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ORIGINAL ARTICLE

Isolation and synthesis of falcitidin, a novel myxobacterial-derived acyltetrapeptide with activity against the malaria target falcipain-2

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A 384-well microtitre plate fluorescence cleavage assay was developed to identify inhibitors of the cysteine protease falcipain-2, an important antimalarial drug target. Bioassay-guided isolation of a MeOH extract from a myxobacterium *Chitinophaga* sp. Y23 isolated from soil collected in Singapore, led to the identification of a new acyltetrapeptide, falcitidin (1), which displayed an IC_{50} value of 6 μM against falcipain-2. The planar structure of 1 was secured by NMR and MS/MS analysis. Attempts to isolate further material for biological testing were hampered by inconsistent production and by a low yield (<100 μg l^{-1}). The absolute configuration of 1 was determined by Marfey's analysis and the structure was confirmed through total synthesis as isovaleric acid-D-His-L-Ile-L-Val-L-Pro-NH_2. Falcitidin (1) is the first member of a new class of falcipain-2 inhibitors and, unlike other peptide-based inhibitors, does not contain reactive groups that irreversibly bind to active cysteine sites.
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Keywords: *Chitinophaga*; falcipain; falcitidin; malaria; myxobacteria; tetrapeptide

INTRODUCTION

Malaria is a mosquito-borne disease caused by protozoa with *Plasmodium falciparum,* being the most lethal species to humans. According to WHO's 2011 World Malaria Report, 216 million cases of malaria were recorded during 2010, from which 665 000 people died.[1] The rapid life cycle of the malaria parasite has led to the emergence of drug resistant strains in a similar manner to other microorganisms. Currently artemisinin is considered the drug-of-last-resort but there are some recent reports that describe a growing number of resistant strains.[2–4] There is, therefore, still a need to identify new antimalarial drugs, especially those with new targets[5] such as falcipain-2,[6–8] a cysteine protease used by *P. falciparum* to help degrade hemoglobin during the trophozoite stage of infection. A recent review has comprehensively described the different classes of falcipain-2 inhibitors that include peptides, peptidomimetics, isoquinolines, thiosemicarbazones and chalcones.[6]

In our search for new antimalarial drug leads, a 384-well microtitre plate assay was developed using the falcipain-2 enzyme derived from the *P. falciparum* Gombak A strain.[9] In this assay, protease activity was monitored by release of the fluorophore 4-amino-7-methyl coumarin (AMC) from the synthetic peptide substrate (Z-Phe-Arg-AMC). Compounds with inhibitory action would prevent the cleavage of the AMC fluorophore, thereby reducing the fluorescence intensity. Although only 2934 extracts were screened due to the limited availability of enzyme, an active MeOH extract was identified from a myxobacterium strain Y23 isolated from a soil sample collected in Singapore (Supplementary Figure S1). Sequencing of 16S rDNA revealed that Y23 was a member of the genus *Chitinophaga*, which is a relatively new genus first described in 1981,[10,11] with a closest match to *Chitinophaga terrae* (94% sequence identity).[12] Although usually found in soil, *C. terrae* has also been reported in an immunocompromised, human bacteremia patient.[13] *Chitinophaga* has been previously reported to produce a series of unique methide antibiotics, the elansolids,[14–16] and a plethora of volatile fatty acids and methyl esters.[17]

Bioassay-guided isolation led to the purification of a small amount (0.5 mg) of a new acyltetrapeptide that was named falcitidin (**1**) and displayed an IC_{50} value of 6 μM against the falcipain-2 enzyme. The absolute configuration of **1** was determined using Marfey's method and the structure confirmed by total synthesis. Falcitidin (**1**) is the

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first representative of a new class of falcipain-2 inhibitors that in contrast to other peptide-based inhibitors does not contain reactive groups such as halomethyl ketones, vinyl sulfones, aldehydes, α-ketoamides and aziridines, which covalently bind to the cysteine in the active site.

RESULTS AND DISCUSSION

Physicochemical properties and structure elucidation of falcitidin (1)

Falcitidin (**1**) ($[\alpha]_D^{31}$ −72 (c 0.28, MeOH)) was assigned a molecular formula of $C_{27}H_{45}N_7O_5$ based upon HRMS data: (−)-ESI-time of flight (TOF)-MS *m/z* of 546.3402 ($C_{27}H_{44}N_7O_5$ requires 546.3404, 0.4 p.p.m.), and (+)-ESI-TOF-MS *m/z* 548.3553 ($C_{27}H_{46}N_7O_5$ requires 546.3560, 1.3 p.p.m.) and *m/z* 570.3379 ($C_{27}H_{45}N_7O_5$ Na requires 570.3379, 0 p.p.m.) (Supplementary Figures S2 and S3). Analysis of the NMR data of falcitidin (**1**) in DMSO-d_6 (Table 1, Supplementary Figure S4) suggested that **1** was a tetrapeptide, comprising of one unit of histidine, isoleucine, valine and proline, with an attached isovaleric acid (3-methylbutanoic acid) unit. It is interesting to note that isovaleric acid has been reported as one of the major volatile fatty acids in *Chitinophaga* sp. Fx7914.[17] Although 2D NMR spectra could not unambiguously determine the position of the proline and valine units due to overlapping resonances, the planar structure of falcitidin (**1**) was secured using (+)-ESI-MS/MS data obtained using an ion-trap MS instrument. Analysis of the b-type MS/MS fragmentations[18] (Figure 1) showed that the C-terminus was an amidated proline (*m/z* 434.5, isovaleric acid-Val-His-Ile-Val). The remaining fragments (*m/z* 335.4, isovaleric acid-His-Ile and *m/z* 222.2, isovaleric acid-His) were in accordance with the proposed structure of falcitidin (**1**) as the acylated tetrapeptide isovaleric acid-His-Ile-Val-Pro-NH_2.

Due to the scarcity of natural material, the all L isomer of falcitidin (**1b**) was synthesized using standard peptide chemistry but was not identical to the natural product, as the ^{1}H NMR spectra were different (Figure 3 and Supplementary Figure S11) and co-injection of **1** and **1b** using C18 HPLC resulted in two peaks (Figure 2b). The absolute configuration of **1** was then determined using Marfey's method.[19,20] We used synthetic **1b** to identify microwave-assisted hydrolysis[21,22] as the optimal hydrolysis method, as conventional hydrolysis was not reproducible on sub-milligram amounts. The naturally occurring falcitidin (**1**) (~250 μg in 0.2 ml of aqueous 6 N HCl) was irradiated at 170 °C for 20 min and all of the individual amino acids of **1** (D-His, L-Ile, L-Val and L-Pro) were resolved by LC-MS after 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide-derivatization (Supplementary Figure S12). Therefore, the structure of falcitidin (**1**) was determined to be isovaleric acid-D-His-L-Ile-L-Val-L-Pro-NH_2.

We next completed a total synthesis for structural correlation to natural **1** (Scheme 1, Supplementary Figures S5–S10). In short, after trifluoroacetic acid (TFA)-mediated removal of the Boc group of the *N*-Boc-proline carboxamide **4**, obtained from **3**, the amino-proline TFA salt was coupled with freshly prepared L-Ile-L-Val **6** using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate/1-hydroxy-7-azabenzotriazole (HATU/HOAt) to give L-Ile-L-Val-L-Pro-NH_2 **7a**.[23] The dipeptide **6** was obtained by coupling L-Ile-NHBoc **2** with the *in situ* prepared bis-trimethylsilane (TMS) ether of L-Val (**5**) under mixed anhydride conditions.[24] The Boc group of tripeptide **7a** was then cleaved using TFA (via **7b**) and coupled to N_α-Fmoc-$N_{(im)}$-trityl-D-histidine using HATU/HOAt to give the Fmoc–tetrapeptide **8**. The Fmoc group of **8** was removed using piperidine[25] and isovaleric acid was attached to **9** using HATU/HOAt to give **10**. The final synthetic target **1** was obtained after trityl deprotection of **10** using TFA in the presence of triisopropylsilane.[26]

Table 1 NMR data for falcitidin (1) (DMSO-d_6, 500 and 125 MHz)

Position	*^{13}C δ*	*^{1}H δ m, J (Hz)*	*COZY*	*gHMBC (H to C)*
Isovaleric acid				
1-CO	171.4[a]	—	—	—
2	44.2	1.95 (m)	Overlap	3, 4, 5
3	24.9	1.92 (m)	Overlap	2, 4, 5
4	21.9[b]	0.77 (d, 6.3)[b]	3	2, 3, 5
5	21.9[b]	0.80 (d, 6.3)[b]	3	2, 3, 4
Histidine				
2-CO	171.1[a]			
NH	—	8.00 (d, 8.2)	α	α, β, 1-CO
α-CH	52.7	4.55 (m)	β, NH	2-CO
β-CH_2	29.4	2.70 (dd, 5.7, 14.8)	Ha-Hb	α, γ, 2-CO
		2.87 (dd, 8.8, 14.8)		
γ-C	132.1 (weak)			
δ2-CH	133.8[a]	7.47 (s)	ε1	ε1
ε1-CH	Not observed	6.79 (s)	δ2	γ
ε2-NH	—	Not observed	—	—
Isoleucine				
3-CO	170.7[a]	—		—
NH	—	7.69 (d, 9.1)	α	2-CO
α-CH	56.4	4.21 (m)[d]	β, NH	β, γ1, γ2, 2-CO
β-CH	36.5	1.64 (m)	γ1	
γ1-CH_3	14.9	0.70 (d, 7.2)	β	α, β, γ2
γ2-CH_2	24.0	0.91 (m), 1.25 (m)	Ha-Hb, β, δ	β, γ1, γ2
δ-CH_3	10.6	0.73 (t, 7.2)	γ2	β, γ2
Valine				
4-CO	169.5[a]	—	—	—
NH	—	7.96 (d, 8.5)	α	3-CO
α-CH	55.5	4.21 (dd, 8.5, 8.5)[d]	β, NH	β, γ1, γ2, 3-CO,4-CO
β-CH	29.4	2.07 (m)	α, γ1, γ2	α, γ1, γ2
γ1-CH_3	17.8[c]	0.86 (d, 6.6)[c]	β	α, β, γ2
γ2-CH_3	18.5[c]	0.88 (d, 6.6)[c]	β	α, β, γ1
Proline				
5-CO	170.6[a]	—	—	—
α-CH	59.1	4.21 (m)[d]	β	β, γ, 5-CO[d]
β-CH_2	23.8	1.77 (m), 1.91 (m)	Ha-Hb	γ, δ
γ-CH_2	28.8	1.77 (m), 1.91 (m)	Ha-Hb	β, δ
δ-CH_2	46.6	3.53 (m), 3.74 (m)	β, γ	β, γ
NH_2	—	6.74 (s), 7.21 (s)	—	α

[a]These resonances were not observed in the ^{13}C NMR spectrum and were obtained from gHMBC data.
[b]These assignments are interchangeable.
[c]These assignments are interchangeable.
[d]These isoleucine and valine proton resonances overlap and hence are indistinguishable by HMBC.

All characterization data and HPLC co-injection studies of synthetic **1** matched that of natural falcitidin (**1**) (Figures 2c and 3, Supplementary Figure S4).

Biological properties of falcitidin (1)

Falcitidin (**1**) displayed an IC_{50} of 6 μM in the falcipain-2 assay. Counter screening of **1** against the aspartic protease plasmepsin-2, which is also a validated antimalarial target, gave an IC_{50} of 50 μM in a fluorescence resonance energy transfer (FRET) assay and 65 μM in a fluorescence polarization assay.[27,28] Falcitidin (**1**) was inactive (IC_{50} > 90 μM) against the serine protease, tryptase.[29]

CONCLUSION

A new inhibitor of the antimalarial target falcipain-2 (IC_{50} 6 μM), falcitidin (**1**), was isolated in small quantities from a myxobacterium *Chitinophaga* sp. collected from a soil sample in Singapore. The planar structure of **1** was secured by NMR and MS/MS analyses but attempts to isolate further material for biological testing were hampered by inconsistent production (even using identical fermentation conditions) and by a low yield (often 0 μg l^{-1} but never >100 μg l^{-1}). Therefore, falcitidin (**1**) needed to be synthesized to help confirm the structure. The synthesized all L isomer **1b** was not identical to the natural product but was a useful tool to optimize the Marfey's amino analysis method. Microwave-assisted hydrolysis and 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide-derivatization established the structure of the naturally occurring peptide as isovaleric acid-D-His-L-Ile-L-Val-L-Pro-NH_2, which was confirmed by total synthesis. A second generation total synthesis of **1** and *in vitro* antimalarial activity of falcitidin analogs will be reported in due course. Falcitidin (**1**) is the first member of a new class of falcipain-2 inhibitors and, unlike other peptide-based inhibitors, does not contain reactive groups that irreversibly bind to active cysteine sites.

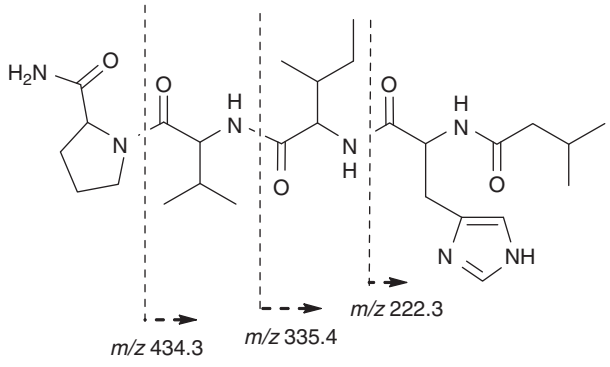


Figure 1 Planar structure of falcitidin (**1**) showing the b-type (+)-ESI-MS/MS fragmentations used for structure elucidation.

METHODS

General

All reactions were performed under Ar atmosphere and stirred magnetically in oven-dried glassware, fitted with rubber septa. CH_2Cl_2 was dried by distilling over CaH_2, whereas tetrahydrofuran (THF) was dried by distilling over sodium benzophenone ketyl. Inorganic salts and acids were used in aqueous solution and are reported in % w/v. All reagents were obtained from Aldrich, Alfa Aesar, GL Biochem or TCI America (Singapore) and used without further purification unless otherwise stated. Vacuum liquid chromatography (VLC) was performed with Waters (Singapore) PREP C18 (55–105 μm particle size, 125 Å), whereas silica flash chromatography was performed using silica gel (25–40-μm particle size). TLC analyses were performed using pre-coated Merck Silica Gel 60 F254 glass slides and visualized with UV light or a ceric ammonium molybdate, $KMnO_4$ and ninhydrin stain. Rf values were obtained by elution in the stated solvent ratios (v/v). All solvent mixtures are reported as (v/v) unless noted otherwise. Preparative HPLC was performed on a Waters system with Millenium[32] software, Waters 996 PDA detector, Waters 600 gradient controller and pump, Waters 717plus autosampler and a Waters fraction collector II using a Luna C18(2) column (5 μ, 150 × 21.2 mm). UV spectra were scanned on a Pharmacia Biotech Ultrospec 2000 (Pharmacia Biotech, Uppsala, Sweden). NMR spectra were collected on a Bruker Avance DRX-500 NMR spectrometer (Bruker, Bremen, Germany), using 5-mm broadband inverse (BBI) (^{1}H, G-COZY, multiplicity-edited gHSQC, and gHMBC) or broadband observe (BBO) (^{13}C spectra) probe-heads equipped with z-gradients or a Bruker 300 MHz spectrometer (Bruker) fitted with a pulse-field gradient probe. Samples were calibrated to the residual protonated solvent signals of DMSO-d_6 (δ_H 2.49 and δ_C 39.5) or TMS for $CDCl_3$. HR-ESI-

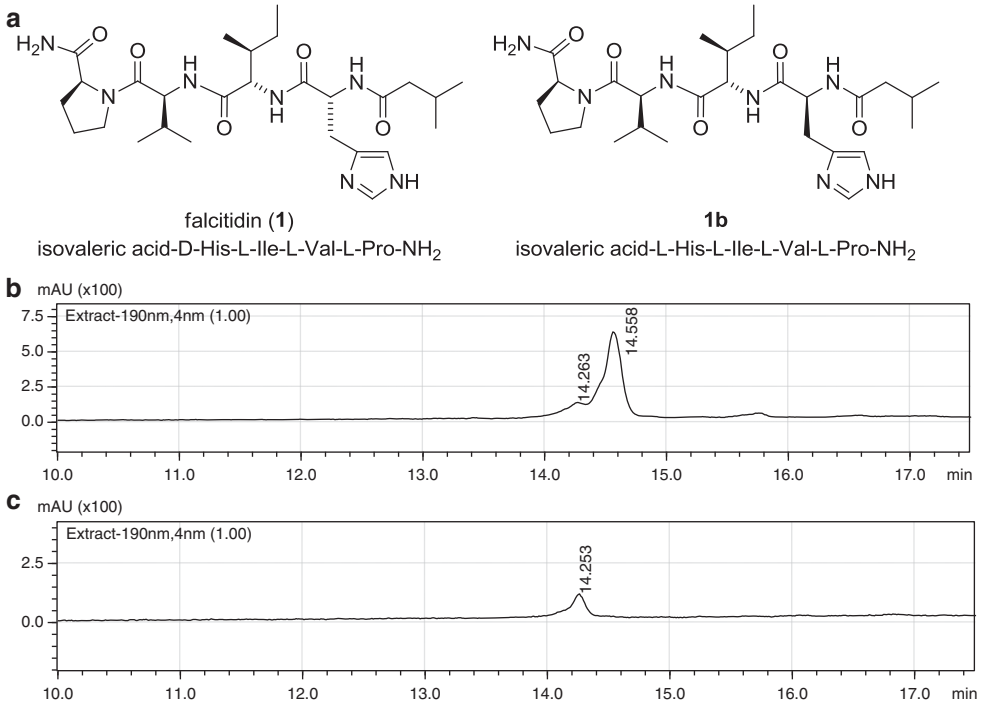


Figure 2 (**a**) Structures of falcitidin (isovaleric acid-D-His-L-Ile-L-Val-L-Pro-NH_2) (**1**) and isovaleric acid-L-His-L-Ile-L-Val-L-Pro-NH_2 (**1b**). (**b**) HPLC chromatogram showing the retention times of natural falcitidin (**1**) (14.3 min) and synthetic **1b** (14.6 min) and; (**c**) co-elution of naturally occurring falcitidin (**1**) (14.3 min) and synthetic isovaleric acid-D-His-L-Ile-L-Val-L-Pro-NH_2 (**1**) (14.3 min).

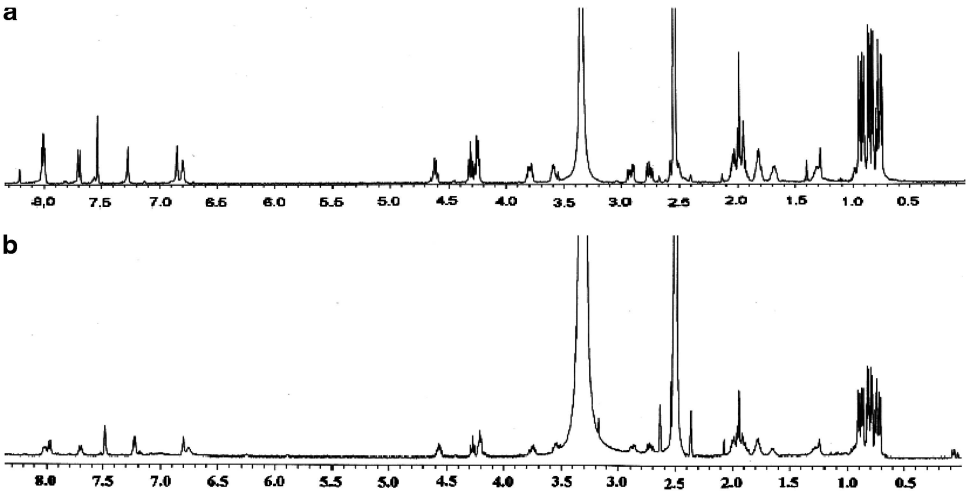
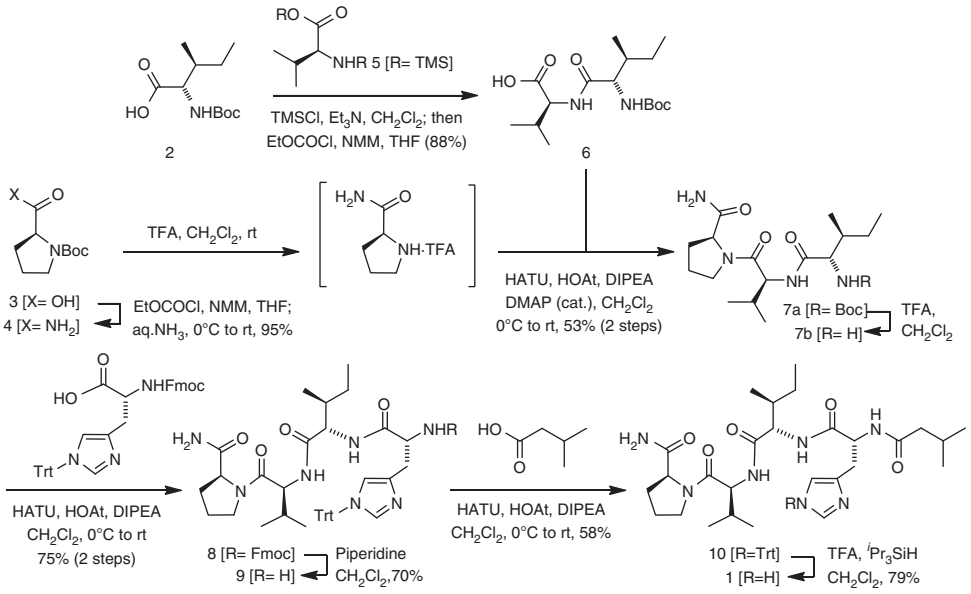


Figure 3 ^{1}H NMR (DMSO-d_6) correlation of (**a**) synthetic **1** with (**b**) natural falcitidin (**1**).



Scheme 1 Total synthesis of falcitidin (**1**) (isovaleric acid-D-His-L-Ile-L-Val-L-Pro-NH_2).

MS values were collected on a Bruker microQTOF-Q mass spectrometer, using sodium formate as an internal standard for both positive- and negative-ionization modes. MS/MS analysis was obtained on an ion-trap Bruker Esquire Mass Spectrometer (Bruker), which allows for the collection of ESI-MS/MS in alternating positive- and negative-ionization modes and is coupled with an Agilent 1100 Series HPLC (Agilent, Palo Alto, CA, USA). The MS analyses of synthetic compounds were recorded on a Finnigan LCQDECA (Thermo Finnigan, San Jose, CA, USA) or Thermo Finnigan MAT900XL mass spectrometer (Thermo Finnigan).

Myxobacteria strain Y23 isolation and fermentation

Strain Y23 was isolated from soil collected underneath a bamboo stand in Singapore. Sequencing of 16S rDNA revealed strain Y23 to be a member of the genus *Chitinophaga* with a closest match (94% sequence identity) to *C. terrae* (accession number AB278570).[12] Y23 was subcultured for 7 days at 28 °C on VY/2 agar, which consisted of Baker's yeast 5 g, $CaCl_2 \bullet 2H_2O$ 1.0 g, agar 15 g in MilliQ water 1 l, pH 7.2, which was autoclaved and then cyanocobalamin 500 μg in 10 ml of sterilized H_2O was added. The actively growing subculture was used to inoculate 250-ml Erlenmeyer flasks that each contained 50 ml of seed medium MD1. The MD1 medium was composed of Casitone 6 g (Difco, Detroit, MI, USA), $CaCl_2 \bullet 2H_2O$ 0.7 g, $MgSO_4 \bullet 7H_2O$ 2 g, cyanocobalamin 500 μg in 10 ml of sterilized H_2O and trace elements 1 ml. The pH of the medium was not adjusted prior to being autoclaved at 121 °C for 30 min. The seed flasks were incubated for 2 days at 28 °C on a rotary shaker with a 50 mm orbit at 200 r.p.m. A seed culture volume of 2.5 ml was used to inoculate 50 ml of the liquid medium in 250 ml flasks containing 0.5 g of Sephabeads SP207 (Mitsubishi Chemical Corp., Tokyo, Japan). The liquid medium was composed of defatted soya flour 7.0 g, Baker's yeast 3 g, $CaCl_2 \bullet 2H_2O$ 1.0 g, $MgSO_4 \bullet 7H_2O$ 1.0 g, glucose 2 g and cyanocobalamin 0.1 mg in 1 l of MilliQ water. The pH was adjusted to 7.2 prior and the medium was autoclaved at 121 °C for 30 min. Fermentation was undertaken at 200 r.p.m. for 5 days at 28 °C.

Extraction and isolation

The broth from 20 × 250 ml flasks (total 5 liter) were mixed with Celite, homogenized for 30 min, filtered under vacuum and the filtrate was separated

by C18 VLC (14.5 × 10 cm) eluted with 0, 20, 30, 40, 50 and 100% MeOH in H_2O (2 liter each fraction). The 100% MeOH fraction (~1 g) was active in the falcipain-2 assay and was further separated by C18 VLC (17 × 4.5 cm) using a 10% step-wise elution from 30% to 100% MeOH in H_2O (400 ml each fraction). Falcipain-2 activity was found in the 60% to 80% MeOH in H_2O fractions, which were combined (80 mg) and separated using a Sephadex LH 20 column (50 × 2 cm, eluent MeOH). The active fraction (8 mg) was separated by C18 preparative HPLC using the solvent A (0.1% HCO_2H in H_2O): solvent B (0.1% HCO_2H in CH_3CN), gradient elution from 100:0 to 95:5 for 2 min and then to 70:30 over 40 min, flow rate 12 ml min^{-1}) to obtain falcitidin (**1**) (0.5 mg, retention time 20 min). The biomass was fractionated in a similar manner as the broth, which yielded ~0.5 mg of peptide of ~85% purity.

Falcipain-2 biological assay

An *in vitro* enzymatic assay was performed using a 15 μg ml^{-1} of the falcipain-2 derived from *P. falciparum* Gombak A strain[9] to cleave 1 μM substrate (Z-Phe-Arg-AMC) peptide (Bachem AG, I1160) in a 384-well black microtitre plate (Griener, 781076) to identify potential inhibitors of falcipain-2. The buffer system contained 0.1 M sodium acetate buffer pH 5.6 (Merck, Singapore), 10 mM DTT (Boehringer Mannheim, 708992, Singapore), 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma, C3023, Singapore) and 20% trehalose (Calbiochem, 625625, Singapore). After mixing the enzyme, substrate and test sample in buffer, the protease reaction was allowed to proceed at 4 °C overnight (16 h). The amount of released AMC was quantified by measuring its fluorescence using an Ultra (Tecan, Männedorf, Switzerland) with excitation at 360 nm and emission at 465 nm. The percentage of (%) enzyme inhibition was calculated using the following formula: ((Sample fluorescence − low fluorescence control)/(high fluorescence control − low fluorescence control)) × 100. This assay had an overall signal to background ratio of 2.7 ± 0.3 and a Z' value of 0.66 ± 0.13, as no standard inhibitor was available when the assay was originally run.

Plasmepsin-2 and tryptase biological assays

The fluorescence resonance energy transfer and fluorescence polarization assay formats used for the plasmepsin-2 assays[27,28] and the fluorescence quenching format used for the tryptase assay[29] were performed as reported previously.

General procedure for cleavage of N-Boc group and amide coupling using HATU

The Boc protected amino acid or peptide compound was dissolved in CH_2Cl_2, and TFA was added slowly at 0 °C and the reaction was left stirring at room temperature for 30 min. After completion of the reaction, excess TFA was removed using rotary evaporation and diethylether was added to precipitate the product. The supernatant diethylether layer was removed and the solid was dissolved in CH_2Cl_2. The α-amino protected amino acid or peptide with free carboxy group was then added to the reaction flask at 0 °C, followed by the addition of DIPEA, HATU and HOAt and a catalytic amount of DMAP. The reaction was stirred for 5 h at room temperature. After completion of the reaction, the reaction mixture was evaporated *in vacuo* and the residue dissolved in EtOAc. The organic layer was washed with saturated $NaHCO_3$ solution, followed by brine solution. The organic layer was finally dried using Na_2SO_4 and evaporated *in vacuo* to give a solid residue that was purified by column chromatography.

Preparation of dipeptide (6)

(*S*)-2-((2*S*,3*S*)-2-(*tert*-butoxycarbonylamino)-3-methylpentanamido)-3-methyl butanoic acid: bis-TMS-valine (**5**) was separately prepared by dissolving L-valine (1.6 g, 13.6 mmol) in 60 ml CH_2Cl_2. Triethyl amine (6.7 ml, 48 mmol) and trimethylsilylchloride (6.1 ml, 48 mmol) were then added (2.0 g, 8.65 mmol). The mixture was refluxed for 2 h. The mixture was then used *in situ* for coupling to the anhydride. Thus, Boc-L-Isoleucine **2** (2.0 g, 8.65 mmol, 1 eq) was dissolved in THF and the solution was maintained at −20 °C using an ice-salt bath. Then, *N*-methyl morpholine (1.2 ml, 10.3 mmol, 1.2 eq) was added followed by the addition of ethyl chlorofomate (1.0 ml, 10.3 mmol, 1.0 eq). The mixture was stirred at −20 °C for 15 to 20 min. At this point, bis-TMS-valine (**5**) was added to the anhydride solution and stirred for 10 h at room temperature. After that, the reaction mixture was concentrated by rotary evaporation. The residue obtained was dissolved in saturated $NaHCO_3$ and the aqueous layer was washed with *n*-hexane. The aqueous bicarbonate layer was acidified with citric acid and then extracted with EtOAc. The organic layer was washed with brine, dried using anhydrous Na_2SO_4 and removed by rotary evaporation to give white solid, which was purified by silica column chromatography (hexane/EtOAc, 2.5:1) to give **6** (2.5 g, 7.5 mmol, 88%) as a white crystalline solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.77–0.83 (m, 6H), 0.87 (m, 6H), 1.08 (m, 1H), 1.37 (s, 9H), 1.58 (m, 1H), 1.98 (m, 2H), 3.84 (*t*, J = 8.2 Hz, 1H), 4.13 (dd, J = 5.7, 8.4 Hz, 1H), 6.73 (d, J = 9.0 Hz, 1H), 7.74 (d, J = 8.4 Hz, 1H), 12.56 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 10.8, 15.3, 17.9, 18.9, 24.3, 28.1, 29.9, 36.3, 56.9, 58.7, 77.9, 155.8, 171.6, 172.7; ESI-MS *m/z* 330.9 $(M+H)^+$, 353.1 $(M+Na)^+$.

Preparation of tripeptide (7a)

Tert-butyl (2*S*,3*S*)-1-((*S*)-1-((*S*)-2-carbamoylpyrrolidin-1-yl)-3-methyl-1-oxobutan-2-ylamino)-3-methyl-1-oxopentan-2-ylcarbamate: Boc-L-proline amide **4** (350 mg, 1.6 mmol) was coupled to the dipeptide **6** (647 mg, 1.95 mmol) according to the general procedure for peptide coupling using HATU/HOAt to give the desired compound **7a** (368 mg, 0.86 mmol, 53%) after silica column purification with $CHCl_3$/MeOH (25:1). ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.77 (m, 6H), 0.85 (m, 6H), 1.04 (m, 1H), 1.37 (s, 9H), 1.65 (m, 1H), 1.75 (m, 2H), 1.82 (m, 4H), 3.54 (m, 1H), 3.70 (m, 1H), 3.81 (t, 1H), 4.20 (1H, m), 4.33 (t, 1H) 6.82 (brs, 2H), 7.24 (s, 1H), 7.72 (d, J = 8.8 Hz, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 10.8, 15.3, 18.1, 19.1, 24.4, 28.1, 29.2, 30.1, 36.5, 46.9, 58.8, 78.0, 79.1, 155.2, 169.6, 171.2, 173.3; ESI-MS *m/z* 427.0 $(M+H)^+$, 449.2 $(M+Na)^+$.

Preparation of Fmoc–tetrapeptide (8)

(9*H*-fluoren-9-yl)methyl(*R*)-1-((2*S*,3*S*)-1-((*S*)-1-((*S*)-2-carbamoylpyrrolidin-1-yl)-3-methyl-1-oxobutan-2-ylamino)-3-methyl-1-oxopentan-2-ylamino)-1-oxo-3-(1-trityl-1H-imidazol-4-yl)propan-2-ylcarbamate (**8**): Tripeptide **7** (160 mg, 0.37 mmol) was coupled to N_α-fmoc-$N_{(im)}$-trityl-D-histidine (256 mg, 0.41 mmol) using the general procedure for HATU coupling. The desired compound tetrapeptide **8** (262 mg, 0.28 mmol, 75%) was obtained after silica column purification using chloroform/methanol (25:1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.75 (m, 12H), 1.02 (m, 1H), 1.18 (m, 1H), 1.41 (m, 1H), 1.87 (6H, m), 3.0 (d, J = 6.2 Hz, 2H), 3.61 (m, 1H), 3.77 (m, 1H), 4.19 (m, 3H), 4.51(m, 3H), 6.54 (brs, 1H), 6.66 (s, 1H), 7.06 (m, 7H), 7.14 (brs, 1H), 7.28 (m, 14H), 7.56 (m, 2H), 7.64 (brd, 1H), 7.72 (m, 2H), 7.79 p.p.m. (brd, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) 11.3, 15.8, 17.9, 19.2, 24.4, 25.0, 27.7, 31.2, 47.0, 47.7, 55.1, 55.8, 57.8, 59.3, 67.2, 75.3, 119.3, 119.8, 125.1, 125.2, 127.0, 127.6, 128.0, 129.6, 138.5, 141.1, 141.2, 142.2, 143.7, 143.9, 171.2, 171.3, 171.7, 173.5; ESI-MS *m/z* 928.2 $(M+H)^+$, 950.4 $(M+Na)^+$.

Preparation of tetrapeptide (9)

(*S*)-1-((*S*)-2-((2*S*,3*S*)-2-((*R*)-2-amino-3-(1-trityl-1*H*-imidazol-4-yl)propanamido)-3-methylpentanamido)-3-methylbutanoyl)pyrrolidine-2-carboxamide (**9**): Fmoc–tetrapeptide **8** (140 mg, 0.15 mmol) was cleaved using piperidine to give the desired compound **9** (77 mg, 0.11 mmol, 70%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.83 (m, 12H), 1.10 (m, 1H), 1.40 (m, 1H), 1.93 (m, 6H), 2.82 (m, 2H), 3.62 (m, 1H), 3.85 (m, 1H), 4.00 (m, 1H), 4.30 (m, 1H), 4.53 (m, 2H), 5.86 (s, 1H), 6.63 (s, 1H), 7.03 (brs, 1H), 7.10 (m, 6H), 7.16 (d, 1H), 7.33 (m, 9H), 7.46 (s, 1H), 7.65 (d, J = 8.2 Hz, 1H), 8.28 (d, J = 8.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.2, 15.7, 18.1, 19.1, 24.6, 24.9, 28.2, 30.6, 32.2, 36.5, 47.6, 54.5, 55.9, 58.4, 59.5, 75.1, 119.5, 127.9, 129.5, 136.7, 138.6, 142.0, 171.3, 171.5, 173.4, 173.7; ESI-MS *m/z* 706.1 $(M+H)^+$, 728.3 $(M+Na)^+$.

Preparation of Trt-falcitidin (10)

(S)-1-((S)-3-methyl-2-((2S,3S)-3-methyl-2-((R)-2-(3-methylbutanamido)-3-(1-trityl-1H-imidazol-4-yl)propanamido)pentanamido)butanoyl)pyrrolidine-2-carboxamide (**10**): Tetrapeptide **9** (74 mg, 0.1 mmol) was coupled to isovaleric acid (10 μl, 0.1 mmol) using the general procedure for HATU coupling. The desired compound **10** (48 mg, 0.06 mmol, 58%) was obtained after silica column purification using chloroform/methanol (25:1). ^{1}H NMR

(500 MHz, $CDCl_3$) δ 0.82 (m, 18H), 1.05 (m, 1H), 1.41 (m, 1H), 1.92 (m, 2H), 2.02 (m, 6H), 2.29 (m, 1H), 2.92 (m, 1H), 3.02 (m, 1H), 3.57 (1H, m), 3.75 (1H, m), 4.36 (m, 1H), 4.53 (m, 2H), 4.64 (q, J = 6.3 Hz, 1H), 6.67 (s, 1H), 6.94 (brs, 1H), 7.02 (brs, 1H), 7.08 (6H, m), 7.32 (m, 11H), 7.98 (brs, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 11.4, 15.8, 17.9, 19.3, 22.4, 22.5, 24.6, 25.0, 26.0, 27.1, 29.6, 29.7, 31.2, 36.8, 45.8, 47.7, 53.2, 55.7, 58.3, 59.3, 75.5, 119.4, 128.1, 129.7, 138.1, 142.1, 171.1, 171.6, 171.7, 173.1, 173.2; ESI-MS *m/z* 790.16 $(M+H)^+$.

Preparation of synthetic falcitidin (1)

(*S*)-1-((*S*)-2-((2*S*,3*S*)-2-((*R*)-3-(1H-imidazol-4-yl)-2-(3-methylbutanamido)-propanamido)-3-methylpentanamido)-3-methylbutanoyl)pyrrolidine-2-carboxamide: Compound **10** (57 mg, 71 μmol) was dissolved in CH_2Cl_2 and then TFA was added followed by triisopropylsilane. After 1 h, the reaction was complete. The solvent was removed *in vacuo*, saturated $NaHCO_3$ added, and the aqueous layer was extracted with EtOAc. The EtOAc was removed by rotary evaporation and the residue was purified by preparative HPLC using the same conditions as the natural products to give **1** as the formate salt (31 mg, 0.057 mmol, 79%). $[\alpha]_D^{31}$ −67 (c 0.84, MeOH) [**1**: $[\alpha]_D^{31}$ −72 (c 0.28, MeOH)]; HPLC co-elution with naturally occurring peptide (Figure 2c); ^1H NMR identical to natural product (Figure 3).

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