoma (RD) cells were seeded in a 96-well cell culture plate (8000 cells/well) in Dulbecco's Modified Eagle's Medium (DMEM) with 2% fetal bovine serum (FBS). Cells were pre-incubated overnight in a 5% CO_2 incubator at 37 °C. The virus (EV71/Shenzhen/120F1/09 at 100×$TCID_{50}$) and the chemical compounds in DMSO were added to the cells and incubated at 37 °C for 72 hours. The final concentration of DMSO in each well was 1%. CCK-8 was added to each well in the end of incubation to determine the cell viability. The optical density in each well was measured at 450 nm. The compound concentration required to reduce the virus-induced cell death by 50% was defined as EC_{50}.

Plasma stability assay

The compound stability was evaluated in CD-1 Mouse Plasma. The compounds were prepared in 10 mM DMSO and store at 2-8 °C. The solution was first diluted to 400 μM DMSO with 50% ACN/H_2O and then to 40 μM DMSO with 20% ACN/H_2O. The plasma was thawed and warmed in water bath and centrifuged at 4,000 rpm for 5 min to remove clots. pH of the plasma was adjusted to pH 7.4 ± 0.1 with either NaOH or phosphoric acid. Five microliters of the compound solution in 40 μM DMSO were mixed with 95 μl of the plasma and incubated for 0, 10, 30, 60, and 120 minutes in duplicates at 37 °C. The incubation was terminated with 300 μl of a stop solution (50% ACN/MeOH containing 100 ng/mL Tolbutamide and 10 ng/mL Buspirone), mixed for 20 min, centrifuged at 3,220 RCF for 20 min, and subjected to LC / MS / MS analysis. The percentage of remaining compounds after incubation in plasma was calculated as the ratio between PAR at a specific time point and PAR at 0 min. PAR is the peak area ratio between the test compound and an internal standard.

Crystallization and structure determination

Crystals of EV71 $3C^{pro}$ were obtained by the methods of hanging-drop vapour diffusion and the droplets were prepared with a 1:1(v:v) mixture of EV71 $3C^{pro}$ (12 mg/ml^{-1}) and reservoir solution consisting of 100 mM Tris–HCl pH 8.5, 25% PEG4000, and 0.8 M lithium chloride. Crystals suitable for data collection were obtained after one week at 289 K.

The complex was prepared by socking the EV71 $3C^{pro}$ crystal with the compound solution in a 1:5 molar ratio and incubating overnight at 277 K. The conditions used for co-crystallization were basically the same as those used for the protein alone.

The crystals were flash-cooled to 100 K under liquid nitrogen for data collection. X-ray diffraction data for the complex were collected using an in house X-ray facility equipped with a Rigaku rotating-anode X-ray generator (λ= 1.5418 Å) and a MAR345dtb detector and processed with the Automar package from MAR Research GmbH. The structure of EV71 $3C^{pro}$ was solved by molecular replacement using the program Phaser (McCoy et al., 2007). The coordinates of EV71 $3C^{pro}$ complex (Protein Data Bank accession code 4GHT; Wu C et al., 2013) were used as the search model. Manual model building and refinement were carried out with Coot (Emsley et al., 2010) and PHENIX[24] .The compound library file was generated using AceDRG (Fei Long et al., 2017). The figures were produced using PyMOL 2.2.3 (Schrödinger). The statistics for data collection and refinement were shown in Table 1.

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Data collection	
Space group	$C222_1$
Unit cell	
a(Å)	64.27
b(Å)	64.64
c(Å)	75.59
α(°)	90.00
β(°)	90.00
γ(°)	90.00
Rmerge (%)	6.9（42.6）
Total observations	38077

Unique reflections	8598
Resolution range (Å)	50.00-2.03（2.11-2.03）
Completeness (%)	95.1
Average I/σ(I)	6.2（1.1）
Multiplicity	3.96（3.61）
Refinement	
R-factor/Rfree	0.1955（0.2589）
no. of atoms	
Protein	1364
Ligand/ion	87
Water	118
B factors (Å^2)	
Protein	40
Ligand/ion	55.2
Water	43.1
r.m.s deviation	
Bond lengths (Å)	0.008
Bond angles (°)	0.017
Ramachandran statistics† (%)	
Most favoured region	91.48
Allowed regions	6.25
Outliers	2.27

Acknowledgements

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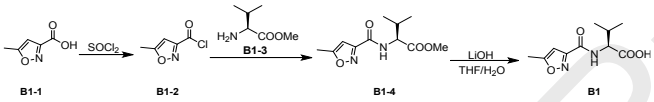
Supplementary Material

General Information.

Reagents and solvents were obtained from commercial suppliers and were used without further purification. ^{1}H NMR measurements were recorded at 400 MHz using a Bruker AMX400instrument. Chromatographic purifications were performed silica gel (300-400 mesh). Preparative HPLC separations were performed on a Boston Green ODS 150*30*5u column using gradient mixtures of water/0.1% TFA and water (10%)/acetonitrile (90%)/0.1% TFA. All of the compounds were established by a variety of LC/MS, and NMR analytical techniques, and purities were >95% for all final products.

General Procedure for preparation of B1.



(S)-methyl 2-amino-3-methylbutanoate (**B1-3).** To a solution of L-Valine (23.43 g, 0.2 mol) in methanol (140 mL) was added $SOCl_2$ (28.4 mL, 0.40 mol) at 25 °C. The resulting mixture was stirred at 80 °C for 12 h. The reaction mixture was concentrated to give **B1-3** as white solid (33.5 g, yield: 100%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (brs, 2H), 3.98 (s, 1H), 3.84 (s, 3H), 2.48-2.47 (m, 2H), 1.17-1.13 (m, 6H).

5-methylisoxazole-3-carbonyl chloride (**B1-2)**. A solution of compound **B1-1** (30 mg, 0.24 mmol) in $SOCl_2$ (1 mL) was stirred at 80°C under N_2 for 2 hrs. The mixture was concentrated to give compound **B1-2** (30 mg, crude). It was used for next step without further purification.

(S)-methyl 3-methyl-2-(5-methylisoxazole-3-carboxamido)butanoate (**B1-4)**. To a solution of compound **B1-2** (1.0 g, 6.0 mol) and Et_3N (1.80 g, 18.0 mol) in dry DCM (10 mL) was added compound **B1-3** (1.04 g, in 5.0 mL DCM) dropwise at 25 °C. The resulting mixture was stirred at 25 °C for 1h. The reaction mixture was concentrated and EtOAc (50 mL) and water (50 mL) was added. The mixture was separated and the organic phase was washed with water (50 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to give **B1-4** as white solid (0.86 g, yield: 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25-7.23 (m, 1H), 6.42 (s, 1H), 4.75-4.65 (m, 1H), 3.77 (s, 3H), 2.50 (s, 3H), 2.35-2.20 (m, 1H), 1.10-0.90 (m, 6H).

(S)-3-methyl-2-(5-methylisoxazole-3-carboxamido)butanoic acid (**B1)**. To a solution of **B1-4** (4.3 g, 17.9 mol) in THF/H_2O (120 mL, 1:1) was added LiOH (0.86 g, 36.0 mmol) at 25 °C. The resulting mixture was stirred at 25 °C for 3h. The reaction mixture was concentrated and acidified to pH = 4 by citric acid, then extracted by DCM (50 mL*3). The organic phase was dried over Na_2SO_4, filtered and concentrated to give **B1** as yellow oil (3.5 g, yield: 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30-7.23 (m, 1H), 6.46 (s, 1H), 4.80-4.65 (m, 1H), 2.49 (s, 3H), 2.40-2.25 (m, 1H), 1.10-0.90 (m, 6H).

General procedure for Scheme 1

Ethyl 2-nitro-3-phenylacrylate (1-2). To a stirred solution of ethyl 2-nitroacetate (88 g, 0.66 mol) and benzaldehyde (100 g, 0.94

mol) in THF (1000 mL) was added $TiCl_4$ (198 g, 1.06 mol) at -10 ℃. The mixture was stirred for 10 min and treated with N-methyl morpholine (284 g, 2.82 mol) dropwise over 20 min. The mixture was allowed to warm to room temperature for 18h. The reaction was quenched with water and extracted with EA (300 mL*3), dried with Na_2SO_4, filtered and concentrated. The crude product was purified by silica gel chromatography (PE / EA = 30 / 1) to give compound **1-2** (55 g, yield: 34.8%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39-7.60 (m, 6H), 4.35-4.51 (m, 2H), 1.33-1.43 (m, 3H).

Ethyl 3-(but-3-en-1-yloxy)-2-nitro-3-phenylpropanoate (1-3). But-3-en-1-ol (3.9 g, 54 mmol) in THF (30 mL) was added KHMDS (54 mL, 1M, 54.0 mmol) at -78 °C under N_2. The resulting mixture was stirred at -78 °C for 20 min. Then 18-crown-6 (14.2 g, 54 mmol) in dry toluene (50mL) was added. The resulting mixture was stirred at -78 °C for 30 min. Then Compound **1-2** (8.0 g, 36 mmol) in dry THF (60 mL) was added dropwise. The resulting mixture was stirred at -78 °C for 3h. Then water (100 mL) was added. The organic phase was separated and the aqueous phase was extracted by EA (50 mL*2). The combined organic phase was dried over Na_2SO_4, filtered and concentrated to give **1-3** as yellow oil (10 g, yield: 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27-7.40 (m, 5H), 5.73-5.80 (m, 1H), 5.01-5.08 (m, 2H), 4.79 (d, J = 4.4 Hz, 1H), 4.12-4.17 (m, 2H), 3.88 (d, J = 4.4 Hz, 1H), 3.31-3.52 (m, 2H), 2.68 (brs., 1H), 2.27-3.35 (m, 2H), 1.18 (t, J = 7.2 Hz, 3H). MS (ESI+) m/z : 280 ([M＋H]$^+$).

Ethyl 2-amino-3-(but-3-en-1-yloxy)-3-phenylpropanoate (1-4). Zn (44.3 g, 0.68 mmol) was added to a mixture of compound **1-3** (10 g, 34 mmol) and HCl (4N, 100 mL) at 0°C under N_2. The resulting mixture was stirred at 25°C for 18 h. Then aq. $NaHCO_3$ was added until pH = 8. The organic phase was separated and the aqueous phase was extracted by EA (150 mL*3). The combined organic phase was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by silica gel chromatography (PE / EA = 5 / 1) to give compound **1-4.** MS (ESI+) m/z : 264 ([M＋H]$^+$).

Ethyl 3-(but-3-en-1-yloxy)-2-((S)-3-methyl-2-(5-methylisoxazole-3-carboxamido)butanamido) -3-phenylpropanoate (1-5). To a solution of compound **1-5** (100 mg, 0.44 mmol) and compound **1-4** (140 mg, 0.53 mmol) in anhydrous THF (5 mL) was added dropwise T_3P (560 mg, 50% in EA, 0.88 mmol) at 0 °C under N_2 and stirred for 12 h. After the reaction was complete, water (10 mL) was added and EA (10 mL*3) was used to extract the product, the organic layer was washed with brine, dried over with Na_2SO_4, filtered and concentrated to give compound **1-5** (150 mg, yield: 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.15-7.23 (m, 8H), 6.35-6.46 (m, 2H), 5.68-5.70 (m, 1H), 4.87-5.00 (m, 3H), 4.59-4.75 (m, 1H), 4.10-4.35 (m, 3H), 3.25-3.47 (m, 2H), 2.22-2.44 (m, 8H), 0.65-0.91 (m, 13H). MS (ESI+) m/z : 472 ([M＋H]$^+$).

3-(but-3-en-1-yloxy)-2-((S)-3-methyl-2-(5-methylisoxazole-3-carboxamido)butanamido)-3-phenylpropanoic acid (1-6). To a solution of compound **1-5** (150 mg, 0.32 mmol) in THF (5 mL) and H_2O (1 mL) was added $LiOH.H_2O$ (54 mg, 1.27 mmol) in portions. The reaction was stirred at 25 °C for 16 hours. After the reaction was complete, water (30 mL) was added, extracted with EA (20 mL*2). The aqueous phase was adjusted pH = 3 with 1N HCl, EA (20 mL*3) was used to extract the product. The organic phase was washed with brine, dried over with Na_2SO_4, concentrated to give **1-6** (100 mg, yield: 71 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16-7.26 (m, 6H), 6.36-6.60 (m, 2H), 5.61-5.78 (m, 1H), 4.79-5.00 (m, 3H), 4.33-4.36 (m, 1H), 3.33-3.50 (m, 2H), 2.40-2.44 (m, 4H), 2.24-2.27 (m, 2H), 0.80-1.29 (m, 8H). MS (ESI+) m/z : 444 ([M＋H]$^+$) .

(4S,E)-ethyl 4-(3-(but-3-en-1-yloxy)-2-((S)-3-methyl-2-(5-methylisoxazole-3-carboxamido) butanamido)-3-phenylpropanamido)-5-((S)-2-oxopyrrolidin-3-yl)pent-2-enoate (1-7). To a solution of compound **1-6** (500 mg, 1.13 mmol), NMM (685 mg, 6.78 mmol) and compound **B3** (444 mg, 1.69 mmol) in dry DCM (10 mL) was added EDCI (434 mg, 2.26 mmol) and HOBT (305 mg, 2.26 mmol) in turns at 0 °C under N_2. The reaction was stirred at 25 °C for 12 h. The reaction mixture was quenched with water at 0°C. extracted with EA（50 mL*3）, and the combined organic phase was washed with brine, dried over Na_2SO_4 and concentrated in vacuum. The residue was purified by prep-HPLC (TFA) to give compound **1-7** (300 mg, yield: 41%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81-9.06 (m, 1H), 6.50-7.33 (m, 9H), 5.62-5.91 (m, 2H), 4.62-5.74 (m, 4H), 4.13-4.19 (m, 2H), 3.33-3.47 (m, 4H), 2.28-2.54 (m, 7H), 1.61-1.95 (m, 3H), 0.53-0.99 (m, 9H). MS (ESI+) m/z : 652 ([M＋H]$^+$).

General procedure for Scheme 2

(4S,E)-ethyl-4-(3-(((E)-5-hydroxypent-3-en-1-yl)oxy)-2-((S)-3-methyl-2-(5-methylisoxazole-3 -carboxamido)butanamido)-3-phenylpropanamido)-5-((S)-2-oxopyrrolidin-3-yl)pent-2-enoate (1-11). To a solution of compound **1-10** (100 mg, 48.5 mmol，prepared using the same procedure of **1-7**) and (E)-but-2-ene-1,4-diol (244 mg, 3 mmol) in DCM (10 mL) was added Grubb's 2nd (20 mg, cat.) at 25 °C. The reaction mixture was stirred at 25 °C for 12 h. The reaction was concentrated and the crude product was purified by prep-HPLC (TFA) to give compound **1-11** (80 mg, yield: 78%) as a white solid. MS (ESI+) m/z : 682 ([M＋H]$^+$).

(4S,E)-4-(3-(((E)-5-hydroxypent-3-en-1-yl)oxy)-2-((S)-3-methyl-2-(5-methylisoxazole-3-carboxamido)butanamido)-3-phenylpropanamido)-5-((S)-2-oxopyrrolidin-3-yl)pent-2-enoic acid (1-12). A solution of compound **1-11** (58 mg, 0.1 mmol) and $LiOH.H_2O$ (17 mg, 0.4 mmol) in THF (4 mL) and H_2O (1 mL) was stirred at 25 °C for 4 hours. The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was concentrated and adjust pH = 3 with HCl (1M), extracted with EA (20 mL*3) and concentrated to give compound **1-12** (50 mg, yield: 91%), which was used for the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56-8.45 (m, 1H), 7.20-7.54 (m, 5H), 6.50-6.97 (m, 2H), 5.62-5.94 (m, 2H), 4.06-5.17 (m, 6H), 3.33-3.75 (m, 6H), 1.83-2.54 (m, 14H), 0.60-1.43 (m, 6H). MS (ESI+) m/z : 654 ([M＋H]$^+$).

N-((2S)-1-(((6S,7E,12E)-4,9-dioxo-6-(((S)-2-oxopyrrolidin-3-yl)methyl)-2-phenyl-1,10-dioxa-5-azacyclopentadeca-7,12-dien-3-yl)amino)-3-methyl-1-oxobutan-2-yl)-5-methylisoxazole-3-carboxamide (Compound 4) To a solution of compound **1-12** (70 mg, 0.11 mmol) and TEA (111 mg, 1.1 mmol) in THF (5 mL) was added 2,4,6-trichlorobenzoyl chloride (134 mg, 0.55 mmol). The reaction mixture was stirred at 15 °C for 1 h and 25 °C for 2 h. The reaction mixture was added dropwise to a mixture of DMAP (268 mg, 2.2 mmol) in toluene (60 mL) at 25 °C under N_2 and stirred at 25 °C for 2 h. 1N HCl (aq.) was added until pH = 3. EA (50 mL*2) was used to extract the product, dried over Na_2SO_4, concentrated in vacuum and the residue was purified with prep-HPLC (TFA) to give ***compound 4*** (3 diastereoisomers, total yield: 33%) as white solid.

Diastereoisomer 1 (the active diastereoisomer)**:** ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52-8.61 (m, 1H), 6.95-7.52 (m, 7H), 6.39-6.49 (m, 1H), 5.30-6.04 (m, 2H), 4.46-4.70 (m, 3H), 3.45-3.52 (m, 1H), 2.87-3.13 (m, 2H), 2.01-2.48 (m, 12H), 1.60-1.65 (m, 1H), 1.20-1.30 (m, 1H), 0.45-0.89 (m, 6H). ^{13}C NMR (400 MHz, CD_3OD) δ 171.9, 170.5, 166.7, 159.5, 158.3, 128.2, 127.9, 126.9, 125.2, 125.0, 122.8, 121.9, 110.6, 103.1, 101.9, 100.6, 81.1, 67.7, 62.2, 59.6, 58.9, 67.5, 57.3, 50.2, 40.1, 39.0, 38.2, 31.7, 17.9, 17.5, 10.6. MS (ESI+) m/z: 636 ([M＋H]$^+$). HRMS (ESI) m/z calcd for $C_{33}H_{41}N_5O_8$ $[M+H]^+$ 636.3028, found 636.3043.

Diastereoisomer 2: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71-7.73 (m, 1H), 7.16-7.35 (m, 5H), 7.18-7.19 (m, 1H), 6.54-6.57 (m, 1H), 5.35-6.06 (m, 2H), 5.05-5.06 (m, 1H), 4.17-4.66 (m, 4H), 3.35-3.65 (m, 2H), 3.02-3.26 (m, 2H), 1.76-2.51 (m, 11H), 0.74-0.89 (m, 7H). MS (ESI+) m/z : 636 ([M＋H]$^+$) . HRMS (ESI) m/z calcd for $C_{33}H_{41}N_5O_8$ $[M+H]^+$ 636.3028, found 636.3035.

Diastereoisomer 3: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60-8.78 (m, 1H), 7.23-7.40 (m, 6H), 6.04-6.92 (m, 2H), 5.46-5.71 (m, 2H), 5.18-5.23 (m, 1H), 4.53-4.63 (m, 3H), 4.19-4.23 (m, 1H), 3.58-3.65 (m, 2H), 3.08-3.20 (m, 2H), 2.28-2.75 (m, 7H), 1.75-1.83 (m, 2H), 0.78-0.85 (m, 4H), 0.39-0.49 (m, 4H). MS (ESI+) m/z : 636 ([M＋H]$^+$). HRMS (ESI) m/z calcd for $C_{33}H_{41}N_5O_8$ $[M+H]^+$ 636.3028, found 636.3040.

Graphical Abstract

We describe here the design, synthesis, and evaluation of a macrocyclic peptidomimetic as a potent agent targeting enterovirus A71 (EV71). The compound has a 15-membered macrocyclic ring in a defined conformation. Yamaguchi esterification reaction was used to close the 15-membered macrocycle instead of the typical Ru-catalyzed ring-closing olefin metathesis reaction. The crystallographic characterization of the complex between this compound and its target, 3C protease from EV71, validated the design and paved the way for the generation of a new series of anti-EV71 agents.

Design, synthesis, and evaluation of a novel macrocyclic anti-EV71 agent

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Peng Li, Siqi Wu, Tianyichen Xiao, Yunlong Li, Zhiming Su, Fei Hao, Jiao Hu, Fusen Lin, Xinsheng Chen, Zhengxian Gu, Tianwei Lin, Haiying He, Jian Li, Shuhui Chen