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Alaninyl variants of the marine natural product halocyamine A and their antibacterial properties

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Abstract

In an effort to explore the antibacterial potential of the marine natural product halocyamine A, a series of analogues including desbromo and alanine-substituted variants were synthesised and evaluated for biological activity against a panel of Gram-positive and –negative bacteria. The analogues were synthesised by a combination of solid-phase peptide synthesis and ruthenium complex/ytterbium triflate catalysed hydroamidation chemistry. Single alanine substitutions ([Ala^1]-halocyamine A and [Ala^2]-halocyamine A) gave only modest increases in activity towards Gram-positive bacteria, while di-alaninyl variants exhibited more potent activity with MIC values of 12.5–50 μM towards the Gram-positive bacteria *S. aureus* and *E. faecalis*. A lipophilic trityl-protected intermediate of [Ala^2]-halocyamine was the most active against the Gram negative bacterium *Escherichia coli*.

1. Introduction

The tunichromes are small, modified peptides isolated from the blood cells of marine organisms belonging to the Class Ascidiacea.[ref1, ref2, ref3] Structurally, these natural products are typified by the presence of a C-terminal decarboxy-$\Delta^{2,3}$-enamide fragment. While the C-terminus amino acid is usually tyrosine, 3,4-dihydroxyphnylalanine (DOPA) or 3,4,5-trihydroxyphenylalanine (TOPA)-derived, the halocyamines (e.g. A, **1**) are unusual in that the C-terminus moiety is derived from 6-bromo-tryptophan. A number of ecological roles have been proposed for the tunichromes, varying from tunic formation (cross-linking)[ref4], acting as a primitive wound repair system, to metal ion sequestration[ref5] or that they act as antimicrobial agents.[ref4] Indeed there is growing body of biochemical evidence that many of these roles are at least feasible.

As part of our ongoing investigation of the chemistry and biology of tunichromes, we have recently reported the synthesis and structural confirmation of the tunichromes Sp-1 [ref6] and halocyamine A (**1**).[ref7] With only modest levels of antibacterial activity being observed for synthetic halocyamine A, we sought to use our robust synthetic methodology to prepare novel halocyamine A analogues that explored the influence of bromine substitution and of the His and DOPA amino acids on the biological activity. Herein we report the synthesis and antibacterial activities of these halocyamine A analogues.

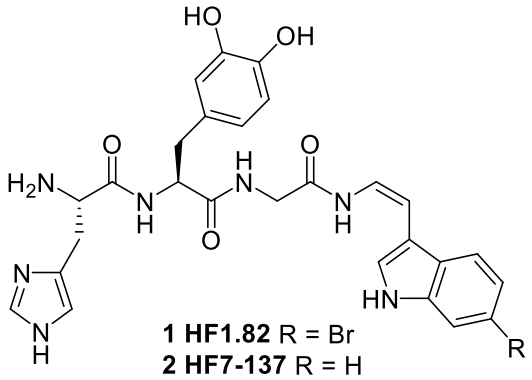


Fig 1. Structures of halocyamine A (**1**) and des-bromo analogue **2**

2. Results and discussion

In our previously reported synthesis of halocyamine A (**1**), the natural product was disconnected at the DOPA-Gly amide bond to give two fragments, Fmoc-His(Trt)-DOPA$(TBS)_2$-OH (**3**) and *Z*-enamide **4** (Fig. 2). [ref7] The two fragments were prepared separately using Fmoc solid phase peptide synthesis (SPPS) and ruthenium-catalysed hydroamidation of an indole-acetylene, respectively.

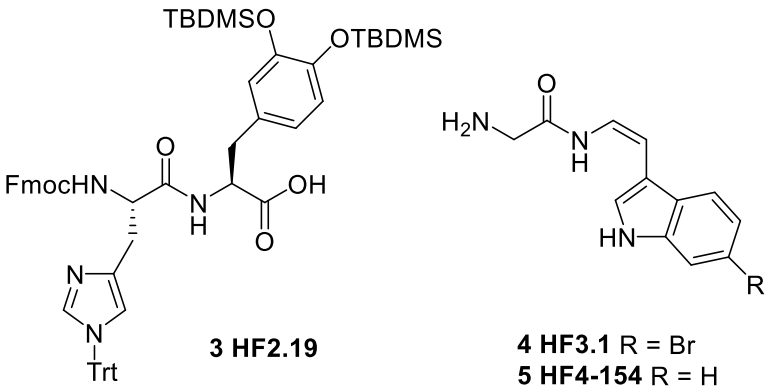
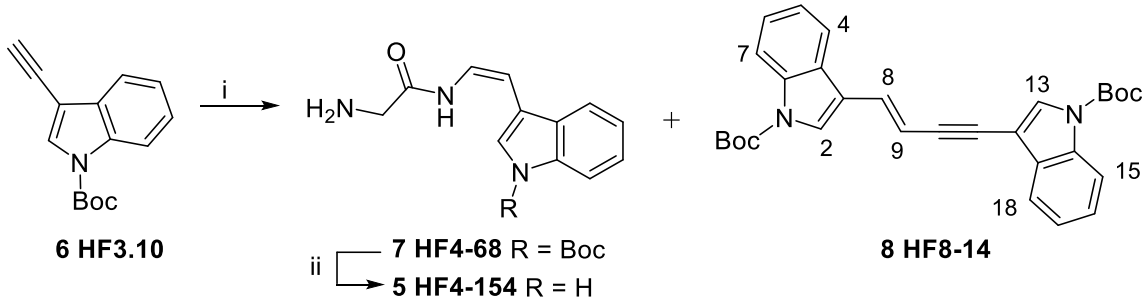


Fig 2. Fragments **3** – **5**.

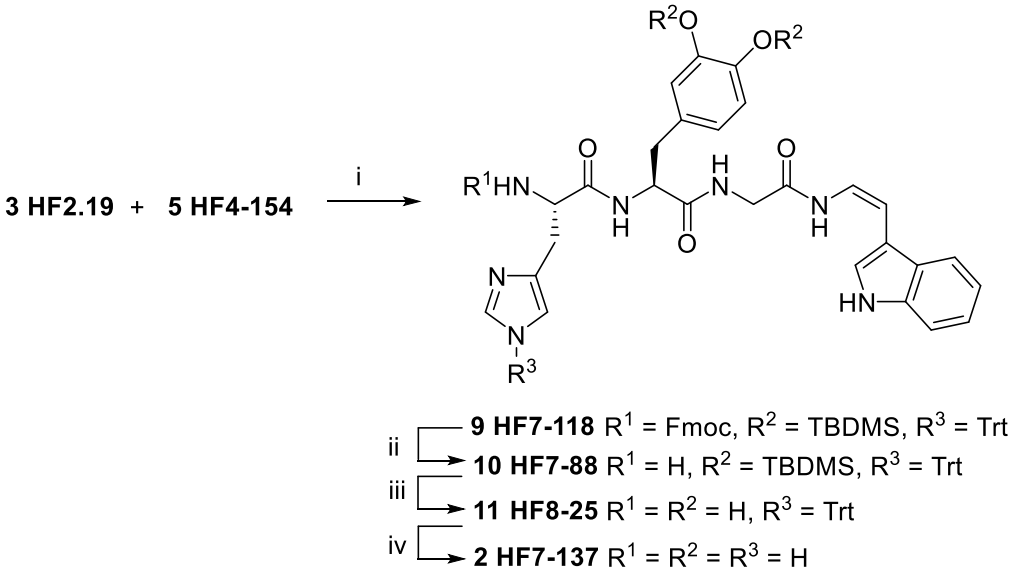
In the current project, we sought to explore the influence, if any, of bromine substitution and each of the amino acids L-His and L-DOPA on the observed antibacterial activity of halocyamine A. To this end, our first target was des-bromo halocyamine A **2**, which required synthesis of indole-enamide **5**. The known indolic alkyne **6** [Ref8 Unsworth] was subjected to hydroamidation with Fmoc-glycinamide using 5 mol% bis(2-methylallyl)(1,5-cyctooctadiene)ruthenium(II) heated at 70 °C for 32 h to give glycyl-*Z*-enamide **7** (69%) and by-product (*E*)-enyne **8**[ref7] (18%) (Scheme 1). Enamide **7** was then deprotected (TFA/CH_2Cl_2) to give **5** in 89% yield.



Scheme 1. *Reagents and conditions*: (i) Fmoc-glycinamide (0.5 eq.), bis(2-methylallyl)(1,5-cycloocta-diene)ruthenium(II) (0.025 eq.), 1,4-bis(dicyclohexylphosphino)butane (0.03 eq.), ytterium triflate (0.02 eq.), DMF, H_2O, 70 °C, 32 h, 69% (**7**) and 18% (**8**); (ii) TFA (60 eq.), CH_2Cl_2, 0 °C, 8 h, 89%.

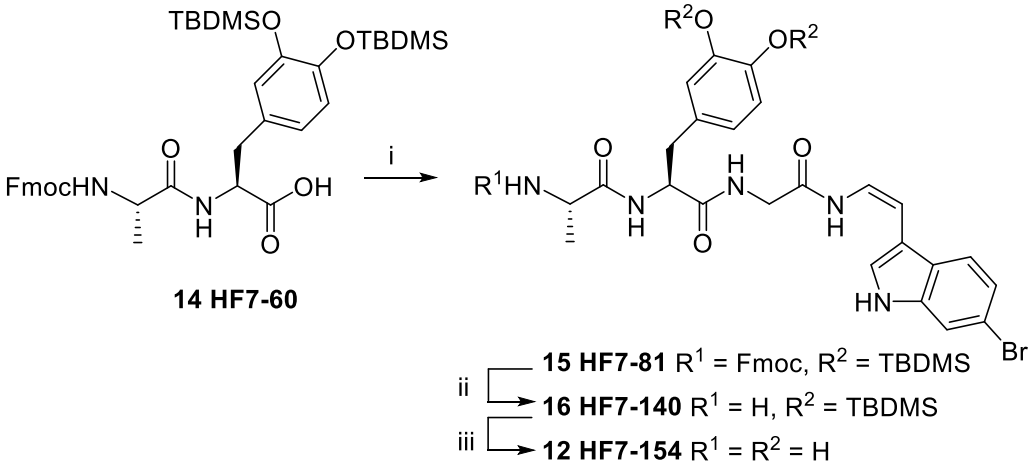
EDC-mediated coupling of enamide **5** and protected dipeptide Fmoc-His(Trt)-DOPA(TBDMS)$_2$-OH **3** [ref7] afforded the protected halocyamine analogue **9** in 39% yield (Scheme 2). Step-wise fmoc deprotection (piperidine, DMF, 20 min) to give **10** (68% yield), followed by desilylation (triethylamine trihydrofluoride, THF, 55 min) to give **11** (69% yield) and lastly, removal of the trityl

group (HCl/HFIP, H_2O, TIS, 1 h) afforded des-bromo halocyamine A **2** as the dihydrochloride salt in 73% yield.



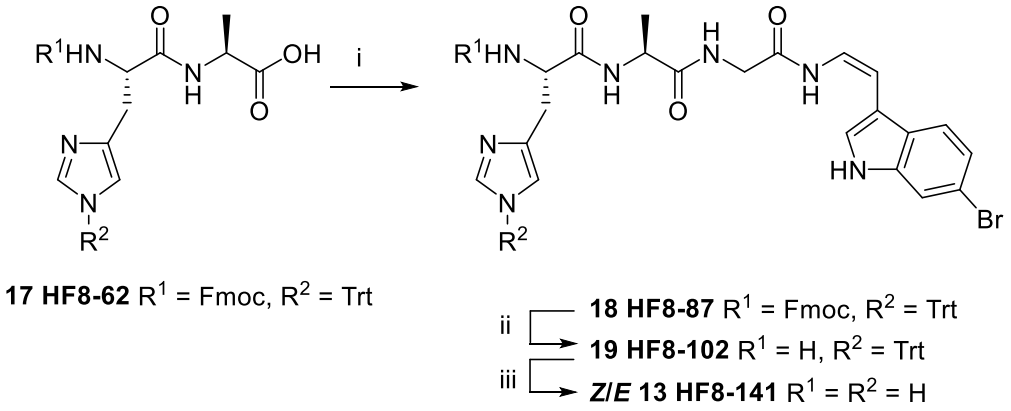
Scheme 2. *Reagents and conditions*: (i) EDC.HCl (1.5 eq.), HOBt (2 eq.), DIPEA (6 eq.), CH_2Cl_2, 9 h, 39%; (ii) piperidine, DMF, r.t. 20 min, 68%; (iii) $Et_3N.3HF$, THF, 0 °C, 55 min, 69%; (iv) 0.01 N HCl/HFIP, H_2O, TIS, r.t., 1 h, 73%.

We next explored the antibacterial influence of the L-histidine and/or L-DOPA residues of halocyamine A by preparing two L-alanine-substituted variants **12** and **13**. The first of these analogues (**12**), where Ala replaced His, required the protected dipeptide Fmoc-Ala-DOPA(TBDMS)$_2$-OH (**14**) which was prepared using standard Fmoc-SPPS methodology in 85% yield (see experimental). Bromo-indole enamide **4**[ref7] was then coupled (EDC, HOBt, DIPEA, 7.5 h) with **14** to give **15** in 42% yield (Scheme 3). Removal of the Fmoc protecting group (piperidine, DMF, 20 min) gave the chromatographically-separable (*Z*)-**16** (36%) and (*E*)-**16** (31%). Desilylation of (*Z*)-**16** (triethylamine trihydrofluoride, THF, 55 min) gave [Ala^1]-halocyamine A **12** in 67% yield.



Scheme 3. *Reagents and conditions*: (i) **4** (1 eq.), EDC.HCl (1.5 eq.), HOBt (2 eq.), DIPEA (6 eq.), CH_2Cl_2, r.t., 7.5 h, 42%; (ii) piperidine, DMF, r.t., 20 min, 36% (*Z*), 31% (*E*); (iii) $Et_3N.3HF$, THF, 0 °C, 55 min, 67%.

A similar reaction sequence was used to prepare the second Ala-variant **13** with Ala replacing the DOPA residue. Dipeptide **17**, prepared by Fmoc-SPPS in 88% yield, was coupled with **4** to give **18** in 44% yield (Scheme 3). Deprotection at the N-terminus (piperidine, DMF, 20 min) gave **19** (86%) which followed by removal of the trityl group (HCl/HFIP, H_2O, TIS, 1 h) afforded [Ala^2]-halocyamine A (**13**) (83%) as an inseparable mixture of *Z*/*E* (1:0.9) isomers.



Scheme 1. *Reagents and conditions*: (i) **4** (1 eq.), EDC.HCl (1.1 eq.), HOBt (1.5 eq.), DIPEA (5 eq.), CH_2Cl_2, DMF, r.t., 8 h, 44%; (ii) piperidine, DMF, r.t., 20 min, 86%; (iii) 0.01 N HCl/HFIP, H_2O, TIS, r.t., 1 h, 83% (*Z*:*E*, 1:0.9).

A final two analogues were prepared that explored substituting both His and DOPA residues for either L-Ala-L-Ala (**20**) or D-Ala-D-Ala (**21**). The requisite fmoc-dipeptides **22** and **23** were prepared by SPSS and then coupled with **4** (EDC, HOBt, DIPEA, 8h) to afford, respectively **24** (33%) or **25**

(34%). Subsequent deprotection (piperidine, DMF, 20 min) gave [Ala[1],Ala[2]]-halocyamine A **20** (60%) or [D-Ala[1],D-Ala[2]]-halocyamine A **21** (69%), respectively.

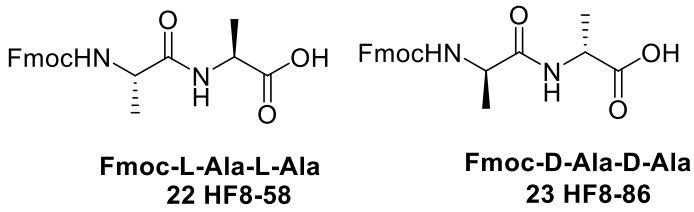
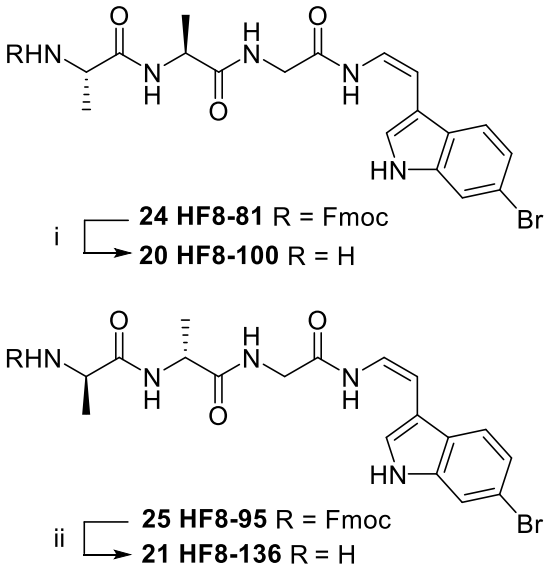


Fig. 3. Fmoc-protected dipeptides **22** and **23**.



Scheme 2. *Reagents and conditions*: (i) piperidine, DMF, r.t., 20 min, 60%; (ii) piperidine, DMF, r.t., 20 min, 69%.

Des-bromo halocyamine A **2**, alanine analogues **12** and **13**, di-alaninyl analogues **20** and **21** and a number of the reaction intermediates (**15**, **16**, **18**, **19**, **24**, **25**) were evaluated against a panel of Gram-negative (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922) and Gram-positive (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212) bacteria and for cytotoxicity towards the mammalian L6 rat skeletal myoblast cell line (Table 1). As we have previously noted, halocyamine A exhibits poor levels of antibacterial activity, with a modest MIC of 100 μM towards *P. aeruginosa* and *E. faecalis*.[ref7] In the present study, the des-bromo analogue **2** failed to exhibit any antibacterial activities, while both of the [Ala[1]] (**12**) and [Ala[2]] (**13**) analogues (the latter tested as an *E*/*Z* mixture of isomers) showed a modest increase in activity towards Gram-positive bacteria (*S. aureus* MIC 100 μM; *E. faecalis* 50 – 100 μM). An interesting finding related to the [Ala[2]] analogue was the enhanced activity towards the Gram-negative bacterium *E. coli* (MIC

25–50 μM) for the fmoc/trityl (**19**) and trityl-protected (**19**) intermediates. Although these same two compounds also exhibited cytotoxicity towards the L6 cell line (IC_{50} 65.6 and 25.6 μM, respectively) the observation of antibacterial activity for these compounds and not for the corresponding de-tritylated analogue (**13**) suggests that the presence of a sterically bulky lipophilic residue at the His-residue position in halocyamine A may be a useful starting point for the discovery of novel Gram-negative antibacterials.

Gram-positive antibacterial activity was observed for both di-alaninyl analogues with the di-L-alaninyl variant **20** exhibiting activity towards *S. aureus* (MIC 12.5 μM) and the di-D-alaninyl analogue **21** exhibiting activity towards both *S. aureus* and *E. faecalis* (both MIC 50 μM). Modest cytotoxicity was observed for these analogues (IC_{50} 115.8 and 142 μM, respectively), equating to a selectivity index (= cytotoxicity IC_{50} / antibacterial MIC) in the case of di-L-alaninyl **20** of close to ten. The antibacterial activity for both di-alaninyl analogues was dependent upon a free amine at the *N*-terminus, as both fmoc-protected precursors (**24** and **25**) were inactive.

Table 1
Summary of biological activities observed for compounds **1**, **2**, **12**, **13**, **15**, **16**, **18**-**21**, **24** and **25**.

Compound	*P. aeruginosa*[a]	*E. coli*[b]	*S. aureus*[c]	*E. faecalis*[d]	L6[e]
1 HaloA	100	>200	>200	100	NT[f]
2 HF7-137 Des	>200	>200	>200	NT	>150
12 HF7-154 Ala[1]	>200	>200	100	50	82.8
13 HF8-141 Ala[2] [g]	>200	>200	100	100	136.8
15 HF7-81 fmoc tbs ala-dopa	>200	>200	>200	NT	NT
16 HF7-140 tbs ala-dopa	>200	>200	25	NT	4.8
18 HF8-99/HF8-87 fmoc-trt-his-ala	>200	50	>200	>200	65.6
19 HF8-102 trt-his-ala	>200	25	25	200	25.6
20 HF8-100/HF8-103 Ala-Ala	>200	200	12.5	200	115.8
21 HF8-136 d-ala-d-ala	>200	>200	50	50	142
24 HF8-81 fmoc Ala-Ala	>200	100	200	>200	48.5
25 HF8-118/HF8-95 fmoc-d-ala-d-ala	>200	>200	100	100	NT

Colistin	1	2			
Chloramphenicol			1.5 – 3	1.5 – 3	
Podophyllotoxin					0.013

[a] *P. aeruginosa* ATCC27853. MIC (μM) values presented as the mean (n = 3).
[b] *E. coli* ATCC25922. MIC (μM) values presented as the mean (n = 3).
[c] *S. aureus* ATCC25923. MIC (μM) values presented as the mean (n = 3).
[d] *E. faecalis* ATCC29212. MIC (μM) values presented as the mean (n = 3).
[e] L6 rat skeletal myoblast cell line. IC_{50} (μM) values presented as the average (n = 2).
[f] Not tested.
[g] Tested as a mixture of *E*/*Z* isomers.

3. Conclusions

In conclusion, we have established that alaninyl analogues of the marine natural product halocyamine A exhibit more pronounced antibacterial activities than the natural product. While single alanine substitutions gave modest increases in activity (to MIC 50–100 μM), di-alaninyl analogues exhibited MIC values of 12.5–50 μM towards the Gram-positive bacteria *S. aureus* and *E. faecalis*. The finding of activity of a His(Trt)-Ala analogue of halocyamine A towards *E. coli* is particularly encouraging, suggesting that exploration of increasingly lipophilic analogues of [Ala^2]-halocyamine could be worthwhile in the search for new molecules active against Gram-negative bacteria.

4. Experimental

4.1. General

Infrared spectra were run as dry films on an FTIR infrared spectrometer fitted with a universal ATR sampling accessory. Optical rotations were recorded using a 0.1 dm cell in methanol. NMR spectra were recorded at either 500 or 400 MHz for ^{1}H nuclei and 125 or 100 MHz for ^{13}C nuclei. Proto-deutero solvent signals were used as internal references (CD_3OD: δ_H 3.31, δ_C 49.0; $CDCl_3$: δ_H TMS 0, δ_C 77.16; DMSO-d_6: δ_H 2.50, δ_C 39.52). ^{1}H NMR data is reported as position (δ), relative integral, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad, obs = obscured), coupling constant (*J*, Hz), and the assignment of the atom. ^{13}C NMR data are reported as position (δ) and assignment of the atom. Assignments were based on 2D NMR data acquired using standard COSY, multiplicity edited HSQC and HMBC pulse sequences. MS data were acquired on a micrOTOF Q II mass spectrometer. Flash column chromatography was carried out on C_2 or C_8 (reversed-phase) or on silica gel (normal phase). All solvents used were distilled analytical grade or better. Chemical reagents used were purchased from standard chemical suppliers. Compounds Fmoc-DOPA(TBDMS)$_2$-OH [Ref9 Sever tet 2001], Fmoc-His(Trt)-DOPA(TBDMS)$_2$-OH **3 HF2.19** [ref7], bromo-indole-enamide **4 HF3.1** [ref7] and indolic alkyne **6 HF3.10** [ref8 Unsworth 2013], were prepared by published routes and data obtained were in agreement with those previously reported.

*4.1.1. tert-Butyl (Z)-3-(2-(2-aminoacetamido)vinyl)-1H-indole-1-carboxylate (**7 HF4-68**) and di-tert-butyl 3,3'-(but-1-en-3-yne-1,4-diyl)(E)-bis(1H-indole-1-carboxylate) (**8 HF8-14**)*

Fmoc-Gly-NH_2 (0.296 g, 1.00 mmol), *tert*-butyl 3-ethynyl-1*H*-indole-carboxylate (0.482 g, 2.00 mmol), bis(2-methylallyl)(1,5-cycloocta-diene)ruthenium(II) (0.016 g, 0.05 mmol), 1,4-bis(dicyclohexylphosphino)butane (0.027 g, 0.06 mmol) and ytterbium triflate (0.025 g, 0.04 mmol) were placed under vacuum and then flushed with N_2 (four times). A degassed DMF (3.00 mL) / water

(108 µL, 6 mmol) mixture was added and the solution stirred under N_2 at 70 ºC for 32 h. The reaction was then quenched with sat. aq. $NaHCO_3$ (30 mL) and the resulting mixture extracted with EtOAc (5 x 20 mL). The combined organic layers were washed with water (30 mL) and brine (30 mL), dried ($MgSO_4$), filtered, and the solvent removed in vacuo. Purification using silica gel column chromatography (eluting with n-hexane to n-hexane/EtOAc 9:1) afforded **8 HF8-14** (0.087 g, 18%) as a brown oil while elution with a more polar solvent mixture (n-hexane/EtOAc 1:1 to EtOAc) gave **7 HF4-68** (0.220 g, 69%) as a yellow oil.

4.1.1.1. ***7 HF4-68***: R_f 0.15 (*n*-hexane/EtOAc 8:2); IR (ATR) ν_{max} 3329, 2973, 2931, 1700, 1451, 1368, 1253, 1149 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.71 (1H, d, *J* = 11.6 Hz, H-4), 8.13 (1H, d, *J* = 7.9 Hz, H-10), 7.76 (1H, s, H-8), 7.58 (1H, d, *J* = 7.9 Hz, H-13), 7.36 (1H, td, *J* = 7.9, 1.2 Hz, H-11), 7.28 (1H, td, *J* = 7.9, 1.2 Hz, H-12), 7.06 (1H, dd, *J* = 11.6, 9.2 Hz, H-5), 5.86 (1H, d, *J* = 9.2 Hz, H-6), 3.47 (2H, s, H_2-2), 1.68 (9H, s, $CO_2C(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.5 (C-3), 149.7 ($CO_2C(CH_3)_3$), 135.1 (C-9a), 129.9 (C-13a), 125.2 (C-11), 123.0 (C-12), 122.6 (C-8), 121.9 (C-5), 119.4 (C-13), 115.9 (C-7), 115.3 (C-10), 100.4 (C-6), 84.0 ($CO_2C(CH_3)_3$), 44.6 (C-2), 28.3 ($CO_2C(CH_3)_3$); (+)-HRESIMS $[M+H]^+$ 316.1650 (calcd. for $C_{17}H_{22}N_3O_3$, 316.1656).

4.1.1.2. ***8 HF8-14***: R_f 0.79 (*n*-hexane/EtOAc 4:1); IR (ATR) ν_{max} 2977, 2933, 2865, 1733, 1451, 1368, 1231, 1147, 1093 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 8.18–8.14 (2H, m, H-7 and H-15), 7.78 (1H, s, H-13), 7.78–7.73 (2H, m, H-4 and H-18), 7.70 (1H, s, H-2), 7.37–7.32 (2H, m, H-6 and H-16), 7.31–7.26 (2H, m, H-5 and H-17), 7.17 (1H, d, *J* = 15.8 Hz, H-8), 6.52 (1H, d, *J* = 15.8 Hz, H-9), 1.66–1.65 (18H, m, $3H_3$-21 and $3H_3$-24); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 149.4 (C-19 or C-22), 149.1 (C-19 or C-22), 136.1 (C-7a), 134.8 (C-14a), 132.6 (C-8), 130.6 (C-18a), 128.5 (C-13), 128.2 (C-3a), 125.2 (C-6 or C-16), 125.0 (C-6 or C-16), 124.8 (C-2), 123.29 (C-5 or C-17), 123.27 (C-5 or C-17), 120.2 (C-4 or C-18), 120.0 (C-4 or C-18), 118.5 (C-3), 115.6 (C-7 or C-15), 115.3 (C-7 or C-15), 108.0 (C-9), 103.9 (C-12), 92.9 (C-10), 84.24 (C-20 or C-23), 84.17 (C-20 or C-23), 83.4 (C-11), 28.19 (3C-21 or 3C-24), 28.17 (3C-21 or 3C-24); (+)-HRESIMS $[M+Na]^+$ 505.2091 (calcd. for $C_{30}H_{30}N_2O_4Na$, 505.2098).

*4.1.2. (Z)-N-(2-(1H-Indol-3-yl)vinyl)-2-aminoacetamide (**5 HF4-154**)*

A solution of **7 HF4-68** (0.17 g, 0.436 mmol) in CH_2Cl_2 (2 mL) was stirred at 0 ºC under N_2 for 5 min before TFA (2 mL, 26.1 mmol) was added dropwise. The solution was then stirred for a further 8 h at 0 ºC. The reaction was quenched with sat. aq. $NaHCO_3$ (20 mL) and extracted with EtOAc (4 x 20 mL). The organic layers were combined and dried in vacuo. The crude black oil was purified by silica gel column chromatography (eluting with EtOAc to CH_2Cl_2/MeOH 9:1) to afford **5**

HF4-154 (0.13 g, 89%) as a black oil. R_f 0.50 (CH_2Cl_2/MeOH 9:1); IR (ATR) ν_{max} 3403, 3295, 1655, 1535, 1499, 1231 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.53 (1H, br s, NH-4), 8.30 (1H, br s, NH-9), 7.65 (1H, d, *J* = 7.5 Hz, H-13), 7.40 (1H, d, *J* = 8.6 Hz, H-10), 7.35 (1H, d, *J* = 2.4 Hz, H-8), 7.27–7.24 (1H, m, H-11), 7.18 (1H, t, *J* = 7.5 Hz, H-12), 6.98 (1H, dd, *J* = 11.5, 9.2 Hz, H-5), 6.00 (1H, d, *J* = 9.2 Hz, H-6), 3.45 (2H, s, H_2-2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.3 (C-3), 135.9 (C-9a), 126.8 (C-13a), 123.0 (C-11), 121.8 (C-8), 120.3 (C-12), 119.7 (C-13), 119.5 (C-5), 112.0 (C-7), 111.3 (C-10), 102.6 (C-6), 44.7 (C-2); (+)-HRESIMS $[M+H]^+$ 216.1130 (calcd. for $C_{12}H_{14}N_3O$, 216.1131).

*4.1.3. (9H-Fluoren-9-yl)methyl ((S)-1-(((S)-1-((2-(((Z)-2-(1H-indol-3-yl)vinyl)amino)-2-oxoethyl)amino)-3-(3,4-bis((tert-butyldimethylsilyl)oxy)phenyl)-1-oxopropan-2-yl)amino)-1-oxo-3-(1-trityl-1H-imidazol-4-yl)propan-2-yl)carbamate (**10 HF7-118**)*

To a solution of dipeptide **3 HF2.19** (0.43 g, 0.42 mmol), enamide **5 HF4-154** (0.09 g, 0.42 mmol), EDC.HCl (0.12 g, 0.63 mmol) and HOBt (0.11 g, 0.84 mmol) in CH_2Cl_2 (3 mL) was added DIPEA (0.44 mL, 2.5 mmol) under N_2 and stirred for 9 h. The reaction mixture was diluted with CH_2Cl_2 (15 mL), washed with water (10 mL) and the organic solvent removed *in vacuo*. Subsequent purification by silica gel column chromatography (eluting with *n*-hexane/EtOAc 9:1 to *n*-hexane/EtOAc 1:1) afforded **9 HF7-118** (0.20 g, 39%) as a yellow oil. $[\alpha]^{26.7}_D$ +16.6 (*c* 1.57, CH_2Cl_2); R_f 0.58 (CH_2Cl_2/MeOH 9:1); IR (ATR) ν_{max} 2930, 2861, 1714, 1670, 1509, 1447, 1297, 1253, 1155, 1130 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 9.02 (2H, br s, NH-20 and NH-28), 8.22 (1H, d, *J* = 10.9 Hz, NH-23), 7.75 (2H, d, *J* = 6.8 Hz, H-FmocAr), 7.73 (1H, d, *J* = 7.3 Hz, H-FmocAr), 7.56 (1H, d, *J* = 7.7 Hz, H-32), 7.52–7.49 (1H, m, 2H-FmocAr), 7.39–7.36 (4H, m, H-6, H-29 and 2H-FmocAr), 7.30–7.25 (7H, m, H-FmocAr and 6H-TrtAr), 7.24–7.20 (3H, m, H-27, H-30 and H-FmocAr), 7.07–7.05 (3H, m, H-31 and 3H-TrtAr), 7.00–6.99 (6H, m, 6H-TrtAr), 6.74–6.73 (2H, m, H-17 and H-24), 6.68 (2H, br s, NH-10 and H-14), 6.56–6.54 (2H, m, H-8 and H-18), 6.01 (1H, d, *J* = 5.9 Hz, NH-1), 5.88 (1H, d, *J* = 9.1 Hz, H-25), 4.76 (1H, br s, H-11), 4.31–4.29 (3H, m, CO_2CH_2CH and H-2), 4.15–4.12 (2H, m, CO_2CH_2CH and H_2-21a), 3.76 (1H, dd, *J* = 16.7, 4.8 Hz, H_2-21b), 3.07 (1H, dd, *J* = 14.2, 7.1 Hz, H_2-12a), 3.00–2.94 (2H, m, H_2-3a and H_2-12b), 2.65 (1H, br s, H_2-3b), 0.97–0.95 (18H, m, $2SiC(CH_3)_3$), 0.17–0.16 (12H, m, $2Si(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.1 (C-19), 170.6 (C-9), 166.6 (C-22), 156.2 (CO_2CH_2CH), 147.3 (C-15), 146.3 (C-16), 143.9 (C-FmocAr), 143.7 (C-FmocAr), 141.4 (2C-FmocAr and 3C-TrtAr), 135.9 (C-28a), 129.8 (3C-TrtAr), 129.7 (6C-TrtAr), 129.2 (C-13), 128.4 (6C-TrtAr), 127.9 (2C-FmocAr), 127.2 (2C-FmocAr), 127.1 (C-32a), 125.2 (2C-FmocAr), 122.9 (C-27), 122.7 (C-30), 122.3 (C-18), 122.0 (C-14), 121.3 (C-17), 120.9 (C-8), 120.2 (2C-FmocAr), 120.0 (C-31), 119.0 (C-32), 118.9 (C-24), 111.5 (C-29), 111.1 (C-26), 103.4 (C-25), 67.4 (CO_2CH_2CH), 54.7 (C-2 and C-11), 47.2 (CO_2CH_2CH), 43.9 (C-21), 36.6 (C-12), 29.5 (C-3),

26.1 (2SiC(CH_3)$_3$), 18.6 (2SiC(CH_3)$_3$), −3.9 (2Si(CH_3)$_2$); (+)-HRESIMS $[M+H]^+$ 1224.5848 (calcd. for $C_{73}H_{82}N_7O_7Si_2$, 1224.5809).

*4.1.4. (S)-N-(2-(((Z)-2-(1H-Indol-3-yl)vinyl)amino)-2-oxoethyl)-2-((S)-2-amino-3-(1-trityl-1H-imidazol-4-yl)propanamido)-3-(3,4-bis((tert-butyldimethylsilyl) oxy)phenyl)propanamide (**10 HF7-88**)*

Piperidine (0.18 mL, 1:4 v/v in DMF) was added to **9 HF7-118** (43.9 mg, 35.9 µmol) and stirred at r.t. under N_2 for 20 min. Water (10 mL) was added to the solution and the aqueous layer was extracted with EtOAc (4 x 20 mL). The organic layers were combined and dried in vacuo. The crude product was purified by silica gel column chromatography (eluting with EtOAc to CH_2Cl_2/MeOH 9:1) to afford **10 HF7-88** (24.5 mg, 68%) as a yellow oil. $[\alpha]^{26.7}_D$ −12.2 (c 2.66, CH_2Cl_2); R_f 0.62 (CH_2Cl_2/MeOH 9:1); IR (ATR) ν_{max} 2931, 2857, 1659, 1509, 1445, 1364, 1251, 1230, 1158, 1132, 1092 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.40 (1H, br s, NH-28), 8.72 (1H, dd, *J* = 6.0, 6.0 Hz, NH-20), 8.24 (1H, d, *J* = 10.9 Hz, NH-23), 7.57 (1H, d, *J* = 7.8 Hz, H-32), 7.36–7.34 (2H, m, H-6 and H-29), 7.31–7.26 (6H, m, 6H-TrtAr), 7.25–7.23 (1H, m, H-27), 7.21–7.17 (1H, m, H-30), 7.13–7.06 (4H, m, H-31, and 3H-TrtAr), 7.02–7.00 (6H, m, 6H-TrtAr), 6.89 (1H, d, J = 7.8 Hz, NH-10), 6.74–6.70 (2H, m, H-17 and H-24), 6.68–6.67 (1H, m, H-14), 6.58–6.55 (2H, m, H-8 and H-18), 5.87 (1H, d, *J* = 9.1 Hz, H-25), 4.69 (1H, ddd, *J* = 7.8, 7.2, 6.4 Hz, H-11), 4.12 (1H, dd, *J* = 16.8, 6.0 Hz, H_2-21a), 3.83 (1H, dd, *J* = 16.8, 6.0 Hz, H_2-21b), 3.50 (1H, dd, *J* = 5.2, 5.2 Hz, H-2), 3.05 (1H, dd, *J* = 14.1, 7.2 Hz, H_2-12a), 2.99 (1H, dd, *J* = 14.1, 7.2 Hz, H_2-12b), 2.85 (1H, dd, *J* = 14.7, 5.2 Hz, H_2-3a), 2.71 (1H, dd, *J* = 14.7, 5.2 Hz, H_2-3b), 0.97–0.96 (18H, m, 2SiC(CH_3)$_3$), 0.17–0.16 (12H, m, 2Si(CH_3)$_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.9 (C-9), 172.3 (C-19), 166.7 (C-22), 147.1 (C-15), 146.1 (C-16), 142.2 (3C-TrtAr), 128.1 (3C-TrtAr), 139.0 (C-6), 136.0 (C-4), 135.8 (C-28a), 129.8 (C-13), 129.7 (6C-TrtAr), 128.3 (6C-TrtAr), 127.1 (C-32a), 123.1 (C-27), 122.6 (C-30), 122.3 (C-18), 122.1 (C-14), 121.3 (C-17), 120.6 (C-8), 119.9 (C-31), 118.9 (C-32), 118.6 (C-24), 111.4 (C-29), 110.8 (C-26), 103.6 (C-25), 75.6 (CAr_3), 54.40 (C-2 or C-11), 54.37 (C-2 or C-11), 43.8 (C-21), 36.6 (C-12), 33.1 (C-3), 26.1 (2SiC(CH_3)$_3$), 18.6 (SiC(CH_3)$_3$), 18.5 (SiC(CH_3)$_3$), −3.9 (2Si(CH_3)$_2$); (+)-HRESIMS $[M+H]^+$ 1002.5152 (calcd. for $C_{58}H_{72}N_7O_5Si_2$, 1002.5128).

*4.1.5. (S)-N-(2-(((Z)-2-(1H-Indol-3-yl)vinyl)amino)-2-oxoethyl)-2-((S)-2-amino-3-(1-trityl-1H-imidazol-4-yl)propanamido)-3-(3,4-dihydroxy phenyl)propanamide (**11 HF8-25**)*

Triethylamine trihydrofluoride (4.59 µL, 28.5 µmol) was added to a solution of **10 HF7-88** (9.5 mg, 9.5 µmol) in THF (0.50 mL) at 0 ºC and stirred under N_2 for 55 min. The reaction mixture was dried under a stream of N_2 and water (15 mL) was added. The aqueous layer was extracted with CH_2Cl_2 (4 x 20 mL) and the organic layers were combined and solvent was removed *in vacuo* to give

a yellow oil. Purification by silica gel column chromatography (eluting with EtOAc to MeOH/CH_2Cl_2, 1:9), afforded **11 HF8-25** (5.1 mg, 69%) as a yellow oil. $[\alpha]^{22.2}_D$ −5.1 (*c* 0.28, CH_2Cl_2); R_f 0.33 (CH_2Cl_2/MeOH 9:1); IR (ATR) ν_{max} 3312, 1657, 1493, 1445, 1281, 1155, 1037 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) δ 7.52, (1H, d, *J* = 7.9 Hz, H-32), 7.41 (1H, br s, H-27), 7.36 (1H, d, *J* = 1.4 Hz, H-6), 7.34–7.31 (10H, m, H-29 and 9H-TrtAr), 7.12–7.09 (1H, m, H-30), 7.09–7.07 (6H, m, 6H-TrtAr), 7.04–7.01 (1H, m, H-31), 6.68 (1H, br s, H-8), 6.64–6.61 (3H, m, H-14, H-17 and H-24), 6.49 (1H, dd, *J* = 8.1, 2.1 Hz, H-18), 5.99 (1H, d, *J* = 9.0 Hz, H-25), 4.54 (1H, dd, *J* = 9.1, 5.3 Hz, H-11), 3.88 (2H, br s, H_2-21), 3.53 (1H, dd, *J* = 5.8, 5.6 Hz, H-2), 3.01 (1H, dd, *J* = 14.1, 5.3 Hz, H_2-12a), 2.80 (1H, dd, *J* = 14.7, 5.6 Hz, H_2-3a), 2.74–2.67 (2H, m, H_2-3b and H_2-12b); ^{13}C NMR (CD_3OD, 125 MHz) δ 175.7 (C-9), 174.6 (C-19), 169.2 (C-22), 146.3 (C-15), 145.3 (C-16), 143.6 (3C-TrtAr), 139.8 (C-6), 137.51 (C-4 or C-28a), 137.48 (C-4 or C-28a), 130.8 (6C-TrtAr), 129.7 (C-13), 129.2 (9C-TrtAr), 128.2 (C-32a), 124.7 (C-27), 123.1 (C-30), 121.7 (C-8 or C-18)*, 121.6 (C-8 or C-18)*, 120.5 (C-31), 119.4 (C-32), 118.8 (C-24), 117.3 (C-14), 116.3 (C-17), 112.4 (C-29), 111.0 (C-26), 106.2 (C-25), 76.8 (*C*Ar_3), 56.3 (C-11), 55.4 (C-2), 44.1 (C-21), 37.9 (C-12), 33.8 (C-3); (+)-HRESIMS $[M+Na]^+$ 774.3411 (calcd. for $C_{46}H_{47}N_4O_6Na$, 774.3388).

*4.1.6. Des-bromo halocyamine A dihydrochloride (**2 HF7-137**)*

A cocktail solution of 0.01 N HCl/HFIP-TIS/H_2O (2 mL, 95:2.5:2.5) was added to **11 HF8-25** (48.6 mg, 0.0628 mmol) and stirred for 1 h. The reaction was dried under a stream of N_2. Purification by C_8 column chromatography (eluting with H_2O to H_2O/MeOH 6:4) afforded **2 HF7-137** (24.4 mg, 73%) as a white powder. m.p. 260 ºC (decomposed); R_f 0.87 (butan-1-ol/acetic acid/H_2O 2:1:1); $[\alpha]^{23.0}_D$ +7.8 (*c* 0.43, MeOH); IR (ATR) ν_{max} 3262, 3024, 2928, 1652, 1494, 1445, 1258, 1184, 1079, 1032 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 11.39 (1H, s, NH-28), 9.06 (1H, d, *J* = 10.1 Hz, NH-23), 8.71 (1H, d, *J* = 18.7 Hz, NH-20), 8.13 (1H, d, *J* = 6.0 Hz, NH-10), 7.71 (1H, d, *J* = 2.0 Hz, H-27), 7.60 (1H, d, *J* = 8.0 Hz, H-32), 7.55 (1H, s, H-6), 7.39 (1H, d, *J* = 8.0 Hz, H-29), 7.12 (1H, t, *J* = 8.0 Hz, H-30), 7.04 (1H, t, *J* = 8.0 Hz, H-31), 6.82 (1H, br s, H-8), 6.65 (1H, dd, *J* = 10.1, 9.6 Hz, H-24), 6.61–6.58 (2H, m, H-14 and H-17), 6.42 (1H, dd, *J* = 8.0, 1.8 Hz, H-18), 5.95 (1H, d, *J* = 9.6 Hz, H-25), 4.44 (1H, br s, H-11), 3.99 (1H, dd, *J* = 18.7, 5.9 Hz, H_2-21a), 3.93 (1H, dd, *J* = 18.7, 5.9 Hz, H_2-21b), 3.38 (1H, obs, H-2), 2.92 (1H, dd, *J* = 13.9, 4.2 Hz, H_2-12a), 2.80 (1H, dd, *J* = 14.5, 4.4 Hz, H_2-3a), 2.68 (1H, dd, *J* = 13.9, 9.0 Hz, H_2-12b), 2.53 (1H, d, *J* = 8.7 Hz, H_2-3b); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 172.0 (C-19), 167.5 (C-22), 144.8 (C-15), 143.7 (C-16), 135.5 (C-28a), 134.9 (C-6), 128.4 (C-13), 126.6 (C-32a), 123.9 (C-27), 121.6 (C-30), 120.0 (C-18), 119.0 (C-31), 118.2 (C-32), 117.8 (C-24), 116.6 (C-14), 115.2 (C-17), 111.5 (C-29), 109.4 (C-26), 102.8 (C-25), 54.9 (C-2), 53.9 (C-11), 42.6 (C-21), 39.8 (C-3), 36.9 (C-12); (+)-HRESIMS $[M+H]^+$ 532.2296 (calcd. for $C_{27}H_{30}N_7O_5$, 532.2303).

*4.1.7. Fmoc-Ala-DOPA(TBDMS)$_2$-OH (**14 HF7-60**)*

A solution of Fmoc-DOPA(TBDMS)$_2$-OH [Ref9 Sever Tet 2001] (0.65 g, 1.0 mmol) in CH_2Cl_2 (15 mL) was added to 2-chlorotrityl chloride resin (2 g, loading at 0.5 mmol/g), followed by DIPEA (0.17 mL, 1.0 mmol). After the resin mixture was agitated for 10 min, DIPEA (0.26 mL, 1.5 mmol) was added and the mixture was further shaken for 1 h. The solution was drained off and the resin was washed with DMF (10 mL). A solution of CH_2Cl_2/MeOH/DIPEA (12.5 mL, 80:15:5) was added to the mixture and shaken for 23 min. The solution was drained and the procedure was repeated. The resin was then washed with DMF (10 mL). Piperidine in DMF (12.5 mL, 1:4) was added to the resin mixture and shaken for 10 min. The liquid was drained off and piperidine washing was repeated for another 20 min. The amino acid-loaded resin was thoroughly washed with DMF (15 mL), isopropanol (15 mL) and *n*-hexane (15 mL). The resin was then dried under vacuum for 30 min and placed in a desiccator overnight. CH_2Cl_2 (30 mL) was added to the resin and left for 1 h. The solution was drained and a solution of HBTU (1.42 g, 3.75 mmol), HOBt (0.570 g, 3.75 mmol), Fmoc-Ala-OH (0.62 g, 2.0 mmol) and DIPEA (0.87 mL, 5.0 mmol) in DMF (3.75 mL) was added. The amino acid resin mixture was agitated for 5 h. The solution was then drained and washed with DMF (20 mL), isopropanol (20 mL) and *n*-hexane (20 mL). 2,2,2-Trifluroethanol in CH_2Cl_2 (12.5 mL, 1:4) was added to the amino acid-loaded resin and agitated for 2 h. The solution was drained and the organic solvent was removed *in vacuo* and dried extensively to afford **14 HF7-60** (0.61 g, 85%) as a yellow foam. m.p. 79–80 ºC; $[\alpha]^{25.1}_D$ +10.2 (*c* 1.59, CH_2Cl_2); R_f 0.38 (EtOAc); IR (ATR) ν_{max} 2932, 2858, 1719, 1665, 1509, 1450, 1423, 1297, 1251, 1127 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.74 (2H, d, *J* = 7.3 Hz, 2H-FmocAr), 7.57 (2H, d, *J* = 7.3 Hz, 2H-FmocAr), 7.38 (2H, t, *J* = 7.3 Hz, 2H-FmocAr), 7.28 (2H, t, *J* = 7.3 Hz, 2H-FmocAr), 6.70 (1H, d, *J* = 8.2 Hz, H-12), 6.65 (1H, d, *J* = 1.9 Hz, H-9), 6.59–6.57 (1H, m, H-13), 6.53 (1H, br s, NH-5), 5.56 (1H, br s, NH-1), 4.75 (1H, ddd, *J* = 6.2, 6.2, 6.2 Hz, H-6), 4.38–4.36 (2H, m, CO_2CH_2CH), 4.23 (1H, br s, H-2), 4.19 (1H, t, *J* = 7.0 Hz, CO_2CH_2CH), 3.07 (1H, dd, *J* =14.0, 6.2 Hz, H_2-7a), 2.98 (1H, dd, *J* = 14.0, 6.2 Hz, H_2-7b), 1.32 (3H, d, *J* = 5.7 Hz, H_3-3), 0.95–0.94 (18H, m, 2SiC(CH_3)$_3$), 0.16–0.14 (12H, m, 2Si(CH_3)$_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 174.6 (C-14), 172.5 (C-4), 156.1 (CO_2CH_2CH), 147.0 (C-10), 146.3 (C-11), 143.8 (2C-FmocAr), 141.4 (2C-FmocAr), 128.7 (C-8), 127.9 (2C-FmocAr), 127.2 (2C-FmocAr), 125.2 (2C-FmocAr), 122.4 (C-13), 122.1 (C-9), 121.2 (C-12), 120.1 (2C-FmocAr), 67.3 (CO_2CH_2CH), 53.5 (C-6), 50.6 (C-2), 47.2 (CO_2CH_2CH), 36.8 (C-7), 26.0 (2SiC(CH_3)$_3$), 18.9 (C-3), 18.5 (2SiC(CH_3)$_3$), −3.9 (2Si(CH_3)$_2$); (+)-HRESIMS $[M+Na]^+$ 741.3381 (calcd. for $C_{39}H_{54}NaN_2O_7Si_2$, 741.3362).

*4.1.8. (9H-Fluoren-9-yl)methyl ((S)-1-(((S)-3-(3,4-bis((tert-butyldimethylsilyl)oxy)phenyl)-1-((2-(((Z)-2-(6-bromo-1H-indol-3-yl)vinyl)amino)-2-oxoethyl) amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl) carbamate (**15 HF7-81**)*

To a solution of enamide **4 HF3.1** (0.14 g, 0.49 mmol), **14 HF7-60** (0.36 g, 0.49 mmol), EDC.HCl (0.14 g, 0.73 mmol) and HOBt (0.13 g, 0.98 mmol) in CH_2Cl_2 (5 mL) under N_2, was added DIPEA (0.51 mL, 2.9 mmol) and the solution was stirred at r.t. for 7.5 h. Water (10 mL) was added and the crude reaction product was extracted with EtOAc (4 x 15 mL). The organic layers were combined, dried ($MgSO_4$), filtered and solvent removed *in vacuo* to give a crude oil. Purification by silica gel column chromatography (eluting with *n*-hexane/EtOAc 9:1 to *n*-hexane/EtOAc 1:1) afforded **15 HF7-81** (112 mg, 42%) as a yellow oil. R_f 0.45 (*n*-hexane/EtOAc 1:1); $[\alpha]^{24.8}_D$ +3.2 (*c* 0.52, CH_2Cl_2); IR (ATR) ν_{max} 3280, 2931, 2858, 1629, 1504, 1447, 1293, 1252, 1160 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 9.15 (1H, br s, NH-23), 8.36 (1H, d, *J* = 10.2 Hz, NH-18), 7.74 (2H, d, *J* = 7.2 Hz, 2H-FmocAr), 7.52 (2H, t, *J* = 7.2 Hz, 2H-FmocAr), 7.44 (1H, d, *J* = 1.2 Hz, H-24), 7.39–7.36 (3H, m, H-27 and 2H-FmocAr), 7.27 (2H, t, *J* = 7.2 Hz, 2H-FmocAr), 7.21 (1H, d, *J* = 1.9 Hz, H-22), 7.18 (1H, dd, *J* = 8.5, 1.2 Hz, H-26), 7.14 (1H, br s, NH-15), 6.94 (1H, d, *J* = 7.5 Hz, NH-5), 6.80 (1H, dd, *J* = 10.2, 9.6 Hz, H-19), 6.68 (1H, d, *J* = 8.2 Hz, H-12), 6.63 (1H, d, *J* = 2.0 Hz, H-9), 6.51 (1H, dd, *J* = 8.2, 2.0 Hz, H-13), 5.87 (1H, d, *J* = 9.6 Hz, H-20), 5.36 (1H, d, *J* = 4.2 Hz, NH-1), 4.62 (1H, ddd, *J* = 7.5, 7.5, 7.5 Hz, H-6), 4.37 (2H, d, *J* = 6.1 Hz, CO_2CH_2CH), 4.15–4.10 (1H, m, CO_2CH_2CH), 4.09–4.03 (2H, m, H-2 and H_2-16a), 3.82–3.78 (1H, m, H_2-16a), 2.96 (1H, dd, *J* = 13.9, 7.5 Hz, H_2-7a), 2.86 (1H, dd, *J* = 13.9, 7.5 Hz, H_2-7b), 1.17 (3H, d, *J* = 7.0 Hz, H_3-3), 0.95–0.93 (18H, m, $2SiC(CH_3)_3$), 0.14–0.13 (12H, m, $2Si(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.6 (C-4), 172.3 (C-14), 166.6 (C-17), 156.7 (CO_2CH_2CH), 147.1 (C-10), 146.3 (C-11), 143.7 (C-FmocAr), 143.6 (C-FmocAr), 141.42 (C-FmocAr), 141.41 (C-FmocAr), 136.7 (C-23a), 129.2 (C-8), 128.0 (2C-FmocAr), 127.3 (2C-FmocAr), 125.9 (C-27a), 125.0 (2C-FmocAr), 123.5 (C-22), 123.2 (C-26), 122.1 (C-9 or C-13), 122.0 (C-9 or C-13), 121.2 (C-12), 120.2 (C-27 and 2C-FmocAr), 119.5 (C-19), 116.2 (C-25), 114.4 (C-24), 110.9 (C-21), 103.4 (C-20), 67.3 (CO_2CH_2CH), 54.6 (C-6), 51.3 (C-2), 47.2 (CO_2CH_2CH), 44.0 (C-16), 37.4 (C-7), 26.0 ($2SiC(CH_3)_3$), 18.6 ($SiC(CH_3)_3$), 18.5 ($SiC(CH_3)_3$), 18.2 (C-3), −4.0 ($2Si(CH_3)_2$); (+)-HRESIMS $[M+Na]^+$ 1016.3451 (calcd. for $C_{51}H_{64}Na^{79}BrN_5O_7Si_2$, 1016.3420).

*4.1.9. (S)-2-((S)-2-Aminopropanamido)-3-(3,4-bis((tert-butyldimethylsilyl)oxy)phenyl)-N-(2-((2-(6-bromo-1H-indol-3-yl)vinyl)amino)-2-oxoethyl)propanamide (**16 HF7-140**)*

Piperidine (0.23 mL, 20% in DMF) was added to **15 HF7-81** (47.0 mg, 0.0473 mmol) and the solution was stirred under N_2 at r.t. for 20 min. EtOAc (15 mL) was added and the mixture was washed with water (5 mL). The aqueous layer was further washed with EtOAc (3 x 15 mL) and then

the organic layers were combined and dried *in vacuo*. The crude product was purified by silica gel column chromatography (eluting with EtOAc to CH_2Cl_2/MeOH 9:1), to afford *Z*-**16 HF7-140Z** (13.0 mg, 36%) and *E*-**16 HF7-140E** (13.0 mg, 31%) as yellow oils.

*4.1.9.1. **Z-16 HF7-140Z***: R_f 0.71 (CH_2Cl_2/MeOH 9:1); $[\alpha]^{22.3}_D$ +22.1 (*c* 1.14, CH_2Cl_2); IR (ATR) ν_{max} 3280, 2931, 1632, 1508, 1472, 1422, 1295, 1252, 1161, 1127 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 9.20 (1H, br s, NH-23), 8.29 (1H, d, *J* = 10.4 Hz, NH-18), 7.78 (1H, d, *J* = 7.8 Hz, NH-5), 7.53 (1H, br s, H-24), 7.43 (1H, d, *J* = 8.1 Hz, H-27), 7.23 (2H, br s, H-22 and H-26), 7.01 (1H, br s, NH-15), 6.83 (1H, dd, *J* = 10.4, 9.6 Hz, H-19), 6.73 (1H, d, *J* = 7.9 Hz, H-12), 6.66 (1H, s, H-9), 6.56 (1H, d, *J* = 7.9 Hz, H-13), 5.91 (1H, d, *J* = 9.6 Hz, H-20), 4.60–4.57 (1H, m, H-6), 4.04 (1H, dd, *J* = 16.7, 4.8 Hz, H_2-16a), 3.81 (1H, d, *J* = 16.7 Hz, H_2-16b), 3.38–3.37 (1H, m, H-2), 2.98 (1H, dd, *J* = 13.6, 5.9 Hz, H_2-7a), 2.88–2.84 (1H, m, H_2-7b), 1.16 (3H, d, *J* = 6.4 Hz, H_3-3), 0.97 (18H, br s, 2SiC(CH_3)$_3$), 0.16 (12H, br s, 2Si(CH_3)$_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 176.5 (C-4), 172.6 (C-14), 166.5 (C-17), 147.1 (C-10), 146.3 (C-11), 136.7 (C-23a), 129.2 (C-8), 125.9 (C-27a), 123.4 (C-22 or C-26), 123.3 (C-22 or C-26), 122.22 (C-9 or C-13), 122.17 (C-9 or C-13), 121.3 (C-12), 120.3 (C-27), 119.7 (C-19), 116.4 (C-25), 114.4 (C-24), 111.1 (C-21), 103.3 (C-20), 54.3 (C-6), 50.7 (C-2), 43.9 (C-16), 37.3 (C-7), 26.1 (2SiC(CH_3)$_3$), 21.4 (C-3), 18.62 (2SiC(CH_3)$_3$), 18.55 (2SiC(CH_3)$_3$), −3.9 (2Si(CH_3)$_2$); (+)-HRESIMS $[M+Na]^+$ 794.2745 (calcd. for $C_{36}H_{54}{}^{79}BrNaN_5O_5Si_2$, 794.2739).

*4.1.9.2. **E-16 HF7-140E***: R_f 0.61 (CH_2Cl_2/MeOH 9:1); $[\alpha]^{22.6}_D$ +16.8 (*c* 0.83, CH_2Cl_2); IR (ATR) ν_{max} 3021, 2881, 1597, 1487, 1480, 1429, 1357, 1268, 1179, 1090 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 8.90 (1H, br d, *J* = 10.2 Hz, NH-18), 8.46 (1H, br s, NH-23), 7.93 (1H, br s, NH-5), 7.55 (1H, d, *J* = 8.5 Hz, H-27), 7.48 (1H, s, H-24), 7.32 (1H, dd, *J* = 14.7, 10.2 Hz, H-19), 7.18 (1H, dd, *J* = 8.5, 1.0 Hz, H-26), 7.10 (1H, s, H-22), 6.84 (1H, br s, NH-15), 6.77 (1H, d, *J* = 8.1 Hz, H-12), 6.70 (1H, d, *J* = 1.9 Hz, H-9), 6.65 (1H, dd, *J* = 8.1, 1.9 Hz, H-13), 6.48 (1H, d, *J* = 14.7 Hz, H-20), 4.30 (1H, ddd, *J* = 7.1, 7.1, 7.1 Hz, H-6), 4.13 (1H, dd, *J* = 16.9, 5.9 Hz, H_2-16a), 3.79 (1H, dd, *J* = 16.9, 5.9 Hz, H_2-16b), 3.51–3.48 (1H, m, H-2), 3.11 (1H, dd, *J* = 13.9, 7.1 Hz, H_2-7a), 2.93 (1H, dd, *J* = 13.9, 7.1 Hz, H_2-7b), 1.27 (3H, d, *J* = 7.0 Hz, H_3-3), 0.98–0.96 (18H, m, 2SiC(CH_3)$_3$), 0.18–0.17 (12H, m, 2Si(CH_3)$_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.3 (C-4), 172.0 (C-14), 166.3 (C-17), 147.2 (C-10), 146.3 (C-11), 137.5 (C-23a), 129.2 (C-8), 124.6 (C-27a), 123.4 (C-26), 122.6 (C-22), 122.1 (C-9 or C-13)*, 122.0 (C-9 or C-13)*, 121.4 (C-12), 121.0 (C-27), 120.4 (C-19), 116.0 (C-25), 114.4 (C-24), 113.4 (C-21), 107.0 (C-20), 56.3 (C-6), 50.7 (C-2), 43.3 (C-16), 36.5 (C-7), 26.1 (2SiC(CH_3)$_3$), 21.2 (C-3), 18.63 (SiC(CH_3)$_3$), 18.57 (SiC(CH_3)$_3$), −3.9 (2Si(CH_3)$_2$); (+)-HRESIMS $[M+Na]^+$ 794.2731 (calcd. for $C_{36}H_{55}{}^{79}BrNaN_5O_5Si_2$, 794.2739).

*4.1.10. (S)-1-(((S)-1-((2-(((Z)-2-(6-Bromo-1H-indol-3-yl)vinyl)amino)-2-oxoethyl)amino)-3-(3,4-dihydroxy phenyl)-1-oxopropan-2-yl)amino)-1-oxopropan-2-aminium 2,2,2-trifluoroacetate (**12 HF7-154**)*

A solution of Z-**16 HF7-140Z** (18.2 mg, 0.0236 mmol) in THF (2 mL) was stirred at 0 ºC under N_2 for 10 min before triethylamine trihydrofluoride (14.0 µL, 0.0856 mmol) was added dropwise. The solution was stirred for 55 min and then dried under a stream of N_2. Purification using C_2 column chromatography [eluting with H_2O/MeOH/TFA (99.99:0:0.01 to 59.99:39.99:0.02)] afforded **12 HF7-154** (10.4 mg, 67%) as a yellow oil. R_f 0.87 (butan-1-ol/acetic acid/H_2O 2:1:1); $[\alpha]^{22.4}_D$ +5.9 (*c* 0.66, MeOH); IR (ATR) ν_{max} 3253, 2923, 1668, 1532, 1456, 1200, 1129, 1023 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 11.46 (1H, s, NH-23), 9.22 (1H, d, *J* = 10.2 Hz, NH-18), 8.53 (1H, d, *J* = 8.5 Hz, NH-5), 8.39 (1H, dd, *J* = 5.7, 5.7 Hz, NH-15), 8.00–7.99 (3H, m, NH_3-1), 7.74 (1H, d, *J* = 2.4 Hz, H-22), 7.58 (1H, d, *J* = 1.7 Hz, H-24), 7.56 (1H, d, *J* = 8.4 Hz, H-27), 7.17 (1H, dd, *J* = 8.4, 1.7 Hz, H-26), 6.69–6.67 (2H, m, H-9 and H-19), 6.61 (1H, d, *J* = 8.1 Hz, H-12), 6.52 (1H, dd, *J* = 8.1, 2.0 Hz, H-13), 5.91 (1H, d, *J* = 9.5 Hz, H-20), 4.51 (1H, ddd, *J* = 4.4, 8.5, 9.6 Hz, H-6), 3.99 (1H, dd, *J* = 16.9, 5.7 Hz, H_2-16a), 3.93 (1H, dd, *J* = 16.9, 5.7 Hz, H_2-16b), 3.78–3.76 (1H, m, H-2), 2.89 (1H, dd, *J* = 13.9, 4.4 Hz, H_2-7a), 2.62 (1H, dd, *J* = 13.9, 9.6 Hz, H_2-7b), 1.32 (3H, d, *J* = 7.0 Hz, H_3-3); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 171.4 (C-14), 169.4 (C-4), 167.5 (C-17), 144.9 (C-10), 143.8 (C-11), 136.4 (C-23a), 128.4 (C-8), 125.7 (C-27a), 124.9 (C-22), 121.9 (C-26), 120.2 (C-27), 120.0 (C-13), 118.7 (C-19), 116.6 (C-9), 115.3 (C-12), 114.3 (C-25), 114.0 (C-24), 109.8 (C-21), 102.1 (C-20), 54.7 (C-6), 48.0 (C-2), 42.2 (C-16), 37.0 (C-7), 17.2 (C-3); (+)-HRESIMS $[M+H]^+$ 544.1173 (calcd. for $C_{24}H_{27}{}^{79}BrN_5O_5$, 544.1190).

*4.1.11. Fmoc-His(Trt)-Ala-OH (**17 HF8-62**)*

A solution of Fmoc-L-Ala-OH (0.93 g, 3.0 mmol) in CH_2Cl_2 (25 mL) and DMF (2 mL) was added to 2-chlorotrityl chloride resin (6 g, loading at 0.5 mmol/g), followed by DIPEA (0.52 mL, 3.0 mmol). After the resin mixture was agitated for 10 min, DIPEA (0.78 mL, 4.5 mmol) was added and the mixture was further shaken for 2 h. The solution was drained off and the resin was washed with DMF (30 mL). A solution of CH_2Cl_2/MeOH/DIPEA (20 mL, 80:15:5) was added to the mixture and shaken for 30 min. The solution was drained and the procedure was repeated. The resin was then washed with DMF (20 mL). Piperidine in DMF (15 mL, 1:4) was added to the resin mixture and shaken for 5 min. The liquid was drained off and piperidine washing was repeated for another 20 min. The amino acid-loaded resin was thoroughly washed with DMF (30 mL), isopropanol (30 mL) and *n*-hexane (30 mL). The resin was then dried under vacuum overnight. CH_2Cl_2 (40 mL) was added to the resin and left for 30 min. The solution was drained and a solution of HBTU (4.27 g, 11.25 mmol), HOBt (1.72 g, 11.25 mmol), Fmoc-His(Trt)-OH (4.65 g, 7.5 mmol) and DIPEA (2.61 mL, 15.0 mmol)

in DMF (11.3 mL) was added to the resin. The amino acid resin mixture was agitated for 5 h. The solution was then drained and washed with DMF (30 mL), isopropanol (30 mL) and *n*-hexane (30 mL). 2,2,2-Trifluroethanol in CH_2Cl_2 (25 mL, 1:4) was added to the amino acid-loaded resin and agitated for 1.5 h. The solution was drained and the organic solvent removed *in vacuo* to afford **17 HF8-62** (1.82 g, 88%) as a yellow foam. m.p. 144.1–145.0 ºC; R_f 0.55 (CH_2Cl_2/MeOH 9:1); $[\alpha]^{20.4}_D$ +10.7 (*c* 1.01, CH_2Cl_2); IR (ATR) ν_{max} 3318, 3063, 1720, 1652, 1526, 1493, 1447, 1237, 1044 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 8.53 (1H, br s, NH-10), 7.76 (2H, d, *J* = 7.4 Hz, 2H-FmocAr), 7.56 (1H, s, H-8), 7.53 (1H, d, *J* = 7.8 Hz, H-FmocAr), 7.51 (1H, d, *J* = 7.8 Hz, H-FmocAr), 7.39 (1H, t, *J* = 7.4 Hz, 2H-FmocAr), 7.29–7.25 (11H, m, 2H-FmocAr, 9H-TrtAr), 7.04–7.02 (6H, m, 6H-TrtAr), 6.67 (1H, s, H-8), 5.88 (1H, br s, NH-1), 4.88 (1H, t, *J* = 10.7 Hz, H-2), 4.47 (1H, dq, *J* = 6.6, 6.6 Hz, H-11), 4.38 (1H, dd, *J* = 9.4, 7.3 Hz, CO_2CH_2CH), 4.07 (1H, t, *J* = 7.3 Hz, CO_2CH_2CH), 3.98 (1H, dd, *J* = 9.4 Hz, CO_2CH_2CH), 3.39 (1H, d, *J* = 13.2 Hz, H-3a), 2.58 (1H, dd, *J* = 13.2, 10.7 Hz, H-3b), 1.54 (1H, d, *J* = 6.6 Hz, H-12); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 176.8 (C-13), 171.0 (C-9), 156.0 (CO_2CH_2CH), 144.2 (C-FmocAr), 143.9 (C-FmocAr), 141.6 (3C-TrtAr), 141.4 (2C-FmocAr), 137.8 (C-6), 135.0 (C-4), 129.8 (6C-TrtAr), 128.6 (3C-TrtAr), 128.4 (6C-TrtAr), 127.8 (2C-FmocAr), 127.3 (2C-FmocAr), 125.6 (C-FmocAr), 125.3 (C-FmocAr), 120.4 (C-8), 120.1 (2C-FmocAr), 76.3 (CAr_3), 67.1 (CO_2CH_2CH), 54.9 (C-2), 50.0 (C-11), 47.3 (CO_2CH_2CH), 32.8 (C-3), 19.2 (C-12); (+)-HRESIMS $[M+H]^+$ 691.2900 (calcd. for $C_{43}H_{39}N_4O_5$, 691.2915).

*4.1.12. Fmoc-Trt-[Ala^2]-halocyamine A (**18 HF8-87**)*

Fmoc-L-His(Trt)-L-Ala-OH **17 HF8-62** (0.243 g, 0.352 mmol), EDC.HCl (0.074 g, 0.387 mmol) and HOBt (0.071 g, 0.527 mmol) were dissolved in CH_2Cl_2 (1.5 mL) and stirred for 1 h at r.t. under N_2. A solution of enamide **4 HF3.1** (0.103 g, 0.352 mmol) in DMF (0.5 mL) was added to the mixture followed by DIPEA (0.31 mL, 1.76 mmol). The solution was stirred at r.t. under N_2 for 7 h. The solution was diluted with EtOAc (10 mL) and washed with water (5 mL). The organic layer was dried (MgSO4), filtered and the solvent removed in vacuo. The crude product was purified by silica gel flash column chromatography (eluting with *n*-hexane/EtOAc 8:2 to *n*-hexane/EtOAc/MeOH 5:4.5:0.5) to afford **18 HF8-87** (0.151 g, 44%) as a yellow oil. R_f 0.36 (EtOAc); $[\alpha]^{20.4}_D$ +15.3 (*c* 1.26, CH_2Cl_2); IR (ATR) ν_{max} 3303, 2160, 1653, 1494, 1448, 1230, 1041, 1002 cm^{-1}; ^1H NMR ($CHCl_3$, 300 MHz) δ 9.34 (1H, s, NH-22), 9.13 (1H, dd, *J* = 6.1, 5.6 Hz, NH-15), 8.23 (1H, d, *J* = 10.8 Hz, NH-17), 7.73–7.71 (2H, m, 2H-FmocAr), 7.52 (1H, d, *J* = 8.2 Hz, H-FmocAr), 7.49 (1H, d, *J* = 8.2 Hz, H-FmocAr), 7.47 (1H, s, H-23), 7.40–7.36 (3H, m, 2H-FmocAr and H-26), 7.34 (1H, s, H-6), 7.29–7.26 (9H, m, 9H-TrtAr), 7.25–7.24 (3H, m, 2H-FmocAr and H-21), 7.21–7.17 (1 H, m, H-25), 7.03–7.02 (6H, m, 6H-TrtAr), 6.76–6.69 (2H, m, NH-10 and H-18), 6.61 (1H, s, H-8), 6.08 (1H, d, *J* = 6.3 Hz, NH-1), 5.80 (1H, d, *J* = 9.2 Hz, H-19), 4.55–4.51 (1H, m, H-11), 4.36–4.33 (3H,

m, CO_2CH_2CH and H-2), 4.15 (1H, t, *J* = 6.3 Hz, CO_2CH_2CH), 4.04 (1H, dd, *J* = 16.5, 6.1 Hz, H_2-15a), 3.87 (1 H, dd, *J* = 16.5, 5.6 Hz, H_2-15b), 3.00–2.84 (2 H, m, H_2-3), 1.31 (3 H, d, *J* = 7.3 Hz, H_3-12); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.5 (C-13), 171.1 (C-9), 167.0 (C-16), 156.4 (CO_2CH_2CH), 143.8 (2 x C-FmocAr), 142.1 (3 x C-TrtAr), 141.4 (2 x C-FmocAr), 138.9 (C-6), 136.6 (C-22a), 135.5 (C-4), 129.7 (6 x C-TrtAr), 128.4 (3 x C-TrtAr), 128.3 (6 x TrtAr), 128.0 (2 x C-FmocAr), 127.2 (2 x C-FmocAr), 125.9 (C-26a), 125.1 (2 x C-FmocAr), 123.6 (C-21), 123.2 (C-25), 120.6 (C-8), 120.3 (C-26), 120.2 (2 x C-FmocAr), 119.4 (C-18), 116.1 (C-24), 114.4 (C-23), 111.0 (C-20), 103.0 (C-19), 75.7 (CAr_3), 67.3 (CO_2CH_2CH), 55.0 (C-2), 49.3 (C-11), 47.2 (CO_2CH_2CH), 43.9 (C-15), 31.0 (C-3), 17.8 (C-12); (+)-HRESIMS $[M+Na]^+$ 966.2957 (calcd. for $C_{55}H_{49}{}^{79}BrNaN_7O_5$, 966.2973).

*4.1.13. Trt-[Ala^2]-halocyamine A (**19 HF8-102**)*

A flask containing **18 HF8-87** (95.0 mg, 98.4 μmol) was flushed with N_2 before piperidine (20% in DMF, 1 mL) was added. The solution was stirred at r.t. for 20 min before EtOAc (20 mL) was added and the mixture was washed with water (10 mL). The aqueous layer was further washed with EtOAc (3 × 10 mL) and the organic layers were combined and dried *in vacuo*. Purification by silica gel column chromatography (eluting with EtOAc to CH_2Cl_2/MeOH 9:1), afforded **19 HF8-102** (62.8 mg, 86%) as a yellow oil. R_f 0.17 (CH_2Cl_2/MeOH 9:1); $[\alpha]^{20.7}{}_D$ -10.1 (*c* 0.24, CH_2Cl_2); IR (ATR) ν_{max} 3276, 1652, 1533, 1492, 1445, 1231, 1130, 1039 cm^{-1}; ^{1}H NMR ($CHCl_3$, 300 MHz) δ 9.80 (1H, s, NH-22), 8.76 (1H, dd, *J* = 5.8, 5.6 Hz, NH-14), 8.27 (1H, d, *J* = 10.2 Hz, NH-17), 7.48 (1H, d, *J* = 1.5 Hz, H-23), 7.38 (2H, d, J = Hz, H-6 and H-26), 7.32–7.28 (11H, m, NH-10, H-21 and 9H-TrtAr), 7.17 (1H, dd, *J* = 8.4, 1.5 Hz, H-25), 7.06–7.03 (6H, m, 6H-TrtAr), 6.74 (1H, dd, *J* = 10.2, 9.8 Hz, H-18), 6.64 (1H, s, H-8), 5.81 (1H, d, *J* = 9.8 Hz, H-19), 4.48–4.43 (1H, m, H-11), 4.05 (1H, dd, *J* = 16.9, 5.8 Hz, H_2-15a), 3.93 (1H, dd, *J* = 16.9, 5.6 Hz, H_2-15b), 3.66 (1H, s, H-2), 2.94 (1H, dd, *J* = 14.6, 4.8 Hz, H_2-3a), 2.84 (1H, dd, *J* = 14.6, 5.5 Hz, H_2-3b), 1.32 (3H, d, *J* = 7.2 Hz, H_3-12); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 174.7 (C-9), 173.6 (C-13), 167.1 (C-16), 142.0 (3C-TrtAr), 139.0 (C-6), 136.7 (C-4), 136.2 (C-22a), 129.7 (6C-TrtAr), 128.3 (6C-TrtAr), 128.2 (3C-TrtAr), 126.0 (C-26a), 123.8 (C-21), 123.1 (C-25), 120.5 (C-8), 120.2 (C-26), 119.3 (C-18), 116.0 (C-24), 114.4 (C-23), 110.8 (C-20), 103.2 (C-19), 75.7 (CAr_3), 54.6 (C-2), 49.4 (C-11), 43.8 (C-15), 33.1 (C-3), 17.8 (C-12); (+)-HRESIMS $[M+H]^+$ 744.2307 (calcd. for $C_{44}H_{39}{}^{79}BrN_7O_3$, 744.2292).

*4.1.14. [Ala^2]-halocyamine A (**13 HF8-141**)*

A cocktail solution of 0.01 N HCl/HFIP-TIS/H_2O (1 mL, 95:2.5:2.5) was added to **19 HF8-102** (62.8 mg, 0.0844 mmol) and stirred for 1 h. The reaction was dried under a stream of N_2. Subsequent purification by C_8 column chromatography (eluting with H_2O to H_2O/MeOH 25:75) afforded **13 HF8-141** as an inseparable 1*Z*:0.9*E* mixture (yellow oil) (39.9 mg, 83%).

The mixture had the following properties: R_f 0.59 (butan-1-ol/acetic acid/H_2O 2:1:1); $[\alpha]^{21.6}_D$ -0.6 (*c* 1.03, MeOH); IR (ATR) ν_{max} 3240, 2963, 1646, 1533, 1410, 1259, 1004 cm^{-1}; (+)-HRESIMS $[M+H]^+$ 502.1195 (calcd. for $C_{21}H_{25}{}^{79}BrN_7O_3$, 502.1197).NMR assignments for each of the isomers were discerned from 2D NMR data.

*4.1.14.1. Z-**13 HF8-141Z***: ^{1}H NMR ($CHCl_3$, 300 MHz) δ 11.54 (1H, s, NH-22), 9.06 (1H, d, *J* = 9.9 Hz, NH-17), 8.80 (1H, br, s, NH-14), 8.20 (1H, d, *J* = 4.4 Hz, NH-10), 7.71 (1H, s, H-21), 7.59–7.54 (3H, m, H-6, H-23 and H-26), 7.20–7.14 (1H, m, H-25), 6.86–6.85 (1H, m, H-8), 6.67 (1H, dd, *J* = 9.9, 9.7 Hz, H-18), 5.92 (1H, d, *J* = 9.7 Hz, H-19), 4.33–4.31 (1H, m, H-11), 3.97–3.94 (2H, m, H_2-15), 3.48–3.40 (1H, m, H-2), 2.92–2.83 (1H, m, H_2-3a), 2.77–2.62 (1H, m, H_2-3b), 1.24 (3H, d, *J* = 7.1 Hz, H_3-12); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 174.1 (C-9), 173.1 (C-13), 167.6 (C-16), 136.4 (C-22a), 135.0 (C-6), 125.7 (C-26a), 124.8 (C-21), 122.1 (C-25), 120.6 (C-26), 118.6 (C-18), 114.3 (C-24), 114.0 (C-23), 109.7 (C-20), 102.3 (C-19), 54.7 (C-2), 48.1 (C-11), 42.5 (C-15), 32.7 (C-3), 18.2 (C-12);

*4.1.14.2. E-**13 HF8-141E***: ^{1}H NMR ($CHCl_3$, 300 MHz) δ 11.26 (1H, s, NH-22), 9.71 (1H, d, *J* = 10.0 Hz, NH-17), 9.07–9.04 (1H, m, NH-14), 8.39 (1H, br, s, NH-10), 7.59–7.54 (3H, m, H-6, H-23 and H-26), 7.47 (1H, d, *J* = 2.0 Hz, H-21), 7.30 (1H, dd, *J* = 14.9, 10.0 Hz, H-18), 7.20–7.14 (1H, m, H-25), 6.86–6.85 (1H, m, H-8), 6.49 (1H, d, *J* = 14.9 Hz, H-19), 4.22–4.15 (1H, m, H-11), 3.86–3.84 (2H, m, H_2-15), 3.48–3.40 (1H, m, H-2), 2.92–2.83 (1H, m, H_2-3a), 2.77–2.62 (1H, m, H_2-3b), 1.28 (3H, d, *J* = 7.0 Hz, H_3-12); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 175.3 (C-9), 172.8 (C-13), 166.5 (C-16), 137.6 (C-22a), 134.9 (C-6), 124.3 (C-26a), 123.9 (C-21), 121.9 (C-25), 120.1 (C-26), 120.0 (C-18), 114.3 (C-24), 114.1 (C-23), 111.9 (C-20), 105.7 (C-19), 54.7 (C-2), 49.1 (C-11), 42.2 (C-15), 32.5 (C-3), 17.6 (C-12)

*4.1.15. Fmoc-L-Ala-L-Ala-OH (**22 HF8-58**)*

A solution of Fmoc-Ala-OH (0.93 g, 3.0 mmol) in CH_2Cl_2 (25 mL) was added to 2-chlorotrityl chloride resin (6 g, loading at 0.5 mmol/g), followed by DIPEA (0.52 mL, 3.0 mmol). After the resin mixture was agitated for 14 min, DIPEA (0.78 mL, 4.5 mmol) was added and the mixture was further shaken for 2.5 h. The solution was drained off and the resin was washed with DMF (20 mL). A solution of CH_2Cl_2/MeOH/DIPEA (40 mL, 80:15:5) was added to the mixture and shaken for 30 min. The solution was drained and the procedure was repeated. The resin was then washed with DMF (20 mL). Piperidine in DMF (15.0 mL, 1:4) was added to the resin mixture and shaken for 10 min. The liquid was drained off and piperidine washing was repeated for another 40 min. The amino acid-loaded resin was thoroughly washed with DMF (35 mL), isopropanol (35 mL) and *n*-hexane (35 mL). The resin was then dried under vacuum for 30 min and placed in a desiccator overnight. CH_2Cl_2 (50 mL) was added to the resin and left for 1 h. The solution was drained and a solution of HBTU (4.27

g, 11.25 mmol), HOBt (1.72 g, 11.25 mmol), Fmoc-Ala-OH (2.34 g, 7.5 mmol) and DIPEA (2.61 mL, 15.0 mmol) in DMF (11.3 mL) was added. The amino acid resin mixture was agitated for 4 h. The solution was then drained and washed with DMF (30 mL), isopropanol (30 mL) and *n*-hexane (30 mL). 2,2,2-Trifluroethanol in CH_2Cl_2 (15 mL, 1:4) was added to the amino acid-loaded resin and agitated for 2 h. The solution was drained and the organic solvent removed *in vacuo* to afford **22 HF8-58** (0.90 g, 78%) as a yellow foam. m.p. 195–196 ºC; R_f 0.50 (CH_2Cl_2/MeOH 9:1); $[\alpha]^{18.9}_D$ -28.9 (*c* 1.53, MeOH); IR (ATR) ν_{max} 3297, 2918, 1692, 1650, 1533, 1450, 1318, 1229 cm^{-1}; ^1H NMR ($CHCl_3$, 500 MHz) δ 8.09 (1H, d, *J* = 7.2 Hz, NH-5), 7.89 (2H, d, *J* = 7.4 Hz, 2H-FmocAr), 7.74 (1H, d, *J* = 7.4 Hz, 1H-FmocAr), 7.72 (1H, d, *J* = 7.4 Hz, 1H-FmocAr), 7.50 (1H, d, *J* = 7.5 Hz, NH-1), 7.41 (2H, t, *J* = 7.4 Hz, H-FmocAr), 7.33 (2H, td, *J* = 7.4, 0.9 Hz, 2H-FmocAr), 4.27–4.15 (4H, m, CO_2CH_2CH, CO_2CH_2CH, H-6), 4.08 (1H, d, *J* = 7.5, 7.2 Hz, H-2), 1.27 (3H, d, *J* = 7.3 Hz, H_3-7), 1.22 (3H, d, *J* = 7.2 Hz, H_3-3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 174.2 (C-8), 172.2 (C-4), 155.6 (CO_2CH_2CH), 143.9 (C-FmocAr), 143.8 (C-FmocAr), 140.7 (2C-FmocAr), 127.6 (2C-FmocAr), 127.1 (2C-FmocAr), 125.3 (2C-FmocAr), 120.1 (2C-FmocAr), 65.6 (CO_2CH_2CH), 49.7 (C-2), 47.5 (C-6), 46.7 (CO_2CH_2CH), 18.2 (C-3), 17.3 (C-7); (+)-HRESIMS $[M+Na]^+$ 405.1424 (calcd. for $C_{21}H_{22}NaN_2O_5$, 405.1421).

*4.1.16. Fmoc-[Ala[1],Ala[2]]-halocyamine A (**24 HF8-81**)*

Fmoc-L-Ala-L-Ala **22 HF8-58** (0.176 g, 0.461 mmol), EDC.HCl (0.097 g, 0.507 mmol) and HOBt (0.093 g, 0.691 mmol) was dissolved in DMF (1.5 mL) and the mixture was stirred for 1 h at r.t. under N_2. A solution of **4 HF3.1** (0.135 g, 0.461 mmol) in DMF (1.5 mL) was added to the mixture followed by DIPEA (0.40 mL, 2.30 mmol). The solution was stirred at r.t. under N_2 for 7 h. The solution was diluted with EtOAc (10 mL) and washed with water (5 mL). The organic layer was dried ($MgSO_4$), filtered and the solvent removed in vacuo. The crude product was purified by silica gel flash column chromatography (eluting with *n*-hexane/EtOAc 8:2 to *n*-hexane/EtOAc/MeOH 5:4.5:0.5) to afford **24 HF8-81** (0.10 g, 33%) as a yellow oil. R_f 0.34 (EtOAc); $[\alpha]^{21.1}_D$ -6.7 (*c* 0.36, MeOH); IR (ATR) ν_{max} 3019, 2978, 1595, 1488, 1451, 754 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 11.43 (1H, s, NH-17), 9.13 (1H, d, *J* = 10.0 Hz, NH-12), 8.14 (1H, t, *J* = 5.7 Hz, NH-9), 8.03 (1H, d, *J* = 7.4 Hz, NH-5), 7.88 (2H, d, *J* = 7.3 Hz, 2H-FmocAr), 7.73–7.40 (3H, m, 2H-FmocAr and H-16), 7.58–7.54 (2H, m, H-18 and H-21), 7.52 (1H, d, *J* = 7.7 Hz, NH-1), 7.41 (2H, t, *J* = 7.3 Hz, 2H-FmocAr), 7.32 (2H, t, *J* = 7.3 Hz, 2H-FmocAr), 7.16 (1H, dd, *J* = 8.4, 1.7 Hz, H-20), 6.66 (1H, dd, *J* = 10.0, 9.7 Hz, H-13), 5.91 (1H, d, *J* = 9.7 Hz, H-14), 4.35–4.31 (1H, m, H-6), 4.27–4.18 (3H, m, CO_2CH_2CH and CO_2CH_2CH), 4.10–4.05 (1H, m, H-2), 3.94 (2H, d, *J* = 5.7 Hz, H_2-10), 1.23 (3H, d, *J* = 6.7 Hz, H_3-7), 1.21 (3H, d, *J* = 6.9 Hz, H_3-3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 172.7 (C-8), 172.2 (C-4), 167.5 (C-11), 155.7 (CO_2CH_2CH), 143.9 (C-FmocAr), 143.8 (C-FmocAr), 140.7 (2C-

FmocAr), 136.4 (C-17a), 127.6 (2C-FmocAr), 127.1 (2C-FmocAr), 125.7 (C-21a), 125.3 (2C-FmocAr), 124.9 (C-16), 121.9 (C-20), 120.1 (2C-FmocAr and C-21), 118.7 (C-13), 114.3 (C-19), 114.0 (C-18), 109.8 (C-15), 102.2 (C-14), 65.6 ($CO_2\mathit{C}H_2CH$), 50.0 (C-2), 48.1 (C-6), 46.6 ($CO_2CH_2\mathit{C}H$), 42.3 (C-10), 18.2 (C-3 or C-7), 18.1 (C-3 or C-7); (+)-HRESIMS $[M+Na]^+$ 680.1474 (calcd. for $C_{33}H_{32}{}^{79}BrNaN_5O_5$, 680.1479).

*4.1.17. [Ala[1],Ala[2]]-halocyamine A (**20 HF8-100**)*

To **24 HF8-81** (19.0 mg, 0.0289 mmol) was added piperidine (20% in DMF, 1 mL) and flushed with nitrogen. The solution was stirred at r.t. for 20 min before EtOAc (20 mL) was added and the mixture was washed with water (10 mL). The aqueous layer was further washed with EtOAc (3 × 10 mL) and the organic layers were combined and dried *in vacuo*. Purification by silica gel column chromatography (eluting with EtOAc to CH_2Cl_2/MeOH 8:2), afforded **20 HF8-100** (7.54 mg, 60%) as a yellow oil. R_f 0.20 (CH_2Cl_2/MeOH 8:2); $[\alpha]^{19.4}{}_D$ +1.6 (*c* 0.26, MeOH); IR (ATR) ν_{max} 3393, 2258, 1655, 1048, 1024 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 11.49 (1H, br s, NH-17), 9.11 (1H, d, *J* = 10.1 Hz, NH-12), 8.26 (1H, d, *J* = 5.7 Hz, NH-9), 8.08 (1H, br s, NH-5), 7.70 (1H, s, H-16), 7.58 (1H, d, *J* = 1.8 Hz, H-18), 7.56 (1H, d, *J* = 8.4 Hz, H-21), 7.16 (1H, dd, *J* = 8.4, 1.8 Hz, H-20), 6.66 (1H, dd, *J* = 10.1, 9.7 Hz, H-13), 5.91 (1H, d, *J* = 9.7 Hz, H-14), 4.33 (1H, d, *J* = 7.2 Hz, H-6), 3.93 (2H, d, *J* = 5.7 Hz, H_2-10), 3.29 (1H, obs, H-2), 1.23 (3H, d, *J* = 7.2 Hz, H_3-3), 1.13 (3H, d, *J* = 6.9 Hz, H_3-3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 175.1 (C-4), 172.9 (C-8), 167.5 (C-11), 136.4 (C-17a), 125.6 (C-21a), 124.9 (C-16), 121.9 (C-20), 120.1 (C-21), 118.7 (C-13), 114.3 (C-19), 114.0 (C-18), 109.7 (C-15), 102.2 (C-14), 50.1 (C-2), 47.7 (C-6), 42.3 (C-10), 21.1 (C-3), 18.5 (C-7); (+)-HRESIMS $[M+H]^+$ 436.0970 (calcd. for $C_{18}H_{23}{}^{79}BrN_5O_3$, 436.0979).

*4.1.18. Fmoc-D-Ala-D-Ala-OH (**23 HF8-86**)*

The synthesis of **23 HF8-86** used Fmoc-D-Ala-OH and the same procedure as described for the synthesis of Fmoc-L-Ala-L-Ala-OH (**22 HF8-58**), to give the product (0.45 g, 79%) as a white solid. m.p. 196–197 ºC; R_f 0.23 (CH_2Cl_2/MeOH 9:1); $[\alpha]^{18.2}{}_D$ +19.7 (*c* 0.35, MeOH); IR (ATR) ν_{max} 3293, 2990, 1688, 1653, 1534, 1450, 1319, 1262 cm^{-1}; ^1H NMR ($CHCl_3$, 400 MHz) δ 12.52 (1H, br s, OH), 8.13 (1H, d, *J* = 7.2 Hz, NH-5), 7.89 (2H, d, *J* = 7.4 Hz, 2H-FmocAr), 7.73 (1H, d, *J* = 7.4 Hz, 1H-FmocAr), 7.72 (1H, d, *J* = 7.4 Hz, 1H-FmocAr), 7.50 (1H, d, *J* = 7.5 Hz, NH-1), 7.41 (2H, t, *J* = 7.4 Hz, H-FmocAr), 7.32 (2H, td, *J* = 7.4, 0.9 Hz, 2H-FmocAr), 4.26–4.17 (4H, m, $CO_2C\mathit{H}_2CH$, $CO_2CH_2C\mathit{H}$, H-6), 4.08 (1H, d, *J* = 7.5, 7.2 Hz, H-2), 1.27 (3H, d, *J* = 7.3 Hz, H_3-7), 1.22 (3H, d, *J* = 7.2 Hz, H_3-3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.0 (C-8), 172.3 (C-4), 155.6 ($\mathit{C}O_2CH_2CH$), 143.9 (C-FmocAr), 143.8 (C-FmocAr), 140.7 (2C-FmocAr), 127.6 (2C-FmocAr), 127.1 (2C-FmocAr), 125.3 (2C-FmocAr), 120.1 (2C-FmocAr), 65.6 ($CO_2\mathit{C}H_2CH$), 49.7 (C-2), 47.4 (C-6), 46.6

(CO_2CH_2CH), 18.2 (C-3), 17.2 (C-7); (+)-HRESIMS $[M+Na]^+$ 405.1423 (calcd. for $C_{21}H_{22}NaN_2O_5$, 405.1421).

*4.1.19. Fmoc-[D-Ala[1],D-Ala[2]]-halocyamine A (**25 HF8-95**)*

Fmoc-D-Ala-D-Ala **23 HF8-86** (0.102 g, 0.266 mmol), EDC.HCl (0.056 g, 0.293 mmol) and HOBt (0.054 g, 0.399 mmol) were dissolved in DMF (1.5 mL) and stirred at r.t. for 1 h under nitrogen. A solution of **4 HF3.1** (0.078 g, 0.266 mmol) in DMF (1.5 mL) was then added followed by DIPEA (0.23 mL, 1.33 mmol). The solution was further stirred at r.t. for 7 h. EtOAc (10 mL) was added to the mixture and washed with water (5 mL). The organic layer was dried ($MgSO_4$), filtered and the solvent removed in vacuo. The crude product was purified by silica gel flash column chromatography (eluting with *n*-hexane/EtOAc 8:2 to *n*-hexane/EtOAc/MeOH 5:4.5:0.5) to afford **25 HF8-95** (60.0 mg, 34%) as a yellow oil. R_f 0.21 (n-hexane/EtOAc/MeOH 5:4.5:0.5); $[\alpha]^{21.5}_D$ +5.6 (*c* 0.32, MeOH); IR (ATR) ν_{max} 3367, 2974, 1656, 1494, 1447, 1231, 1130 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 11.43 (1H, s, NH-17), 9.13 (1H, d, *J* = 9.9 Hz, NH-12), 8.14 (1H, d, *J* = 5.8 Hz, NH-9), 8.03 (1H, d, *J* = 7.7 Hz, NH-5), 7.89 (2H, d, *J* = 7.2 Hz, 2H-FmocAr), 7.74–7.70 (3H, m, 2H-FmocAr and H-16), 7.58–7.54 (2H, m, H-18 and H-21), 7.52 (1H, d, *J* = 7.1 Hz, NH-1), 7.41 (2H, t, *J* = 7.2 Hz, 2H-FmocAr), 7.33 (2H, t, *J* = 7.2 Hz, 2H-FmocAr), 7.16 (1H, dd, *J* = 8.5, 1.8 Hz, H-20), 6.67 (1H, d, *J* = 9.9, 9.7 Hz, H-13), 5.91 (1H, d, *J* = 9.7 Hz, H-14), 4.35–4.31 (1H, m, H-6), 4.27–4.21 (3H, m, CO_2CH_2CH and CO_2CH_2CH), 4.10–4.05 (1H, m, H-2), 3.94 (2H, d, *J* = 5.8 Hz, H_2-10), 1.23 (3H, d, *J* = 6.9 Hz, H_3-7), 1.21 (3H, d, *J* = 7.2 Hz, H_3-3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 172.7 (C-8), 172.2 (C-4), 167.4 (C-11), 155.7 (CO_2CH_2CH), 143.9 (C-FmocAr), 143.8 (C-FmocAr), 140.7 (2C-FmocAr), 136.4 (C-17a), 127.6 (2C-FmocAr), 127.1 (2C-FmocAr), 125.7 (C-21a), 125.3 (2C-FmocAr), 124.8 (C-16), 121.9 (C-20), 120.1 (2C-FmocAr and C-21), 118.7 (C-13), 114.3 (C-19), 114.0 (C-18), 109.7 (C-15), 102.1 (C-14), 65.6 (CO_2CH_2CH), 49.9 (C-2), 48.0 (C-6), 46.6 (CO_2CH_2CH), 42.3 (C-10), 18.2 (C-3 or C-C-7), 18.1 (C-3 or C-C-7); (+)-HRESIMS $[M+Na]^+$ 680.1464 (calcd. for $C_{33}H_{32}{}^{79}BrNaN_5O_5$, 680.1479).

*4.1.20. [D-Ala[1],D-Ala[2]]-halocyamine A (**21 HF8-136**)*

Piperidine (20% in DMF, 1 mL) was added to **25 HF8-95** (34.2 mg, 0.52 mmol) and stirred at r.t. under N_2 for 20 min. EtOAc (20 mL) was added and the mixture was washed with water (10 mL). The aqueous layer was further washed with EtOAc (3 × 10 mL) and the organic layers were combined and dried *in vacuo*. Purification by silica gel column chromatography (eluting with EtOAc to CH_2Cl_2/MeOH 8:2), afforded **21 HF8-136** (15.7 mg, 69%) as a clear film. R_f 0.22 (CH_2Cl_2/MeOH 8:2); $[\alpha]^{21.4}_D$ -0.5 (*c* 1.59, CH_2Cl_2); IR (ATR) ν_{max} 3274, 2921, 2854, 1646, 1532, 1449, 1398, 1235, 1054 cm^{-1}; ^1H NMR (CD_3OD, 300 MHz) δ 7.55 (1H, d, *J* = 1.5 Hz, H-18), 7.51 (1H, d, *J* = 0.8 Hz,

H-16), 7.47 (1H, d, *J* = 8.5 Hz, H-21), 7.18 (1H, dd, *J* = 8.5, 1.5 Hz, H-20), 6.76 (1H, d, *J* = 9.2 Hz, H-13), 6.00 (1H, dd, *J* = 9.2, 0.76 Hz, H-14), 4.36 (1H, d, *J* = 7.1 Hz, H-6), 3.97 (2H, s, H_2-10), 3.48 (1H, d, *J* = 6.9 Hz, H-2), 1.33 (3H, d, *J* = 7.1 Hz, H_3-7), 1.26 (3H, d, *J* = 6.9 Hz, H_3-3); ^{13}C NMR (CD_3OD, 75 MHz) δ 177.0 (C-3), 175.7 (C-8), 169.4 (C-11), 138.4 (C-17a), 127.2 (C-21a), 125.5 (C-16), 123.6 (C-20), 121.0 (C-21), 120.0 (C-13), 116.4 (C-19), 115.3 (C-18), 111.4 (C-15), 105.5 (C-14), 51.2 (C2), 50.4 (C-6), 43.9 (C-10), 20.6 (C-3), 17.9 (C-7); (+)-HRESIMS $[M+Na]^+$ 458.0800 (calcd. for $C_{18}H_{22}{}^{79}BrNaN_5O_3$, 458.0798).

4.2. Antibacterial assays[ref10]

The antibacterial activity of the compounds was studied by determination of minimum inhibitory concentrations (MICs) using the standard broth dilution method in accordance with the NCCLS guidelines M7-A2. Briefly, the MICs were determined with an inoculum of 10^5 CFU in 200 μL of MH broth containing twofold serial dilutions of each drug. The MIC was defined as the lowest concentration of drug that completely inhibited visible growth after incubation for 18 h at 37 °C. To determine all MICs, the measurements were independently repeated at least three times. Minimum inhibitory concentration of positive control: colistin [*P. aeruginosa* (1 μM), *E. coli* (2 μM)], streptomycin [*P. aeruginosa* (21.5 μM), *E. coli* (21.5 μM), *S. aureus* (21.5 μM), and *E. faecalis* (21.5 μM)] and chloramphenicol [*S. aureus* (1.5–3 μM), and *E. faecalis* (1.5–3 μM)].

4.3. In vitro cytotoxicity assay[ref11]

Assays were performed in 96-well microtiter plates, each well containing 100 μL of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum, and 4 × 10^4 L6 cells (a primary cell line derived from rat skeletal myoblasts). Serial drug dilutions of seven 3-fold dilution steps covering a range from 90 to 0.123 μg/mL were prepared. After 72 h of incubation, the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Alamar Blue solution (10 μL) was then added to each well and the plates incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analysed using the microplate reader software Softmax Pro. Podophyllotoxin was the reference drug used.

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Supplementary data

Copies of ^{1}H and ^{13}C NMR spectra for all products. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/. These data include MOL files and InChiKeys of the most important compounds described in this article.

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