Influence of Chain Length on the Activity of Tripeptidomimetic Antagonists for CXC Chemokine Receptor 4 (CXCR4)

Markus Baumann[a], Mohammad Musarraf Hussain[a†], Nina Henne[a], Daniel Moya Garrote[a], Stefanie Karlshøj[b], Torgils Fossen[a], Mette M. Rosenkilde[b], Jon Våbenø[c*], Bengt Erik Haug[a*]

[a] Department of Chemistry and Centre for Pharmacy, University of Bergen, Allégaten 41, NO-5007 Bergen, Norway.
[b] Laboratory for Molecular Pharmacology, Department of Neuroscience and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen, Denmark.
[c] Department of Pharmacy, Faculty of Health Sciences, UiT The Arctic University of Norway, Breivika, NO-9037 Tromsø, Norway.

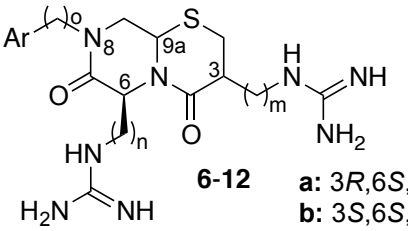
[†]Current affiliation:
MMH: Department of Pharmacy, Jagannath University, Dhaka 1100, Bangladesh.

Keywords: CXCR4 antagonist, peptidomimetic, scaffold

Abstract

Here we report a series of close analogues of our recently published scaffold-based tripeptidomimetic CXCR4 antagonists, containing positively charged guanidino groups in R^1 and R^2, and an aromatic group in R^3. While contraction/elongation of the guanidine carrying side chains (R^1 and R^2) resulted in loss of activity, introduction of bromine in position 1 on the naphth-2-ylmethyl moiety (R^3) resulted in an EC_{50} of 61 μM (mixture of diastereoisomers) against wild-type CXCR4; thus, the antagonistic activity of these tripeptidomimetics seems to be amenable to optimization of the aromatic moiety. Moreover, for analogues carrying a naphth-2-ylmethyl substituent, we observed that a Pictet-Spengler like cyclization side reaction depended on the nature of the R^1 substituent.

Graphical abstract



6-12 **a:** 3*R*,6*S*,9a*S*
b: 3*S*,6*S*,9a*R*

1. Introduction

The G protein coupled CXC chemokine receptor 4 (CXCR4) and its only known endogenous ligand, the 68-residue CXC chemokine ligand 12 (CXCL12), are involved in development and chemotaxis of leucocytes and lymphocytes,[1-3] as well as in embryogenesis by participating in vascularization of the gastrointestinal tract,[4] neural development[5] and haematopoiesis.[6] Since the CXCR4/CXCL12 axis also has been shown to have a pathological role in HIV, cancer and rheumatoid arthritis[7] CXCR4 is an attractive drug target, and several small-molecule CXCR4 antagonists have been described in the literature.[7-9] Plerixafor, the only small-molecule CXCR4 antagonist that has been approved so far, is used for mobilization of hematopoietic stem cells prior to autologous bone marrow transplantation in patients with Hodgkin disease, non-Hodgkin's lymphoma and multiple myeloma.[10, 11] Other small molecule CXCR4 antagonists are undergoing clinical trials; among them is the orally available AMD11070, which is investigated for its efficacy against HIV infection.[12]

In addition to the small-molecule antagonists, a large number of potent peptide antagonists for CXCR4 have been identified.[13-17] From a drug discovery perspective, a series of head-to-tail cyclized pentapeptides originally reported by Fujii and co-workers,[18] i.e. cyclo(-L/D-Arg^1-Arg^2-2-Nal^3-Gly^4-D-Tyr^5-) (**1** and **2**, Figure 1A) represents a particularly attractive starting point. Extensive SAR studies of **1** and **2**[19] indicate that the main activity of the cyclopentapeptide CXCR4 antagonists resides in the L/D-Arg^1-Arg^2-2-Nal^3 tripeptide fragment, which again suggests that tripeptidomimetic compounds based on this motif are interesting as small-molecule CXCR4 antagonists. We have recently reported a novel class of Arg-Arg-2-Nal tripeptidomimetic CXCR4 antagonists,[20] which were based on the synthetically accessible bicyclic scaffold A (3,6,8-trisubstituted 4,7-dioxo-1-thia-5,8-diaza-bicyclo[4.4.0]decane, Figure 1B).[21] Our prototype compounds **3a** and **3b** (Figure 1C) were shown to have EC_{50} values of 64 μM and 80 μM, respectively,[20] and in the current study we have investigated the immediate SAR of these hit compounds by synthesizing close analogues with varying length of the three side chains and different aromatic moieties in the R^3-side chain.

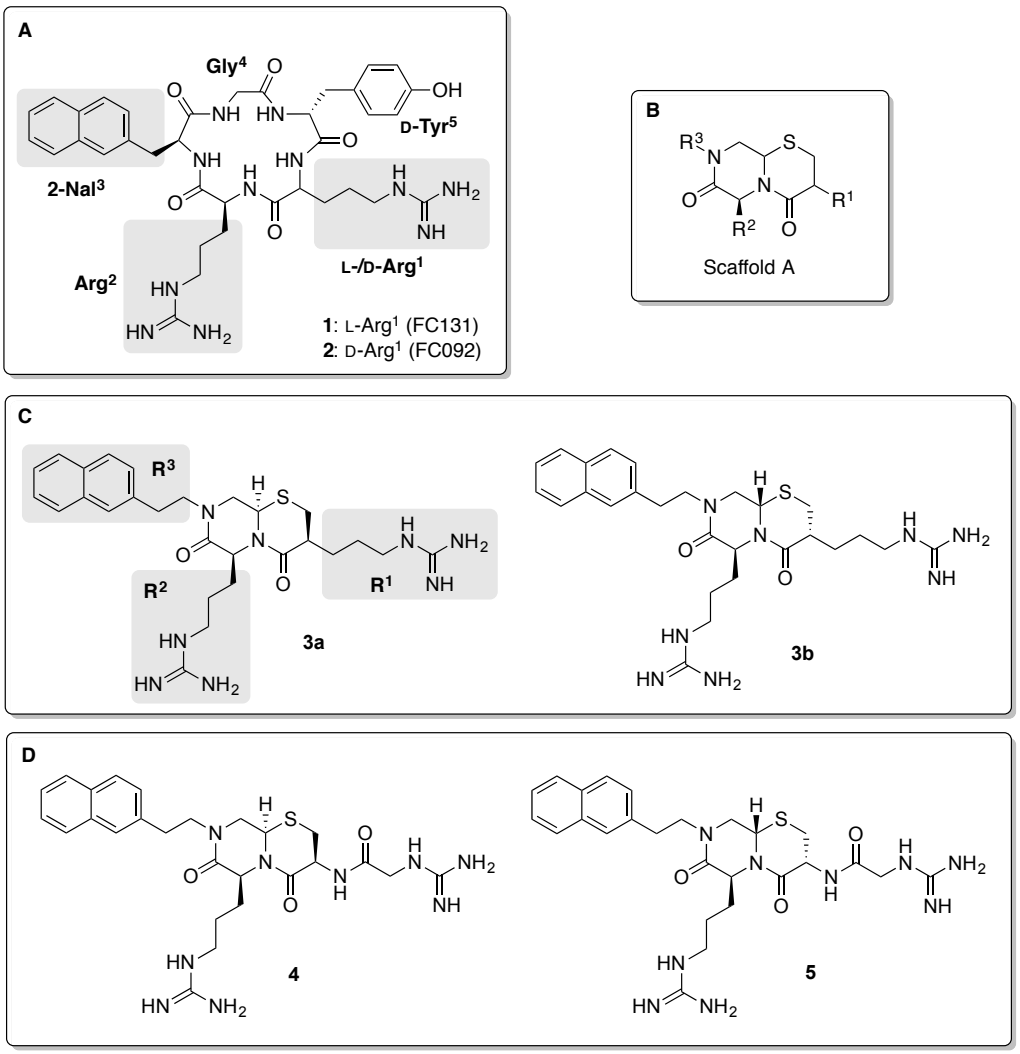


Figure 1. Structures of (A) the cyclic pentapeptide CXCR4 antagonists **1** and **2** reported by Fujii *et al.*[18]; (B) the bicyclic scaffold A employed for our tripeptidomimetic CXCR4 antagonists; (C) the active tripeptidomimetic hit compounds **3a** and **3b**[20]; (D) the inactive analogues **4** and **5** containing an amide bond in the R^1-side chain.[20]

2. Results and discussion

2.1. Design and SAR – Our prototype hit compounds **3a** and **3b** contain Arg-side chains in R^1 and R^2 and a 2-naphthyl group with an ethylene spacer in R^3 (Figure 1C). Analogues with an amide group in the R^1 side chain can be conveniently synthesized by employing appropriately protected Cys residues as building blocks; however, since we previously found **4** and **5** (Figure 1D) to be inactive,[20] this strategy was abandoned. Instead, our synthetic strategy for the target compounds **6–12** in the current work (Table 1) was based on a racemic building block for introduction of the R^1-side chain (see Chemistry below), giving both the (3*R*,6*S*,9a*S*) and the (3*S*,6*S*,9a*R*) diastereoisomer (**a** and **b**, respectively). The antagonistic potency of **6–12** on human CXCR4 was determined by a functional assay as previously described.[22]

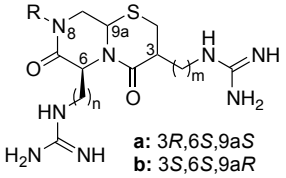
First, compounds with two (**6a** and **6b**) and four carbon atoms (**7a** and **7b**) in the R^1 side chain were synthesized in order to determine the importance of side chain length. However, none of these four analogues showed biological activity, demonstrating that contraction/elongation of the R^1 side chain is unfavourable.

The length of the R^2 side chain was then increased by one carbon atom (**8a** and **8b**), but this elongation resulted in loss of biologic activity. Compounds **9a** and **9b**, where a shorter R^1 side chain was incorporated to compensate the longer R^2 side chain, were also included; however, these compounds were similarly inactive.

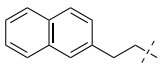
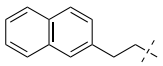
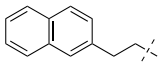
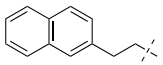
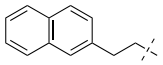
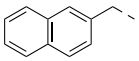
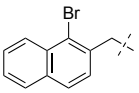
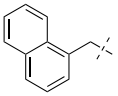
Next, the R^3 side chain spacer was shortened to one carbon atom (**10a**/**10b** and **11a**/**11b**). Neither diastereoisomers of **10** or **11** were separable by preparative RP-HPLC, thus both were tested as the respective diastereomeric mixtures. Compound **10a,b** did not show biological activity; however, the C-1 bromine-substituted analogue **11a,b** had an EC_{50} value of 61 μM. The bromine was introduced to avoid a suspected Pictet-Spengler type side reaction in the synthesis of these analogues (see Chemistry below), and since this substitution simultaneously affects the steric, electronic, and hydrophobic properties of the aromatic moiety, it is difficult to isolate the exact cause of the difference in activity between **10** and **11**. Still, the fact that **11a,b** is equipotent with **3b** indicates that there is room for further optimization of the R^3-side chain.

Finally, we included **12a** and **12b**, which are the 1-substituted isomers of **10a** and **10b**. Compounds **12a** and **12b** were also tested as a mixture of diastereoisomers and did not show biological activity.

Table 1. Structures of compounds **3** and **6-12** and antagonistic potency on human CXCR4.

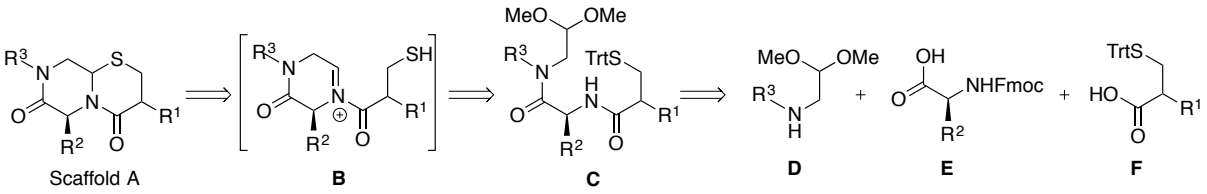


a: 3*R*,6*S*,9a*S*
b: 3*S*,6*S*,9a*R*

Compd	m (R^1)	n (R^2)	R^3	log EC_{50} ± SEM	EC_{50} (µM)
3a[a]	3	3		−4.20 ± 0.12	64
3b[a]				−4.10 ± 0.31	80
6a	2	3		> −4	>100
6b				> −4	>100
7a	4	3		> −4	>100
7b				> −4	>100
8a	3	4		> −4	>100
8b				> −4	>100
9a	2	4		> −4	>100
9b				> −4	>100
10a, 10b[b]	3	3		> −4	>100
11a, 11b[b]	3	3		−4.21 ± 0.02	61
12a, 12b[b]	3	3		> −4	>100

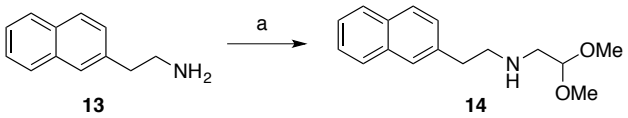
[a] Known compounds; biological data taken from Zachariassen *et al.*[20]; [b] Inseparable diastereoisomers, tested as the diastereomeric mixture.

2.2. Chemistry – All target compounds in this study are based on the 6,6-fused bicyclic ring system of scaffold A (Scheme 1), which can be prepared from linear precursor **C**. Synthesis of the linear precursors requires the coupling of three building blocks (**D** – **F**) bearing each of the side chains. Treatment of the linear precursor **C** with a TFA cocktail liberates the aldehyde from the dimethyl acetal, which is then attacked by the amide nitrogen to form the *N*-acyliminium ion **B**. Finally, nucleophilic attack by the thiol results in the formation of the bicyclic ring system.



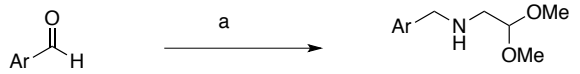
Scheme 1. Retrosynthetic strategy for synthesis of the bicylic scaffold A.

For the synthesis of **6a**,**b** – **9a**,**b** the required secondary amine **14** carrying the R^3 side chain was prepared by alkylation of primary amine **13** using bromoacetaldehyde dimethyl acetal in DMF (Scheme 2).



Scheme 2. Synthesis of **14**. *Reagents and conditions:* (a) $BrCH_2CH(OMe)_2$, K_2CO_3, DMF, 80 °C (52%).

Towards the synthesis of bicycles **10a**,**b**, **11a,b** and **12a,b** secondary amines **18-20** were prepared from the appropriate aldehydes **15-17** by reductive amination through reaction with 2,2-dimethoxyethylamine followed by reduction with $NaBH_4$ (Scheme 3).

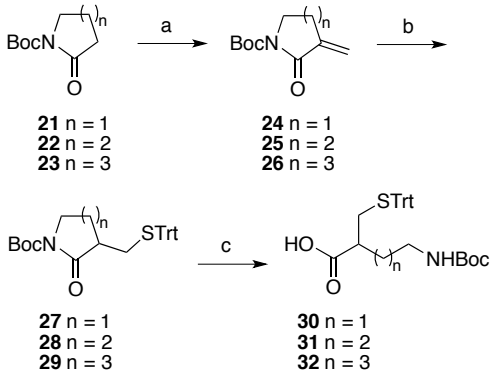


15 Ar = Naphth-2-yl
16 Ar = 1-Bromo-naphth-2-yl
17 Ar = Naphth-1-yl

18 Ar = Naphth-2-yl
19 Ar = 1-Bromo-naphth-2-yl
20 Ar = Naphth-1-yl

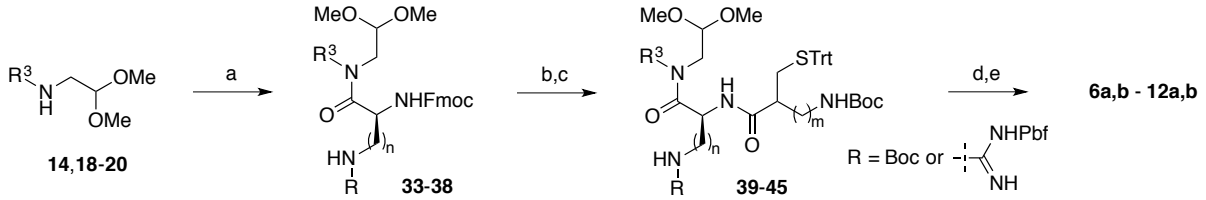
Scheme 3. Reductive aminations. *Reagents and conditions:* (b) i) $NH_2CH_2CH(OMe)_2$, 90-100 °C; ii) $NaBH_4$, rt (44-87%).

The different building blocks bearing the R^1 side chain were prepared starting from Boc-protected lactams **21-23** (Scheme 4). α-Methylenation through release of trifluoroacetate[23] gave Michael acceptors **24-26**, which after reaction with Ph_3CSH using Et_3N as base gave the racemic Michael products **27-29** in good yields. Subsequent hydrolysis with LiOH gave the racemic carboxylic acids **30-32**.



Scheme 4. Synthesis of carboxylic acid building blocks. *Reagents and conditions:* (a) 1. $CF_3CO_2CH_2CF_3$, LiHMDS, 20 – 30 minutes, rt; 2. $(CH_2O)_n$, toluene, 80 °C, 2 h (41 – 57% over 2 steps); (b) Ph_3CSH, Et_3N, CH_2Cl_2, rt, 23 – 44 h (39 – 59%); (c) 1 M aq LiOH, THF, rt, 1 – 3 h (64 – 81%).

The linear precursor were prepared by coupling the secondary amines **14**, **18**, **19** and **20** with Fmoc-Arg(Pbf)-OH, Fmoc-Orn(Boc)-OH or Fmoc-Lys(Boc)-OH to give coupling products **33-38** (Scheme 5). Next, the Fmoc-group was removed using diethylamine and the free amines were coupled with the racemic carboxylic acids **30, 31** or **32** to yield the linear precursors **39-45** as inseparable diastereoisomeric mixtures. Further, treatment with a mixture of TFA, thioanisole and water lead to global deprotection and formation of the bicyclic ring system.[24] Finally, the R^1 side chain amines (also R^2 for **40** and **41**) were guanidinylated to give the final crude products.

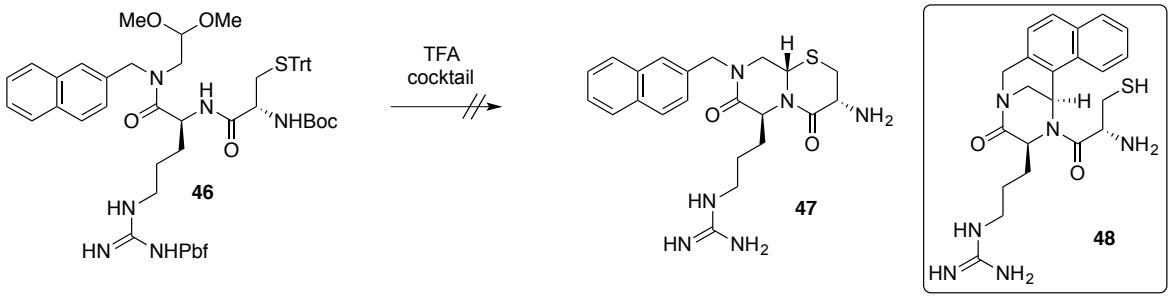


Scheme 5. Synthesis of bicycles. *Reagents and conditions:* (a) Fmoc-Arg(Pbf)-OH, Fmoc-Orn(Boc)-OH or Fmoc-Lys(Boc)-OH, HATU, DIPEA, CH_2Cl_2 or DMF, rt (70-85%); (b) Et_2NH, CH_2Cl_2, rt; (c) HATU or HBTU, DIPEA, CH_2Cl_2, rt (17%-75% over two steps); (d) TFA/thioanisole/H_2O, rt; (e) 1*H*-pyrazole-1-carboxamidene x HCl, DIPEA, DMF (6-31% over 2 steps).

All final compounds were purified by RP-HPLC, however the diastereoisomers of **10**, **11** and **12** proved for all practical purposes to be inseparable. For **6-9**, the two diastereoisomers that were formed were separable by RP-HPLC and analysis of the purified compounds by NMR spectroscopy clearly showed that they were single diastereoisomers. As the stereochemistry at C6 was fixed through incorporation of appropriately protected (*S*)-Arg, (*S*)-Orn or (*S*)-Lys (Scheme 5), the configuration at C3 and C9a (see Tables 1 and S1) could be determined by the 2D ^{1}H ROESY experiment as described previously.[20, 25] For all compounds that were

isolated as single diastereoisomers, strong cross-peaks between H3 and H9a were observed. Further, for the diastereoisomer that displayed the longest retention time of the pairs, strong cross-peaks between H9a and Hβ/Hγ of the R^2 side-chain were always observed (see Table S1 for further details). These findings indicate that all compounds have the same configurations as reported for hit compounds **3a** and **3b**,[20] and that the (3*R*,6*S*,9a*S*) diastereoisomer (**6a-9a**) always eluted first and the (3*S*,6*S*,9a*R*) diastereoisomer (**6b-9b**) always eluted last from the C_{18}-column for the separable diastereoisomeric pairs.

The bromine of **11a,b** was originally introduced as a protecting group as we observed a side reaction in an attempted synthesis of bicycle **47** (Scheme 6).

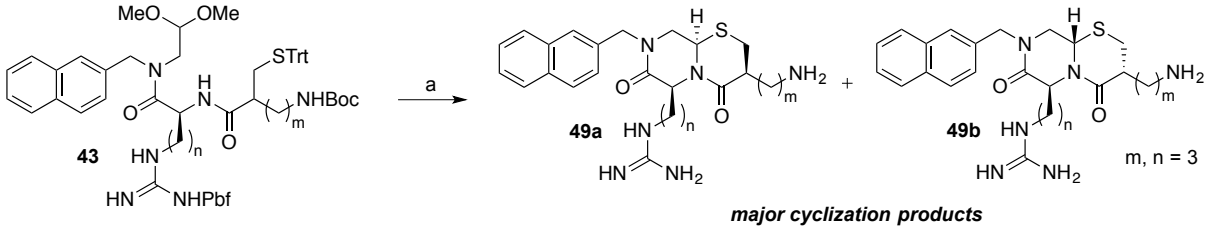


Scheme 6. Attempted synthesis of **47**.

In our attempted synthesis of compound **47** (see SI for experimental details), compound **48** was instead the only product that could be isolated in pure form. Inspection of the ^{1}H NMR spectrum of the isolated compound quickly revealed that it was not the desired product as the signals in the aromatic region integrated to 6 protons instead of 7. The combined information obtained from the 2D ^{1}H-^{13}C HMBC and HSQC spectra revealed the presence of four quaternary carbon signals in the aromatic region. The structure of **48** was elucidated by extensive use of 2D NMR. Thus, it would seem that after formation of the *N*-acyliminium ion **B** (Scheme 1), the cyclization can take two different paths; either the thiol attacks the carbon atom in the double bond to yield the desired cyclization product,[21] or an aromatic substitution reaction, i.e. a Pictet Spengler-type reaction, takes place.[26] The 2D ^{1}H-^{1}H ROESY spectrum of **48** revealed no ROE between the bridge-head proton and the R^2 side chain, thus the nucleophilic attack on the acyliminium ion has occurred from the same side as the R^2 side chain.

In order to avoid the Pictet-Spengler product, bromine was introduced in the 1-position of the naphthalene group by starting with 1-bromo-2-naphthaldehyde (**16**) when preparing the building block bearing the R^3 side chain (Scheme 3). We envisioned that the bromine could

be removed from the cyclized product by treatment with hydrogen gas in the presence of Pd/C.[27] The protection of the naphthalene proved however to be unnecessary, as the cyclization pathway was found to be highly dependent on the nature of the R^1 side chain (see SI for details) and cyclization of linear precursors **43**, which contains a naphth-2-ylmethyl R^3 side chain and a three carbon spacer in the R^1 side chain, gave the desired bicycles as the major products (Scheme 7). Similar chemoselectivity was observed when the R^1 side chain was omitted (see SI for details), indicating that the electron withdrawing ammonium substituent (or Boc-protected amine if Boc deprotection is slower than cyclization) of the N-acyliminium ion formed from **46** (Scheme 6) decreases the nucleophilicity of the thiol.



Scheme 7. Successful cyclization. *Reagents and conditions:* (a) TFA/thioanisole/H_2O (90:5:5)

3. Conclusions

In summary, we have investigated close analogues of our earlier reported hit compounds based on a tripeptidomimetic scaffold for CXCR4 antagonists by varying the spacer length for the three side chains. Our results suggests that a three-carbon spacer is optimal for both of the guanidine carrying side chains, while the aromatic group in the R^3 position can still be optimized. Interestingly, we found that a side reaction of analogues carrying a naphth-2-ylmethyl substituent through a Pictet-Spengler like cyclization reaction was dependent on the nature of the R^1 substituent. Finally, introducing bromine in position 1 on the naphtha-2-ylmethyl moiety gave a mixture of inseparable diastereoisomers with an EC_{50} of 61 μM against wild-type CXCR4, thus the antagonistic activity of these tripeptidomimetics seems to be amenable to changes on the aromatic moiety.

4. Experimental

4.1. Chemistry - All reagents and starting materials were purchased from Sigma-Aldrich and used as delivered unless otherwise stated. Boc-protected lactam **22** was prepared following a literature procedure.[28] Anhydrous toluene and THF were obtained from an anhydrous solvent delivery system (SDS-800 from mBraun) at the Department of Chemistry, University of

Bergen. CH_2Cl_2 was dried over molecular sieves. For analysis by thin layer chromatography, aluminium sheets coated with Merck KGaA silica gel (60 F_{254}) were used. The TLC plates were visualized using either ultraviolet light or by immersion in a solution of 2% ninhydrin in ethanol supplemented with 10 drops of concentrated sulphuric acid pr 100 mL solution, followed by heating. Purification by flash column chromatography was performed on Merck KGaA Kieselgel (230 – 400 mesh). All final compounds were purified by semi-preparative RP-HPLC eluting with mixtures of acetonitrile and H_2O (both containing 0.1% TFA). Fractions of equal purity were pooled and lyophilized. All tested compounds were analysed by RP-HPLC and found to be of > 95% purity (sum of isomers for inseparable diastereoisomers).

4.1.1. 2,2-Dimethoxy-*N*-(2-(naphthalen-2-yl)ethyl)ethan-1-amine (**14**)

To a stirring solution of 2-(naphthalen-2-yl)ethan-1-amine (0.39 g, 2.28 mmol) in anhydrous DMF (10 mL), K_2CO_3 (0.32 g, 2.34 mmol) and bromoacetaldehyde dimethyl acetal (0.38 mL, 3.33 mmol) were added and the resulting suspension was then heated at 80 °C for 26 h. The reaction was quenched with H_2O (20 mL), extracted with EtOAc (3 x 20 mL), washed with brine (20 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure to give the crude product as an orange oil (475 mg). The crude product was purified by flash column chromatography on silica gel (EtOAc/MeOH 9:1) to give the title compound as a yellow oil (307 mg, 52%). R_f (EtOAc/MeOH 9:1) = 0.15; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.82-7.75 (m, 3H), 7.65 (s, 1H), 7.48 – 7.40 (m, 2H), 7.34 (dd, J = 8.4, 1.7, 1H), 4.48 (t, J = 5.5, 1H), 3.35 (s, 6H), 2.99 (s, 4H), 2.80 (d, J = 5.5, 2H), 2.17 (bs, 1H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 137.3, 133.7, 132.2, 128.2, 127.7, 127.5, 127.3, 127.1, 126.1, 125.4, 103.8, 54.2, 51.2, 51.1, 36.5; HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{16}H_{22}NO_2$: 260.1651; found: 260.1651.

4.1.2. 2,2-Dimethoxy-*N*-(naphthalen-2-ylmethyl)ethan-1-amine (**18**)

A mixture of 2-naphthaldehyde (845 mg, 5.4 mmol) and aminoacetaldehyde dimethyl acetal (0.6 mL, 5.5 mmol) was heated at 100 °C for 20 min and then cooled to room temperature. The reaction mixture was then diluted with EtOH (4 mL) and $NaBH_4$ (207 mg, 5.5 mmol) was added in small portions to the stirring mixture. The reaction mixture was stirred for 22 h before the EtOH was removed and the residue was portioned between H_2O (10 mL) and EtOAc (40 mL). The organic phase was washed with H_2O (2 x 10 mL), dried over $MgSO_4$, filtered and concentrated to give the crude product as yellow oil (1.262 g). Purification by

flash column chromatography on silica gel (EtOAc/MeOH 92.5:7.5) gave the title compound as yellow oil (893 mg, 67%). R_f (EtOAc/MeOH 92.5:7.5) = 0.31; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.83 – 7.74 (m, 4H), 7.47 – 7.40 (m, 3H), 4.50 (t, J = 5.5, 1H), 3.97 (s, 2H), 3.36 (s, 6H), 2.79 (d, J = 5.5, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 137.6, 133.5, 132.7, 128.1, 127.7, 127.7, 126.6, 126.5, 126.0, 125.6, 104.0, 54.0, 54.0, 50.6; HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{15}H_{20}NO_2$: 246.1494; found: 246.1497.

4.1.3. *N*-((1-Bromonaphthalen-2-yl)methyl)-2,2-dimethoxyethan-1-amine (**19**)

The title compound was prepared from 1-bromo-2-naphthaldehyde (202 mg, 0.9 mmol) following the protocol described for **18** above. The crude product (yellowish oil, 243 mg) was purified by flash column chromatography on silica gel (EtOAc) to give the title compound as yellowish oil (124 mg, 44%). R_f (EtOAc) = 0.27; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.33 (dd, 8.5, 1H), 7.85 – 7.76 (m, 2H), 7.62 – 7.56 (m, 1H), 7.55 – 7.48 (m, 2H), 4.52 (t, J = 5.5, 1H), 4.14 (s, 2H), 3.37 (s, 6H), 2.80 (d, J = 5.5, 2H), 1.80 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 137.4, 134.0, 132.6, 128.2, 127.8, 127.7, 127.5, 127.3, 126.4, 124.0, 103.9, 54.7, 54.0, 50.4; HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{15}H_{19}O_2N^{79}Br$: 324.0599; found: 324.0599; HRMS (ESI): m/z $[M + Na]^+$ calcd for $C_{15}H_{18}O_2N^{79}BrNa$: 346.0419; found: 346.0415.

4.1.4. 2,2-Dimethoxy-*N*-(naphthalene-1-ylmethyl)ethanamine (**20**)

The title compound was prepared from 1-naphthaldehyde (0.55 mL, 4.0 mmol) following the protocol described for **18** above, except that the temperature in the first step was 90 °C. The crude product (red/brown oil, 855 mg, 87%) was used without further purification. R_f (EtOAc) = 0.23; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.12 (d, J = 8.3, 1H), 7.89 – 7.70 (m, 2H), 7.55 – 7.36 (m, 4H), 4.51 (t, J = 5.5, 1H), 4.25 (s, 2H), 3.35 (s, 6H), 2.86 (d, J = 5.5, 2H), 1.62 (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 135.9, 134.1, 132.0, 128.9, 128.0, 126.3, 126.2, 125.8, 125.6, 123.8, 104.1, 54.1, 51.7, 51.2; HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{15}H_{20}NO_2$: 246.1489; found: 246.1493.

4.1.5. *tert*-Butyl 3-methylene-2-oxopyrrolidene-1-carboxylate (**24**)

A solution of **21** (0.27 mL, 1.58 mmol) in dry THF (7 mL) was transferred to a stirring solution of LiHMDS (1 M in THF, 3.24 mL, 3.24 mmol) at 0 °C via cannulation. The reaction mixture was allowed to warm up to room temperature over 38 minutes before 2,2,2-trifluoroethyl trifluoroacetate (0.40 mL, 2.99 mmol) was added. Stirring was continued for additional 20 minutes at room temperature before the reaction was quenched with saturated

NH_4Cl (25 mL) and extracted with EtOAc (3 x 30 mL). The combined organic phases were dried over $MgSO_4$, filtered and concentrated to yield a dark yellow oil (305 mg). The oil was dissolved in dry toluene (24 mL) and paraformaldehyde (1.18 g, 40.3 mmol) and K_2CO_3 (472 mg, 3.41 mmol) were added to the solution. The reaction mixture was heated at 108 °C for 2 h before quenched with saturated NH_4Cl (40 mL) and extracted with EtOAc (3 x 30 mL), dried over $MgSO_4$, filtered and concentrated to give the crude product as yellow oil (312 mg). Purification by flash column chromatography on silica gel (hexanes/EtOAc 2:1) gave the title compound (178 mg, 57%). R_f (hexanes/EtOAc 2:1) = 0.25; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.19 (t, J = 2.8, 1H), 5.48 (t, J = 2.5, 1H), 3.73 (t, J = 7.3, 2H), 2.78 – 2.72 (m, 2H), 1.55 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 166.6, 151.0, 139.2, 120.0, 83.2, 43.1, 28.2, 23.3; HRMS (ESI): m/z $[M + Na]^+$ calcd for $C_{10}H_{15}NO_3Na$: 220.0950; found: 220.0949; m/z $[2M + Na]^{2+}$ calcd $C_{20}H_{30}N_2O_6Na$: 417.2002; found: 417.2025.

4.1.6. *tert*-Butyl 3-methylene-2-oxopiperidine-1-carboxylate (**25**)

The title compound was prepared from **22** (933 mg, 4.7 mmol) following the protocol described for **24** above, except that stirring in the second step was carried out at 80 °C for 2 h (instead of 108 °C for 2 h). The crude product (yellow oil, 568 mg) was purified by flash column chromatography on silica gel (hexanes/EtOAc 4:1) to give the title compound as colorless oil (404 mg, 41%). R_f = (hexanes/EtOAc 4:1) = 0.26. Analytical data were in accordance with those reported previously.[20]

4.1.7. *tert*-Butyl 3-methylene-2-oxoazepane-1-carboxylate (**26**)

The title compound was prepared from **23** (0.30 mL, 1.4 mmol) following the protocol described for **24** above, except that stirring in the second step was carried out at 80 °C for 2 h (instead of 108 °C for 2 h). The crude product (yellow oil, 0.3674 g) was purified by flash column chromatography on silica gel (Hexane/EtOAc 1:1) to give the title product as colorless oil (301 mg, 57%). R_f = (hexanes/EtOAc 1:1) = 0.64; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.77 (d, J = 1.3, 1H), 5.39 (d, J = 1.3, 1H), 3.71 – 3.64 (m, 2H), 2.47 – 2.39 (m, 2H), 1.85 – 1.61 (m, 4H), 1.53 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): 172.6, 152.8, 147.3, 123.3, 82.7, 46.1, 32.6, 29.2, 28.19, 28.15; HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{12}H_{20}O_3N$: 226.1443; found: 226.1441.

4.1.8. *tert*-Butyl 2-oxo-3-((tritylthio)methyl)pyrrolidine-1-carboxylate (**27**)

To a stirring solution of **24** (251 mg, 1.27 mmol) in dry CH_2Cl_2 (20 mL) was added Et_3N (0.4 mL, 2.87 mmol) and triphenylmethanethiol (705 mg, 2.55 mmol). The reaction mixture was stirred for 24 h at room temperature before it was washed with H_2O (20 mL). The solvent was removed under reduced pressure to yield the crude product as yellow oil (1.26 g). Purification by flash column chromatography on silica gel (hexanes/EtOAc 4:1) gave the title compound as white foam (237 mg, 39%). R_f (hexanes/EtOAc 4:1) = 0.24; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.44 – 7.39 (m, 6H), 7.31 – 7.25 (m, 6H), 7.23 – 7.18 (m, 3H), 3.63 (ddd, J = 11.1, 8.6, 2.3, 1H), 3.49 – 3.41 (m, 1H), 2.78 (dd, J = 12.1, 4.0, 1H), 2.45 – 2.34 (m, 1H), 2.20 (dd, J = 12.0, 10.4, 1H), 2.14 – 2.05 (m, 1H), 1.49 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 172.9, 149.4, 143.7, 128.7, 127.1, 125.9, 82.1, 66.0, 43.4, 42.4, 31.7, 27.2, 23.1; HRMS (ESI): m/z = $[M+Na]^+$ calcd for $C_{29}H_{31}NO_3SNa$: 496.1922; found: 496.1933.

4.1.9. *tert*-Butyl 2-oxo-3-(tritylthiomethyl)piperidine-1-carboxylate (**28**)

The title compound was prepared from **25** (379 mg, 1.8 mmol) following the protocol described for **27** above (44 h reaction time). The crude product (yellow oil, 1.52 g) was purified by flash column chromatography on silica gel (hexanes/EtOAc 4:1) to give the title compound as transparent oil (348 mg, 40%). R_f (hexanes/EtOAc 4:1) = 0.32. Analytical data were in accordance with those reported previously.[20]

4.1.10. *tert*-Butyl 2-oxo-3-((tritylthio)methyl)azepane-1-carboxylate (**29**)

The title compound was prepared from **23** (301 mg, 1.3 mmol) following the protocol described for **27** above (26 h reaction time). The crude product (1.03 g) was purified by flash column chromatography on silica gel (hexanes/EtOAc 8.5:1.5) to give the title compound as white foam (398 mg, 59%). R_f (hexanes/EtOAc 8.5:1.5) = 0.26; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.51 – 7.45 (m, 6H), 7.31 – 7.24 (m, 6H), 7.22 – 7.17 (m, 3H), 4.12 – 4.03 (m, 1H), 2.96 – 2.82 (m, 2H), 2.21 (dd, J = 13.7, 7.5, 1H), 1.80 – 1.66 (m, 3H), 1.60 – 1.52 (m, 1H), 1.49 (s, 9H), 1.39 – 1.17 (m, 3H); ^{13}C NMR (151 MHz, $CDCl_3$): δ = 175.8, 153.2, 145.0, 129.8, 128.0, 126.7, 83.0, 67.0, 46.2, 45.1, 34.2, 30.0, 28.3, 28.2, 28.1; HRMS (ESI): m/z $[M + Na]^+$ calcd for $C_{31}H_{35}NO_3SNa$: 524.2235; found: 524.2237.

4.1.11. 4-((*tert*-Butoxycarbonyl)amino)-2-((tritylthio)methyl)butanoic acid (**30**)

To a solution of **27** (237 mg, 0.50 mmol) in THF (10 mL) was added aqueous LiOH (1 M, 3.0 mL, 3.0 mmol) and the resulting mixture was stirred at room temperature for 5 h. The solvent was removed under reduced pressure and the aqueous residue was acidified to pH 4 with 10%

citric acid. The mixture was extracted with CH_2Cl_2 (20 mL and 3 x 20 mL), washed with brine (30 mL), dried over $MgSO_4$, filtered and concentrated to give the crude product (209 mg). The crude product was purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH 95:5) gave the title compound as white foam (156 mg, 64%). R_f (CH_2Cl_2/MeOH 95:5) = 0.23; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.43 – 7.39 (m, 6H), 7.31 – 7.25 (m, 6H), 7.23 – 7.18 (m, 3H), 4.56 (bs, 1H), 3.02 – 2.91 (m, 2H), 2.69 – 2.58 (m, 1H), 2.26 – 2.18 (m, 1H), 2.07 – 1.98 (m, 1H), 1.75 – 1.49 (m, 2H), 1.42 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 178.7, 156.4, 144.7, 129.7, 128.1, 126.9, 79.8, 67.1, 42.8, 38.3, 33.4, 32.3, 28.5; HRMS (ESI): *m*/*z* $[M + Na]^+$ calcd for $C_{29}H_{33}NO_4SNa$: 514.2028; found: 514.2027.

4.1.12. 5-(*tert*-Butoxycarbonylamino)-2-((tritylthio)methyl)pentanoic acid (**31**)

The title compound was prepared from lactam **28** (348 mg, 0.7 mmol) following the protocol described for **30** above (3 h reaction time). The crude product (yellow foam, 326 mg) was purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH 95:5) to give the title compound as yellow oil (245 mg, 68%). R_f (CH_2Cl_2/MeOH 95:5) = 0.30. Analytical data were in accordance with those reported previously.[20]

4.1.13. 6-(*tert*-Butoxycarbonylamino)-2-(tritylthiomethyl)hexanoic acid (**32**)

The title compound was prepared from lactam **29** (364 mg, 0.73 mmol) following the protocol described for **30** above, except the reaction time was 1.5 h and the pH was adjusted to ~ 3 (instead of 4) during the aqueous work-up. The crude product (yellow oil, 369 mg) was purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH 95:5) to give the title compound as white foam (306 mg, 81%). R_f (CH_2Cl_2/MeOH 95:5) = 0.31; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.43 – 7.39 (m, 6H), 7.30 – 7.24 (m, 6H), 7.22 – 7.17 (m, 3H), 4.49 (bs, 1H), 3.05 – 2.92 (m, 2H), 2.60 (dd, *J* = 12.5, 8.2, 1H), 2.23 – 2.16 (m, 1H), 2.10 – 2.01 (m, 1H), 1.43 (s, 9H), 1.40 – 1.24 (m, 4H), 1.14 – 1.02 (m, 2H); ^{13}C NMR (150.9 MHz, $CDCl_3$); δ = 179.9, 156.1, 144.7, 129.8, 128.1, 126.8, 79.3, 67.1, 45.1, 40.4, 33.1, 31.5, 29.8, 28.6, 24.2; HRMS (ESI): *m*/*z* $[M + Na]^+$ calcd for $C_{31}H_{37}NO_4SNa$: 542.2341; found: 542.2343.

4.1.14. General protocol A: Coupling of the R^2 and R^3 fragments

To a stirring solution of the secondary amine (1.40 mmol) in dry CH_2Cl_2 (5 mL) was added Fmoc-protected amino acid (1.40 mmol), HATU (545, 1.43 mmol) and DIPEA (0.75 mL, 4.31 mmol). The reaction mixture was stirred for 23 h before the solvent was removed and the residue was partitioned between H_2O (10 mL) and EtOAc (10 mL). The aqueous phase was

extracted with EtOAc (2 x 10 mL) and the combined organic phases were washed with 1 M $KHSO_4$ (20 mL), H_2O (20 mL), saturated $NaHCO_3$ (20 mL) and brine (20 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated to yield the crude product, which was purified by flash column chromatography on silica gel.

4.1.15. (9*H*-fluoren-9-yl)methyl (*S*)-(1-((2,2-dimethoxyethyl)(2-(naphthalen-2-yl)ethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)carbamate (**33**)

The title compound was prepared from secondary amine **14** (362 mg, 1.40 mmol) and Fmoc-Arg(Pbf)-OH (911 mg, 1.40 mmol) following general protocol A. The crude product (yellow foam, 1.190 g) was purified by flash column chromatography on silica gel (EtOAc/hexanes 8:2) to give the title compound as white foam (1.060 g, 85%). R_f (EtOAc/hexanes 8:2) = 0.23; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m/z* $[M + H]^+$ calcd for $C_{50}H_{60}N_5O_8S$: 890.4163; found: 890.4163; *m/z* $[M + Na]^+$ calcd for $C_{50}H_{59}N_5O_8SNa$: 912.3982; found 912.3972.

4.1.16. (9*H*-fluoren-9-yl)methyl *tert*-Butyl (5-((2,2-dimethoxyethyl)(2-(naphthalen-2-yl)ethyl)amino)-5-oxopentane-1,4-diyl)(*S*)-dicarbamate (**34**)

The title compound was prepared from secondary amine **14** (307 mg, 1.18 mmol) and Fmoc-Orn(Boc)-OH (539 mg, 1.19 mmol) following general protocol A. The crude product was purified by flash column chromatography on silica gel (EtOAc/hexane 6:4) to give the title compound as white foam (678 mg, 82%, retains EtOAc). R_f (EtOAc/hexane 6:4) = 0,31; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m/z* $[M + Na]^+$ calcd for $C_{41}H_{49}N_3O_7Na$: 718.3468; found: 718.3457.

4.1.17. (9*H*-fluoren-9-yl)methyl *tert*-butyl (6-((2,2-dimethoxyethyl)(2-(naphthalen-2-yl)ethyl)amino)-6-oxohexane-1,5-diyl)(*S*)-dicarbamate (**35**)

To a stirring solution of Fmoc-Lys(Boc)-OH (551 mg, 1.2 mmol) in anhydrous DMF (2.3 mL) was added HATU (447 mg, 1.2 mmol) and DIPEA (0.21 mL, 1.2 mmol). The reaction mixture was stirred for 30 min at room temperature. Secondary amine **14** (305 mg, 1.2 mmol) in anhydrous DMF (1.5 mL) was added and the reaction mixture was stirred for 20 h at room temperature. The solvent was removed and the aqueous work-up was carried out as described for general protocol A. The crude product (890 mg) was purified by flash column chromatography on silica gel (EtOAc/hexanes 8.5:1.5) to give the title compound as pale

yellow foam (678 mg, 71%). R_f (EtOAc/hexanes 8.5:1.5) = 0.58; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m/z* $[M + Na]^+$ calcd for $C_{42}H_{51}N_3O_7Na$: 732.3625; found: 732.3627.

4.1.18. *(9H*-Fluoren-9-yl)methyl (*S*)-(1-((2,2-dimethoxyethyl)(naphthalen-2-ylmethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)carbamate (**36**)

The title compound was prepared from secondary amine **18** (847 mg, 3.45 mmol) and Fmoc-Arg(Pbf)-OH (2.29 g, 3.53 mmol) following general protocol A (17 h reaction time). The crude product (yellow foam, 3.68 g) was purified by flash column chromatography on silica gel (EtOAc/hexanes 9:1) to give the title compound as white foam (2.110 g, 70%). R_f (EtOAc/hexanes 9:1) = 0.39; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m/z* [M + H] calcd for $C_{49}H_{58}N_5O_8S$: 876.4006; found: 876.4008.

4.1.19. (9*H*-fluoren-9-yl)methyl (*S*)-(1-(((1-bromonaphthalen-2-yl)methyl)(2,2-dimethoxyethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)carbamate (**37**)

The title compound was prepared from secondary amine **19** (99 mg, 0.3 mmol) and Fmoc-Arg(Pbf)-OH (414 mg, 0.6 mmol) following general protocol A. The crude product (yellowish foam, 485 mg) was purified by flash column chromatography on silica gel (EtOAc/hexanes, 77:23) to give the title compound as white foam (214 mg, 77%). R_f (EtOAc/hexanes 8:2) = 0.38; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m/z* $[M + H]^+$ calcd for $C_{49}H_{57}O_8N_5BrS$: 954.3111; found: 954.3113; HRMS (ESI): *m/z* $[M + Na]^+$ calcd for $C_{49}H_{56}O_8N_5BrSNa$: 978.2910; found: 978.2925.

4.1.20. (9*H*-fluoren-9-yl)methyl (*S*)-(1-((2,2-dimethoxyethyl)(naphthalen-1-ylmethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)carbamate (**38**)

HATU (411 mg, 1.1 mmol) and DIPEA (0.19 mL, 1.1 mmol) were added to a stirred solution of Fmoc-Arg(Pbf)-OH (636 mg, 1.0 mmol) in dry DMF (3 mL) under an argon atmosphere. The mixture was stirred for 30 minutes at room temperature before secondary amine **20** (265 mg, 1.1 mmol) in dry DMF (2 mL) was added drop wise to the reaction mixture, and stirring continued for 16 hr. The reaction mixture was partitioned between H_2O (10 mL) and EtOAc

(10 mL), and the organic phase was further washed with H_2O (2 x 15 mL) and saturated NaCl, dried over $MgSO_4$, filtered and concentrated to give the crude product. Purification by flash column chromatography (EtOAc/hexanes 4:1) gave the title compound as white foam (545 mg, 64%, retains EtOAc). R_f (EtOAc/hexanes 4:1) = 0.24; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m*/*z* $[M + Na]^+$ calcd for $C_{49}H_{57}N_5O_8SNa$: 898.3826; found: 898.3823.

4.1.21. General protocol B: Fmoc-deprotection

To a stirring solution of the Fmoc-protected amine (0.06 M in CH_2Cl_2) was added Et_2NH (50 equiv) and the resulting mixture was stirred at room temperature for 2-3 h (monitored by TLC). The solvent was removed under reduced pressure and the residue was used in the next step without purification.

4.1.22. General protocol C: Formation of linear precursors

To a stirring solution the carboxylic acid carrying the R^1 side chain (0.31 mmol) in dry CH_2Cl_2 (2 ml) was added HATU (128 mg, 0.34 mmol) and DIPEA (0.15 ml, 0.86 mmol). The reaction mixture was stirred at room temperature for 40 minutes. Fmoc-deprotected amine (see general protocol B, 0.32 mmol) in dry CH_2Cl_2 (12 mL) was added and the stirring continued for 42 h. The solvent was evaporated to give a red residue, which was partitioned between H_2O (10 mL) and EtOAc (10 mL). The aqueous phase was extracted with EtOAc (3 x 10 mL) and the organic phases were combined and washed with 1 M $KHSO_4$ (20 mL), H_2O (20 mL), saturated $NaHCO_3$ (20 mL) and brine (20 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated to yield the crude product, which was purified by flash column chromatography on silica.

4.1.23. *tert*-Butyl (4-(((*S*)-1-((2,2-dimethoxyethyl)(2-(naphthalen-2-yl)ethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)amino)-4-oxo-3-((tritylthio)methyl)butyl)carbamate (**39**)

The title compound was prepared from carboxylic acid **30** (154 mg, 0.31 mmol) and Fmoc-deprotected amine **33** (see general protocol B, 0.32 mmol) following general protocol C. The crude product (red foam, 460 mg) was purified by flash column chromatography on silica gel (EtOAc/hexanes 8:2) to give an inseparable mixture of the two diastereoisomers of **39** (240 mg, 67%). R_f (EtOAc/hexanes 8:2) = 0.22; NMR analyses did not give useful information due

to formation of rotamers; HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{64}H_{81}N_6O_9S_2$: 1141.5506; found: 1141.5525; *m*/*z* [M + Na]$^+$ calcd for $C_{64}H_{80}N_6O_9S_2Na$: 1163.5326; found: 1163.5325.

4.1.24. *tert*-Butyl ((4*S*)-4-(6-((*tert*-butoxycarbonyl)amino)-2-((tritylthio)methyl)hexanamido)-5-((2,2-dimethoxyethyl)(2-(naphthalen-2-yl)ethyl)amino)-5-oxopentyl)carbamate (**40**)

The title compound was prepared from carboxylic acid **32** (267 mg, 0.51 mmol) and Fmoc-deprotected amine **34** (see general protocol B, 0.59 mmol) following general protocol C. The crude product (yellow solid, 676 mg) was purified by flash column chromatography on silica gel (EtOAc/hexane 6:4) to give an inseparable mixture of the two diastereoisomers of **40** as white foam (171 mg, 34%). R_f (EtOAc/hexane 6:4) = 0.29; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m/z* [M + Na]$^+$ calcd for $C_{57}H_{74}N_4O_8SNa$: 997.5125; found: 997.5123.

4.1.25. *tert*-Butyl ((5*S*)-5-(5-((*tert*-butoxycarbonyl)amino)-2-((tritylthio)methyl)pentanamido)-6-((2,2-dimethoxyethyl)(2-(naphthalen-2-yl)ethyl)amino)-6-oxohexyl)carbamate (**41**)

The title compound was prepared from carboxylic acid **31** (244 mg, 0.48 mmol) and Fmoc-deprotected amine **35** (see general protocol B, 0.68 mmol) following general protocol C. The crude product (498 mg) was purified by flash column chromatography on silica gel (EtOAc/hexanes 6:4) to give an inseparable mixture of the two diastereoisomers of **41** as transparent oil (158 mg, 34%). R_f (EtOAc/hexanes 6:4) = 0.34; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m*/*z* [M + Na]$^+$ calcd for $C_{57}H_{74}N_4O_8SNa$: 997.5125; found 997.5128.

4.1.26. *tert*-Butyl (5-(4-((*tert*-butoxycarbonyl)amino)-2-((tritylthio)methyl)butanamido)-6-((2,2-dimethoxyethyl)(2-(naphthalene-2-yl)ethyl)amino)-6-oxohexyl)carbamate (**42**)

The title compound was prepared from carboxylic acid **30** (325 mg, 0.48 mmol) and Fmoc-deprotected amine **35** (see general protocol B, 0.66 mmol) following general protocol C. The crude product (brown oil, 352 mg) was purified by flash column chromatography on silica gel (EtOAc/hexanes 6:4) to give to give an inseparable mixture of the two diastereoisomers of **42** as transparent oil (205 mg, 45 %). R_f (EtOAc/hexanes 6:4) = 0.40; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m*/*z* [M + Na]$^+$ calcd for $C_{56}H_{72}N_4O_8SNa$: 983.4969; found 983.4970.

4.1.27. *tert*-Butyl (5-(((*S*)-1-((2,2-dimethoxyethyl)(naphthalen-2-ylmethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)amino)-5-oxo-4-((tritylthio)methyl)pentyl)carbamate (**43**)

The title compound was prepared from carboxylic acid **31** (469 mg, 0.93 mmol) and Fmoc-deprotected amine **36** (see general protocol B, 0.94 mmol) following general protocol C. The crude product (red foam) was purified by flash column chromatography on silica gel (EtOAc/hexanes 9:1) to give to give an inseparable mixture of the two diastereoisomers of **43** as white foam (707 mg, 67%). R_f (EtOAc/hexanes 9:1) = 0.33; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m*/*z* $[M + Na]^+$ calcd for $C_{64}H_{80}O_9N_6S_2Na$: 1163.5326; found: 1163.5325.

4.1.28. *tert*-Butyl (5-(((*S*)-1-(((1-bromonaphthalen-2-yl)methyl)(2,2-dimethoxyethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)amino)-5-oxo-4-((tritylthio)methyl)pentyl)carbamate (**44**)

The title compound was prepared from carboxylic acid **31** (112 mg, 0.22 mmol) and Fmoc-deprotected amine **37** (see general protocol B, 0.19 mmol) following general protocol C. The crude product (white foam, 384 mg) was purified by flash column chromatography on silica gel (EtOAc/hexanes 8.5:1.5) to give an inseparable mixture of the two diastereoisomers of **44** as white foam (114 mg, 38 %). R_f (EtOAc/hexanes 8.5:1.5) = 0.27; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $C_{64}H_{80}BrN_6O_9S_2$: 1219.4612 and 1221.4591; found: 1219.4630 and 1221.4609.

4.1.29. *tert*-Butyl (5-(((*S*)-1-((2,2-dimethoxyethyl)(naphthalen-1-ylmethyl)amino)-1-oxo-5-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)pentan-2-yl)amino)-5-oxo-4-((tritylthio)methyl)pentyl)carbamate (**45**)

The title compound was prepared from carboxylic acid **31** (87 mg, 0.17 mmol) and Fmoc-deprotected amine **38** (see general protocol B, 0.18 mmol) following general protocol C (18 h reaction time), except HBTU was used as coupling reagent. The crude product was purified by flash column chromatography on silica gel (EtOAc/hexanes 3:1) to give to give an inseparable mixture of the two diastereoisomers of **45** as white foam (0.150 mg, 75%, retains EtOAc). R_f (EtOAc/hexanes) = 0.24; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m*/*z* $[M + Na]^+$ calcd for $C_{64}H_{80}N_6O_9S_2Na$: 1163.5326; found: 1163.5425.

4.1.30. *tert*-Butyl ((7*S*,10*R*)-3-methoxy-5-(naphthalen-2-ylmethyl)-6,9-dioxo-7-(3-(3-((2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-yl)sulfonyl)guanidino)propyl)-13,13,13-triphenyl-2-oxa-12-thia-5,8-diazatridecan-10-yl)carbamate (**46**)

The title compound was prepared from Boc-Cys(Trt)-OH (502 mg, 1.08 mmol) and Fmoc-deprotected amine **36** (see general protocol B, 0.98 mmol) following general protocol C (72 h reaction time), except that HCTU was used as coupling reagent. The crude product (orange foam, 1.19 g) was purified by flash column chromatography on silica gel (EtOAc/hexanes 9:1) to give the title compound as yellow oil (752 mg, 63%). R_f (EtOAc/hexanes 9:1) = 0.34; NMR analyses did not give useful information due to formation of rotamers; HRMS (ESI): *m*/*z* $[M + Na]^+$ calcd for $C_{61}H_{74}N_6O_9S_2Na$: 1121.4856; found: 1121.4859.

4.1.31. General procedure for global deprotection and cyclization

The linear precursor was dissolved in a mixture of TFA, thioanisole and H_20 (90:5:5, 100 mL per mmol) and the resulting mixture was stirred at room temperature for 2-4 h. The TFA was removed under reduced pressure and crude product was precipitated by addition of cold diethyl ether. The diethyl ether was removed and the residue was dried in vacuo to give the crude product as a white solid.

4.1.32. 1-(3-((3*S*,6*S*,9a*R*)-3-(2-guanidinoethyl)-8-(2-(naphthalen-2-yl)ethyl)-4,7-dioxohexahydro-2*H*,6*H*-pyrazino[2,1-*b*][1,3]thiazin-6-yl)propyl)guanidine (**6a**)

Linear precursor **39** (240 mg, 0.21 mmol) was deprotected and cyclized following the general procedure. The crude product (113 mg out of 283 mg) was dissolved in dry DMF (5 mL) and 1*H*-pyrazole-1-carboxamidine hydrochloride (171 mg, 1.17 mmol) and DIPEA (0.20 mL, 1.15 mmol) were added and the mixture was stirred under inert atmosphere for 66 h during which the reaction was monitored by RP-HPLC. Diethyl ether (25 mL) was then added and the mixture was cooled at 4 °C and stirred for an additional hour, resulting in the precipitation of a white solid (390 mg). The crude product was purified by semi-preparative RP-HPLC to give the title compound as a fluffy white solid (13.0 mg, 17%). ^{1}H NMR (600 MHz, CD_3OD): δ = 7.85 – 7.78 (m, 3H), 7.70 (s, 1H), 7.49 – 7.43 (m, 3H), 4.85 (m, 1H), 4.68 (HSQC, 1H), 4.17 – 4.11 (m, 1H), 3.71 – 3.60 (m, 3H), 3.23 – 3.17 (m, 3H), 3.14 – 3.05 (m, 2H), 3.00 – 2.94 (m, 1H), 2.91 – 2.85 (m, 1H), 2.67 – 2.55 (m, 2H), 2.05 – 1.98 (m, 1H), 1.75 – 1.68 (m, 1H), 1.67 – 1.60 (m, 1H), 1.54 – 1.46 (m, 1H), 1.33 – 1.23 (m, 2H); ^{13}C NMR (150.9 MHz, CD_3OD): δ = 174.1, 170.2, 158.8, 158.5, 137.2, 135.0, 133.9, 129.3, 128.7, 128.6, 128.4, 128.4, 127.4, 126.8, 57.3, 53.7, 48.8 (HSQC), 48.6 (HSQC), 41.4, 40.5, 40.5, 34.6, 31.2, 30.7,

29.8, 26.3; HRMS (ESI): *m*/*z* [M + 2H]$^{2+}$ calcd for $C_{26}H_{38}N_8O_2S$: 263.1414; found: 263.1418. Purity = 97.9% (UV 220 nm)

4.1.33. 1-(3-((3*R*,6*S*,9a*S*)-3-(2-guanidinoethyl)-8-(2-(naphthalen-2-yl)ethyl)-4,7-dioxohexahydro-2*H*,6*H*-pyrazino[2,1-*b*][1,3]thiazin-6-yl)propyl)guanidine (**6b**)

Bicycle **6b** was prepared as described for **6a** above. The crude product was purified by semi-preparative RP-HPLC to give the title compound as a fluffy white solid (18.6 mg, 23%). ^{1}H NMR (600 MHz, CD_3OD): δ = 7.83 – 7.78 (m, 3H), 7.69 (s, 1H), 7.48 – 7.42 (m, 3H), 5.15 (t, *J* = 4.8, 1H), 4.87 (HSQC, 1H), 4.04 – 3.98 (m, 1H), 3.73 (dd, *J* = 13.9, 4.0, 1H), 3.62 – 3.56 (m, 1H), 3.53 (dd, *J* = 14.0, 5.8, 1H), 3.24 – 3.20 (m, 2H), 3.11 – 3.07 (m, 2H), 3.06 – 2.97 (m, 3H), 2.84 – 2.78 (m, 1H), 2.60 (t, *J* = 12.1, 1H), 2.03 – 1.95 (m, 1H), 1.70 – 1.63 (m, 3H), 1.41 – 1.35 (m, 2H); ^{13}C NMR (150.9 MHz, CD_3OD); δ = 173.8, 169.3, 158.7, 158.5, 137.3, 135.0, 133.8, 129.3, 128.7, 128.5, 128.5, 128.5, 127.2, 126.7, 56.6, 53.0, 50.7, 49.3 (HSQC), 41.6, 41.4, 40.4, 34.4, 31.3, 30.1, 28.6, 26.2; HRMS (ESI): *m*/*z* [M + 2H]$^{2+}$ calcd for $C_{26}H_{38}N_8O_2S$: 263.1414; found: 263.1419. Purity = 98.0% (UV 220 nm)

4.1.34. 1-(3-((3*S*,6*S*,9a*R*)-3-(4-guanidinobutyl)-8-(2-(naphthalen-2-yl)ethyl)-4,7-dioxohexahydro-2*H*,6*H*-pyrazino[2,1-*b*][1,3]thiazin-6-yl)propyl)guanidine (**7a**)

Linear precursor **40** (134 mg, 0.14 mmol) was deprotected and cyclized following the general procedure. The crude product (134 mg) was dissolved in dry DMF (10 mL) and1*H*-pyrazole-1-carboxamidine hydrochloride (165 mg, 1.13 mmol) and DIPEA (0.20 mL, 1.15 mmol) were added. The mixture was stirred under argon atmosphere for 66 h during which the reaction was monitored by RP-HPLC. Diethyl ether (25 mL) was then added and the mixture was cooled at 4 °C and stirred for an additional hour, resulting in the precipitation of a brown oil (157 mg). The crude product was purified by semi-preparative RP-HPLC to give the title compound as a white fluffy solid (8.5 mg, 16%). ^{1}H NMR (600 MHz, CD_3OD): δ = 7.86 – 7.77 (m, 3H), 7.68 (s, 1H), 7.49 – 7.43 (m, 3H), 4.66 (p, *J* = 8.0, 2H), 4.09 – 4.03 (m, 1H), 3.72 – 3.67 (m, 1H), 3.63 – 3.59 (m, 2H), 3.20 – 3.15 (m, 3H), 3.10 (t, *J* = 6.9, 2H), 3.02 – 2.89 (m, 2H), 2.55 (t, *J* = 11.4, 1H), 2.40 – 2.34 (m, 1H), 1.88 – 1.79 (m, 1H), 1.66 – 1.56 (m, 3H), 1.54 – 1.46 (m, 1H), 1.45 – 1.35 (m, 3H), 1.35 – 1.23 (m, 2H); ^{13}C NMR (150.9 MHz, CD_3OD); δ = 174.3, 170.4, 158.7, 158.5, 137.3, 134.9, 133.8, 129.3, 128.7, 128.7, 128.4, 128.4, 127.3, 126.8, 57.1, 53.8, 49.0 (HSQC), 49.0 (HSQC), 43.1, 42.3, 41.5, 34.6, 31.3, 30.7, 29.9, 29.9, 26.3, 25.4; HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{28}H_{41}N_8O_2S$: 553.3073; found: 553.3071. Purity = 97.7% (UV 220 nm)

4.1.35. 1-(3-((3*R*,6*S*,9a*S*)-3-(4-guanidinobutyl)-8-(2-(naphthalen-2-yl)ethyl)-4,7-dioxohexahydro-2*H*,6*H*-pyrazino[2,1-*b*][1,3]thiazin-6-yl)propyl)guanidine (**7b**)

Bicycle **7b** was prepared as described for **7a** above. The crude product was purified by semi-preparative RP-HPLC to give the title compound as a fluffy white solid (7.1 mg, 13%). ^{1}H NMR (600 MHz, CD_3OD): δ = 7.85 – 7.77 (m, 3H), 7.69 (s, 1H), 7.49 – 7.40 (m, 3H), 5.13 – 5.09 (m, 1H), 4.88 (HSQC, 1H), 3.98 (p, *J* = 7.2, 1H), 3.67 (dd, *J* = 13.7, 4.0, 1H), 3.59 (p, *J* = 6.6, 1H), 3.52 (dd, *J* = 13.7, 6.2, 1H), 3.17 (t, *J* = 7.1, 2H) 3.11 – 3.01 (m, 5H), 2.74 – 2.68 (m, 1H), 2.57 (t, *J* = 12.0, 1H), 1.93 – 1.86 (m, 1H), 1.72 – 1.64 (m, 2H), 1.64 – 1.55 (m, 2H), 1.46 – 1.35 (m, 5H); ^{13}C NMR (150.9 MHz, CD_3OD); δ = 174.0, 169.4, 158.6, 158.5, 137.3, 135.0, 133.8, 129.3, 128.7, 128.5, 128.5, 128.4, 127.2, 126.7, 56.6, 52.7, 51.2, 49.4 (HSQC), 44.1, 42.3, 41.6, 34.7, 31.3, 30.1, 29.9, 28.3, 26.2, 25.1; HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $C_{28}H_{41}N_8O_2S$: 553.3073; found: 553.3072. Purity = 96.3% (UV 220 nm)

4.1.36. 1-(4-((3*S*,6*S,9aR*)-3-(3-guanidinopropyl)-8-(2-(naphthalen-2-yl)ethyl)-4,7-dioxohexahydro-2*H*,6*H*-pyrazino[2,1-*b*][1,3]thiazin-6-yl)butyl)guanidine (**8a**)

Linear precursor **41** (158 mg, 0.16 mmol) was deprotected and cyclized following the general procedure. The crude product (212 mg) was dissolved in dry DMF (10 mL) and 1*H*-pyrazole-1-carboxamidine hydrochloride (132 mg, 0.90 mmol) and DIPEA (0.16 mL, 0.90 mmol) were added. The mixture was stirred for 72 h at room temperature during which the reaction was monitored by RP-HPLC. Diethyl ether (40 mL) was then added and the mixture was cooled to 4 °C and stirred for an additional hour, resulting in the precipitation of a brown oil (163 mg). The crude product was purified by semi-preparative RP-HPLC to give the title compound as a white fluffy solid (5.5 mg, 9%). ^{1}H NMR (600 MHz, CD_3OD): δ = 7.86 – 7.76 (m, 3H), 7.69 (s, 1H), 7.49 – 7.43 (m, 3H), 4.17 – 4.67 (m, 1H), 4.64 – 4.60 (m, 1H), 4.14 – 4.07 (m, 1H), 3.69 – 3.62 (m, 1H), 3.62 – 3.57 (m, 2H), 3.20 – 3.15 (m, 3H), 3.12 – 3.07 (m, 2H), 2.92 (t, *J* = 7.2, 2H), 2.57 (t, *J* = 11.5, 1H), 2.41 (sx, *J* = 6.0, 1H), 1.87 – 1.80 (m, 1H), 1.68 – 1.57 (m, 2H), 1.57 – 1.51 (m, 1H), 1.50 – 1.36 (m, 3H), 1.32 – 1.23 (m, 1H), 1.20 – 1.11 (m, 1H), 1.08 – 1.00 (m, 1H); ^{13}C NMR (150.9 MHz, CD_3OD); δ = 173.9, 170.5, 158.6 (HMBC), 158.5 (HMBC), 137.3, 134.9, 133.9, 129.3, 128.7 (2C), 128.5, 128.4, 127.3, 126.8, 57.7, 53.7, 48.9 (HSQC), 48.8 (HSQC), 42.9, 42.4, 42.2, 34.6, 33.4, 29.9, 29.0, 28.8, 27.8, 24.1; HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $C_{28}H_{41}N_8O_2S$: 553.3073; found: 553.3071, *m*/*z* $[M + 2H]^{2+}$ calcd for $C_{28}H_{42}N_8O_2S$: 277.1570; found: 277.1567. Purity = 95.4% (UV 220 nm)

4.1.37. 1-(4-((3*R*,6*S*,9a*S*)-3-(3-guanidinopropyl)-8-(2-(naphthalen-2-yl)ethyl)-4,7-dioxohexahydro-2*H*,6*H*-pyrazino[2,1-*b*][1,3]thiazin-6-yl)butyl)guanidine (**8b**)

Bicycle **8b** was prepared as described for **8a** above. The crude product was purified by semi-preparative RP-HPLC to give the title compound as a fluffy white solid (10 mg, 16%). ^{1}H NMR (600 MHz, CD_3OD): δ = 7.84 – 7.78 (m, 3H), 7.70 (s, 1H), 7.48 – 7.41 (m, 3H), 5.16 – 5.13 (m, 1H), 4.83 (HSQC, 1H), 4.09 – 4.02 (m, 1H), 3.71 (dd, *J* = 14.0, 4.0, 1H), 3.57 – 3.50 (m, 2H), 3.18 (t, *J* = 7.0, 2H), 3.12 – 3.04 (m, 3H), 3.00 – 2.96 (m, 2H), 2.79 – 2.72 (m, 1H), 2.59 (t, *J* = 12.1, 1H), 1.93 – 1.85 (m, 1H), 1.69 – 1.54 (m, 4H), 1.50 – 1.42 (m, 2H), 1.42 – 1.34 (m, 1H), 1.26 – 1.18 (m, 1H), 1.17 – 1.09 (m, 1H); ^{13}C NMR (150.9 MHz, CD_3OD); δ = 173.7, 169.6, 158.7, 158.6, 137.3, 135.0, 133.8, 129.2, 128.7, 128.5, 128.5, 128.5, 127.2, 126.6, 57.0, 53.0, 50.7, 49.2 (HSQC), 43.6, 42.4, 42.2, 34.4, 32.8, 29.1, 28.8, 28.7, 27.5, 24.0; HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $C_{28}H_{41}N_8O_2S$: 553.3073; found: 553.3071. Purity = 98.1% (UV 220 nm)

4.1.38. 1-(4-((3*S*,6*S*,9a*R*)-3-(2-guanidinoethyl)-8-(2-(naphthalene-2-yl)ethyl)-4,7-dioxohexahydro-2*H*,6*H*-pyrazino[2,1-*b*][1,3]thiazine-6-yl)butyl)guanidine (**9a**)

Linear precursor **42** (185 mg, 0.19 mmol) was deprotected and cyclized following the general procedure. The crude product (188 mg) was dissolved in dry DMF (10 mL) and 1*H*-pyrazole-1-carboxamidine hydrochloride (121 mg, 0.83 mmol) and DIPEA (0.14 mL, 0.83 mmol) were added. The mixture was stirred for 90 h at room temperature during which the reaction was monitored by RP-HPLC. Diethyl ether (40 mL) was then added and the mixture was cooled to 4 °C and stirred for an additional hour, resulting in the precipitation of a brown oil (173 mg). The crude product was purified by semi-preparative RP-HPLC to give the title compound as a white fluffy solid (4.7 mg, 6%). ^{1}H NMR (600 MHz, CD_3OD): δ = 7.86 – 7.79 (m, 3H), 7.71 (s, 1H), 7.50 – 7.43 (m, 3H), 4.88 (dd, *J* = 11.0, 4.9; 1H), 4.63 (t, *J* = 7.9, 1H), 4.25 – 4.18 (m, 1H), 3.69 (dd, *J* = 13.8, 5.1, 1H), 3.64 – 3.55 (m, 2H), 3.23 – 3.16 (m, 3H), 3.14 – 3.04 (m, 2H), 2.92 – 2.84 (m, 2H), 2.67 – 2.56 (m, 2H), 2.04 – 1.96 (m, 1H), 1.75 – 1.68 (m, 1H), 1.56 – 1.49 (m, 1H), 1.47 – 1.40 (m, 1H), 1.40 – 1.32 (m, 1H), 1.25 – 1.17 (m, 1H), 1.15 – 1.07 (m, 1H), 1.02 – 0.93 (m, 1H); ^{13}C NMR (150.9 MHz, CD_3OD); δ = 173.9, 170.4, 158.8, 158.5, 137.2, 135.0, 133.7, 129.3, 128.7, 128.7, 128.5, 128.5, 127.3, 126.7, 57.8, 53.7, 48.6 (HSQC), 48.6 (HSQC), 42.1, 40.5, 40.5, 34.7, 33.4, 31.2, 29.9, 29.1, 24.1; HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $C_{27}H_{39}N_8O_2S$: 539.2917; found: 539.2920, *m*/*z* $[M + 2H]^{2+}$ calcd for $C_{27}H_{40}N_8O_2S$: 270.1492; found: 270.1504. Purity = 98.5% (UV 220 nm)

4.1.39. 1-(4-((3*R*,6*S*,9a*S*)-3-(2-guanidinoethyl)-8-(2-(naphthalene-2-yl)ethyl)-4,7-dioxohexahydro-2*H*,6*H*-pyrazino[2,1-*b*][1,3]thiazine-6-yl)butyl)guanidine (**9b**)

Bicycle **9b** was prepared as described for **9a** above. The crude product was purified by semi-preparative RP-HPLC to give the title compound as a fluffy white solid (5.3 mg, 7%). ^{1}H NMR (600 MHz, CD_3OD): δ = 7.84 – 7.77 (m, 3H), 7.69 (s, 1H), 7.48 – 7.39 (m, 3H), 5.17 (m, 1H), 4.84 (HSQC, 1H), 4.09 – 4.02 (m, 1H), 3.73 (dd, *J* = 14.0, 3.8, 1H), 3.58 – 3.49 (m, 2H), 3.24 – 3.18 (m, 2H), 3.11 – 3.07 (m, 2H), 3.04 (dd, *J* = 12.1, 5.6, 1H), 2.99 – 2.94 (m, 2H), 2.83 – 2.76 (m, 1H), 2.59 (t, *J* = 12.1, 1H), 2.02 – 1.94 (m, 1H), 1.71 – 1.64 (m, 1H), 1.62 – 1.55 (m, 2H), 1.50 – 1.41 (m, 1H), 1.41 – 1.33 (m, 1H), 1.26 – 1.17 (m, 1H), 1.17 – 1.08 (m, 1H); ^{13}C NMR (150.9 MHz, CD_3OD); δ = 173.7, 169.4, 158.7, 158.5, 137.3, 135.0, 133.8, 129.2, 128.7, 128.5, 128.5, 128.5, 127.2, 126.7, 57.1, 53.0, 50.4, 49.2 (HSQC), 42.1, 41.2, 40.4, 34.4, 32.8, 31.2, 29.1, 28.7, 24.0; HRMS (ESI): *m*/*z* $[M + H]^+$ calcd for $C_{27}H_{39}N_8O_2S$: 539.2917; found: 539.2919, *m*/*z* $[M + 2H]^{2+}$ calcd for $C_{27}H_{40}N_8O_2S$: 270.1492; found: 270.1493. Purity = 96.0% (UV 220 nm).

4.1.40. 1,1'-(((3*RS*,6*S*,9a*RS*)-8-(naphthalen-2-ylmethyl)-4,7-dioxohexahydro-2*H*,6*H*-pyrazino[2,1-*b*][1,3]thiazine-3,6-diyl)bis(propane-3,1-diyl))diguanidine (**10a,b**)

Linear precursor **45** (123 mg, 0.11 mmol) was deprotected and cyclized following the general procedure. The crude product (90 mg) was dissolved in DMF (10 mL) and 1*H*-pyrazole-1-carboxamidine hydrochloride (103 mg, 0.70 mmol) and DIPEA (0.1 mL, 0.57 mmol) were added. The mixture was stirred under argon atmosphere for 69 h during which the reaction was monitored by RP-HPLC. Diethyl ether (30 mL) was then added and the mixture was cooled at 4 °C and stirred for an additional hour, resulting in the precipitation of a brown solid (118 mg). The crude product was purified by semi-preparative RP-HPLC to give the title compound as inseparable diastereoisomers as fluffy white solid (25.5 mg, 31%). ^{1}H NMR (500 MHz, CD_3OD): δ = 7.90 – 7.78 (m, 8H), 7.54 – 7.46 (m, 4H), 7.42 (dd, *J* = 8.5, 1.7, 2H), 5.25 – 5.21 (m, 2H), 5.16 (dd, *J* = 10.9, 5.6, 1H), 5.10 (dd, *J* = 9.2, 6.0, 1H), 4.89 (HSQC), 4.77 (d, *J* = 14.6, 1H), 4.67 (d, *J* = 14.6, 1H), 3.81 – 3.63 (m, 4H), 3.26 (HSQC), 3.19 (q, *J* = 7.5, 4H), 3.05 (dd, *J* = 11.7, 5.1, 1H), 2.84 – 2.69 (m, 3H), 2.64 (t, *J* = 11.4, 1H), 2.04 – 1.85 (m, 6H), 1.77 – 1.59 (m, 8H), 1.59 – 1.45 (m, 2H); ^{13}C NMR (150.9 MHz, CD_3OD); δ = 174.5, 173.9, 170.8, 169.7, 158.7, 158.6, 134.87, 134.85, 134,80, 134.6, 134.5 (2C), 130.0, 129.8, 128.84, 128.82, 128.74, 128.72, 128.6, 128.5, 127.6, 127.5, 127.4, 127.3, 127.1, 126.8, 57.3, 56.8, 54.1, 52.9, 51.2, 50.9, 50.0, 47.7, 43.8, 42.9, 42.2, 42.38, 41.8, 41.7, 31.0, 30.3,

30.0, 28.9, 28.8, 28.4, 27.7, 27.4, 26.7, 26.5; HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{26}H_{37}N_8O_2S$: 525.2760; found: 525.2764. Purity = 95% (sum of diastereoisomers, UV 220).

4.1.41. 1,1'-(((3*RS*,6*S,*9a*RS*)-8-((1-bromonaphthalen-2-yl)methyl)-4,7-dioxohexahydro-2*H*,6*H*-pyrazino[2,1-*b*][1,3]thiazine-3,6-diyl)bis(propane-3,1-diyl))diguanidine (**11a,b**)

Linear precursor **44** (112 mg, 0.1 mmol) was deprotected and cyclized following the general procedure. The crude product (31 mg out of 33 mg) was dissolved in dry DMF (3 mL) and 1*H*-pyrazole-1-carboxamidine hydrochloride (32 mg, 0.2 mmol) and DIPEA (0.2 mL, 1.2 mmol) were added and stirring continued for 72 h (monitored by RP-HPLC) under inert atmosphere at room temperature. Diethyl ether (25 mL) was added to the mixture and stirring continued up to one additional hour on ice/water bath. The crude product precipitated as a white solid. The solvent was removed to give the crude product as a white sticky material (31 mg). The crude product was purified by semi-preparative RP-HPLC to give the title compounds as a yellowish fluffy powder (6 mg, 8 %). ^{1}H NMR (600 MHz, CD_3OD): δ = 8.35-8.31 (m, 2H), 7.94-7.88 (m, 4H), 7.69-7.63 (m, 2H), 7.62-7.57 (m, 2H), 7.46-7.42 (m, 2H), 5.27-5.21 (m, 2H), 5.14-5.08 (m, 2H), 5.08, 5.01 (ABq, J_{AB} = 15.2, 2H), 4.95-4.90 (m, 3H), 3.82-3.76 (m, 2H), 3.69 (dd, *J* = 13.8, 5.3, 1H), 3.65 (dd, *J* = 13.8, 6.4, 1H), 3.29-3.25 (m, 4H), 3.22-3.17 (m, 4H), 3.04 (dd, *J* = 11.8, 5.0, 1H), 2.87-2.78 (m, 2H), 2.78-2.71 (m, 1H), 2.66 (app t, *J* = 11.4, 1H), 2.07-1.98 (m, 1H), 1.98-1.90 (m, 5H), 1.78-1.60 (m, 8H), 1.60-1.46 (m, 2H); ^{13}C NMR (150.9 MHz, CD_3OD); δ = 174.5, 173.9, 170.8, 169.8, 158.66, 158.65, 158.63, 135.6 (2C), 134.5, 134.3, 133.63, 133.60, 127.7, 129.6, 129.43, 129.41, 129.1, 129.0, 128.2, 128.14, 128.11, 128.1, 127.8, 127.6, 124.8, 124.6, 57.3, 56.9, 54.1, 52.8, 51.9, 51.8, 50.5, 48.1, 43.9, 42.9, 42.42, 42.38, 41.8, 41.7, 31.0, 30.3, 30.1, 28.93, 28.85, 28.4, 27.7, 27.4, 26.7, 26.5; HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{26}H_{36}{}^{79}BrN_8O_2S$: 603.1865; found: 603.1862; *m*/*z* [M + H]$^+$ calcd for $C_{26}H_{36}{}^{81}BrN_8O_2S$: 605.1845; found: 605.1853. Purity = 96.8% (sum of diastereoisomers, UV 220).

4.1.42. 1,1'-(((3*RS*,6*S,*9a*RS*)-8-(naphthalen-1-ylmethyl)-4,7-dioxohexahydro-2*H*,6*H*-pyrazino[2,1-*b*][1,3]thiazine-3,6-diyl)bis(propane-3,1-diyl))diguanidine ((**12a,b**)

The linear precursor **43** (1.556 g, 1.58 mmol) was deprotected and cyclized following the general procedure. The crude product (718 mg out of 989 mg) was guanidinylated following the procedure described for **6a** to give the crude product as yellow foam (821 mg). A small sample was purified by semi-preparative RP-HPLC to give the title compounds as a fluffy white solid. ^{1}H NMR (600 MHz, CD_3OD): δ = 8.12-8.07 (m, 2H), 7.96-7.87 (m, 4H), 7.56-

7.45 (m, 8H), 5.32-5.24 (m, 2H), 5.15 (dd, *J* = 6.2, 4.1, 1H), 5.11 (dd, *J* = 9.0, 6.1, 1H), 5.06-5.01 (m, 2H), 4.97-4.89 (m, 2H), 3.64 (dd, *J* = 13.8, 5.3, 1H), 3.57 (dd, *J* = 13.9, 4.2, 1H), 3.50 (dd, *J* = 13.9, 6.3, 1H), 3.45 (dd, *J* = 13.8, 11.4, 1H), 3.26-3.21 (m, 5H), 3.20-3.14 (m, 4H), 3.00 (dd, *J* = 12.2, 5.5, 1H), 2.79-2.70 (m, 2H), 2.67-2.58 (m, 2H), 2.01-1.81 (m, 6H), 1.76-1.42 (m, 9H), 1.40-1.35 (m, 1H); ^{13}C NMR (150.9 MHz, CD_3OD); δ = 174.4, 173.9, 170.2, 169.2, 158.63, 158.59, 135.56, 135.53, 133.0, 132.8, 132.5, 132.3, 130.4, 130.3, 130.1, 130.0, 129.3, 129.2, 127.7, 127.6, 127.3, 127.2, 126.5, 126.3, 124.7, 124.6, 57.4, 56.9, 54.0, 52.9, 49.2 (HSQC), 49.0 (HSQC), 48.8 (HSQC), 48.5 (HSQC), 48.3, 46.7, 43.8, 42.9, 42.43, 42.38, 41.77, 41.72, 30.9, 30.2, 30.0, 28.89, 28.82, 28.4, 27.7, 27.4, 26.7, 26.5; HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{26}H_{37}N_8O_2S$: 525.2760; found: 525.2765. Purity = 97.0% (sum of diastereoisomers, UV 220).

4.1.43. 1-(3-((3*S*,6*S*,9a*R*)-3-(3-aminopropyl)-8-(naphthalen-2-ylmethyl)-4,7-dioxohexahydro-2*H*,6*H*-pyrazino[2,1-*b*][1,3]thiazin-6-yl)propyl)guanidine (**49a,b**)

The linear precursor **43** (132 mg, 0.12 mmol) was cyclized following the general procedure to give 71 mg of crude product. Purification by semi-preparative RP-HPLC gave the title compounds as a fluffy white solid (18.9 mg, 30%). ^{1}H NMR (600 MHz, CD_3OD): δ = 7.89-7.83 (m, 6H), 7.81 (d, *J* = 9.1, 2H), 7.53-7.47 (m, 4H), 7.42 (d, *J* = 8.3, 2H), 5.25-5.22 (m, 1H), 5.17 (dd, *J* = 10.9, 5.3, 1H), 5.09 (dd, *J* = 8.1, 6.6, 1H), 4.93-4.87 (m, 3H), 4.77 (d, *J* = 14.4, 1H), 4.67 (d, *J* = 14.6, 1H), 3.82-3.63 (m, 4H), 3.30-3.22 (m, 5H), 3.05 (dd, *J* = 12.0, 5.1, 1H), 2.97-2.91 (m, 4H), 2.85-2.79 (m, 2H), 2.73 (t, *J* = 11.9, 1H), 2.65 (t, *J* = 11.5, 1H), 2.04-1.87 (m, 6H), 1.83-1.67 (m, 8H), 1.60-1.48 (m, 2H); ^{13}C NMR (150.9 MHz, CD_3OD); δ = 174.6, 174.1, 171.0, 169.9, 158.96, 158.92, 135.2, 135.14, 135.09, 134.9, 134.80, 134.79, 130.3, 130.1, 129.15, 129.13, 129.03, 129.01, 128.9, 128.8, 127.9, 127.8, 127.7, 127.6, 127.5, 127.1, 57.6, 57.1, 54.4, 53.3, 51.5, 51.2, 50.3, 48.0, 43.9, 43.1, 42.1, 42.0, 40.9, 40.8, 31.3, 30.7, 30.3, 28.9, 28.8, 28.7, 27.0, 26.78, 26.77, 26.4; HRMS (ESI): *m*/*z* [M + H]$^+$ calcd for $C_{25}H_{35}N_6O_2S$: 483.2542; found: 483.2545.

4.1.44. 1-(3-((1*R*,3*S*)-2-(*L*-cysteinyl)-4-oxo-1,3,4,6-tetrahydro-2*H*-1,5-methanonaphtho[1,2-*f*][1,4]diazocin-3-yl)propyl)guanidine (**48**)

Linear precursor **46** (74 mg, 0.91 mmol) was cyclized following the general procedure to give 80 mg of crude product. Purification by semi-preparative RP-HPLC gave the title compound as a fluffy white solid (1.62 mg, 4%). ^{1}H NMR (600 MHz, CD_3OD): δ = 8.23 (d, *J* = 8.6, 1H), 7.84 (d, *J* = 7.9, 1H), 7.82 (d, *J* = 8.6, 1H), 7.61-7.57 (m, 1H), 7.52-7.48 (m, 1H), 7.26 (d, *J* =

8.5, 1H), 5.24 (d, J = 18.3, 1H), 4.98 (dd, J = 7.8, 5.4, 1H), 4.86 (overlap with residual water, 1H), 4.42 (d, J = 18.3, 1H), 3.95 (dd, J = 14.7, 1.7, 1H), 3.76 (dd, J = 12.1, 4.2, 1H), 3.70 (dd, J = 14.7, 1.7, 1H), 3.54 (dd, J = 14.3, 4.2, 1H), 3.31 (overlap with solvent peak, 1H), 3.18 (dd, J = 14.3, 12.1, 1H), 2.11-2.06 (m, 1H), 1.82-1.71 (m, 3H); ^{13}C NMR (150.9 MHz, CD_3OD); δ = 172.1, 169.3, 158.6, 134.1, 132.2, 132.0, 130.3, 129.6, 128.7, 127.8, 127.0, 125.3, 124.5, 55.4, 50.7, 48.1, 46.8, 46.2, 42.2, 37.8, 29.4, 26.1; HRMS (ESI): m/z $[M + H]^+$ calcd for $C_{22}H_{29}N_6O_2S$: 441.2073; found: 441.2070.

4.2. Biology - COS-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with Glutamax (1% glutamine), supplemented with 10% FBS, 180U/ml penicillin and 45µg/ml streptomycin in a 10% CO_2/90% humidified atmosphere. The cells were transfected by the calcium phosphate precipitation method, where plasmid DNA (10 µg of receptor cDNA and 15 µg of the chimeric $G\alpha_i$ signal-converting G protein $G\alpha_{\delta 6qi4myr}$) was mixed with TE buffer (10 mM Tris-HCl, 2 mM Na_2EDTA, pH 7.5) and 30 µl $CaCl_2$ (2 M). This mixture was added to 240 µl HEPES buffered saline (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.2). The mixture was allowed to stand for 45 minutes at room temperature for precipitation. Together with 150 µl chloroquine (2 mg/ml), it was thereafter added to 5 ml culture media to the 6 × 10^6 COS-7 cells seeded the day before. The transfection was stopped after 5 hours by replacing the culture media, and the cells were incubated overnight.

The scintillation proximity-based inositol-phosphate accumulation assay (SPA-IP) was used to measure the potency of the potential antagonists. One day after transfection, the COS-7 cells were seeded in wells (35,000 cells/well) and were incubated for 24 hours with Myo-[2-^{3}H(N)]-inositol (5 µl/ml) in 100 µl of culture media per well in 96-well plate. The subsequent day, the cells were washed twice in Hank's balanced salt solution (HBSS, Invitrogen, U.K.) and incubated for 90 minutes in HBSS supplemented with 10 mM LiCl at 37 °C in the presence of the potential antagonists and CXCL12. Amounts of CXCL12 added induced up to 80% of full receptor activation. Cell lysis was induced by addition of 40 µl formic acid (10 mM) to each well, followed by incubation on ice for 30 minutes. For quantification of [^{3}H]inositol-phosphates in the formic acid cell lysates, 80 µl of a Ysi-poly-D-Lys coated SPA bead suspension (12.5 µg/µl in H_2O, PerkinElmer) were added to 35 µl cell lysates in a Pico-Plate-96 white plate. The plates were sealed, agitated for 30 minutes and centrifuged (5 minutes, 1500 rpm). The SPA beads were allowed to settle and react with the extract for 8

hours before the radioactivity was measured in a Packard Top Count NXT^{TM} scintillation counter (PerkinElmer, MA, USA). All determinations were made in duplicate.

Acknowledgements

Financial support for this project was obtained from the University of Bergen (M.B. and B.E.H.), Norwegian PhD School of Pharmacy (travel grant to M.B.), UiT The Arctic University of Norway (J.V.), and from the University of Copenhagen and the Danish Medical Research Council, the Hørslev Foundation, the AP-Møller Foundation and the Danish Research Council | Health and Disease (S.K. and M.M.R.). The authors would like to thank Dr. Zack G. Zachariassen at UiT The Arctic University of Norway, Dr. Bjarte Holmelid at the University of Bergen and Maibritt S. Baggesen at the University of Copenhagen for excellent technical support.

Supplementary data

Supplementary data (summary of NMR data for stereochemical assignment, summary of cyclization results with experimental details) associated with this article can be found, in the online version, at http://

References

1. Bleul, C. C.; Farzan, M.; Choe, H.; Parolin, C.; Clark-Lewis, I.; Sodroski, J.; Springer, T. A. *Nature* **1996,** *382*, 829.
2. Oberlin, E.; Amara, A.; Bachelerie, F.; Bessia, C.; Virelizier, J.-L.; Arenzana-Seisdedos, F.; Schwartz, O.; Heard, J.-M.; Clark-Lewis, I.; Legler, D. F.; Loetscher, M.; Baggiolini, M.; Moser, B. *Nature* **1996,** *382*, 833.
3. D'Apuzzo, M.; Rolink, A.; Loetscher, M.; Hoxie, J. A.; ClarkLewis, I.; Melchers, F.; Baggiolini, M.; Moser, B. *Eur. J. Immunol.* **1997,** *27*, 1788.
4. Tachibana, K.; Hirota, S.; Iizasa, H.; Yoshida, H.; Kawabata, K.; Kataoka, Y.; Kitamura, Y.; Matsushima, K.; Yoshida, N.; Nishikawa, S.; Kishimoto, T.; Nagasawa, T. *Nature* **1998,** *393*, 591.
5. Ma, Q.; Jones, D.; Borghesani, P. R.; Segal, R. A.; Nagasawa, T.; Kishimoto, T.; Bronson, R. T.; Springer, T. A. *Proc. Natl. Acad. Sci. U. S. A.* **1998,** *95*, 9448.
6. Zou, Y. R.; Kottmann, A. H.; Kuroda, M.; Taniuchi, I.; Littman, D. R. *Nature* **1998,** *393*, 595.
7. Debnath, B.; Xu, S.; Grande, F.; Garofalo, A.; Neamati, N. *Theranostics* **2013,** *3*, 47.
8. Mosley, C. A.; Wilson, L. J.; Wiseman, J. M.; Skudlarek, J. W.; Liotta, D. C. *Expert Opin. Ther. Pat.* **2009,** *19*, 23.
9. Våbenø, J.; Haug, B. E.; Rosenkilde, M. M. *Future Med. Chem.* **2015,** *7*, 1261.
10. Brave, M.; Farrell, A.; Ching Lin, S.; Ocheltree, T.; Pope Miksinski, S.; Lee, S. L.; Saber, H.; Fourie, J.; Tornoe, C.; Booth, B.; Yuan, W.; He, K.; Justice, R.; Pazdur, R. *Oncology* **2010,** *78*, 282.

11. Steen, A.; Rosenkilde, M. M. In *Novel Developments in Stem Cell Mobilization;* Fruehauf, S., Zeller, W. J., Calandra, G., Eds.; Springer, 2012, pp. 23.
12. Moyle, G.; DeJesus, E.; Boffito, M.; Wong, R. S.; Gibney, C.; Badel, K.; MacFarland, R.; Calandra, G.; Bridger, G.; Becker, S.; Study, X. A. C. T. *Clin. Infect. Dis.* **2009,** *48*, 798.
13. Nakamura, T.; Furunaka, H.; Miyata, T.; Tokunaga, F.; Muta, T.; Iwanaga, S.; Niwa, M.; Takao, T.; Shimonishi, Y. *J. Biol. Chem.* **1988,** *263*, 16709.
14. Miyata, T.; Tokunaga, F.; Yoneya, T.; Yoshikawa, K.; Iwanaga, S.; Niwa, M.; Takao, T.; Shimonishi, Y. *J. Biochem.* **1989,** *106*, 663.
15. Nakashima, H.; Masuda, M.; Murakami, T.; Koyanagi, Y.; Matsumoto, A.; Fujii, N.; Yamamoto, N. *Antimicrobial Agents and Chemotherapy* **1992,** *36*, 1249.
16. Tamamura, H.; Arakaki, R.; Funakoshi, H.; Imai, M.; Otaka, A.; Ibuka, T.; Nakashima, H.; Murakami, T.; Waki, M.; Matsumoto, A.; Yamamoto, N.; Fujii, N. *Bioorg. Med. Chem.* **1998,** *6*, 231.
17. Tamamura, H.; Xu, Y.; Hattori, T.; Zhang, X.; Arakaki, R.; Kanbara, K.; Omagari, A.; Otaka, A.; Ibuka, T.; Yamamoto, N.; Nakashima, H.; Fujii, N. *Biochem. Biophys. Res. Commun.* **1998,** *253*, 877.
18. Fujii, N.; Oishi, S.; Hiramatsu, K.; Araki, T.; Ueda, S.; Tamamura, H.; Otaka, A.; Kusano, S.; Terakubo, S.; Nakashima, H.; Broach, J. A.; Trent, J. O.; Wang, Z. X.; Peiper, S. C. *Angew. Chem. Int. Edit* **2003,** *42*, 3251.
19. Ueda, S.; Oishi, S.; Wang, Z. x.; Araki, T.; Tamamura, H.; Cluzeau, J.; Ohno, H.; Kusano, S.; Nakashima, H.; Trent, J. O.; Peiper, S. C.; Fujii, N. *J. Med. Chem.* **2007,** *50*, 192.
20. Zachariassen, Z. G.; Thiele, S.; Berg, E. A.; Rasmussen, P.; Fossen, T.; Rosenkilde, M. M.; Våbenø, J.; Haug, B. E. *Bioorg. Med. Chem.* **2014,** *22*, 4759.
21. Vojkovský, T.; Weichsel, A.; Patek, M. *J. Org. Chem.* **1998,** *63*, 3162.
22. Mungalpara, J.; Thiele, S.; Eriksen, Ø.; Eksteen, J.; Rosenkilde, M. M.; Våbenø, J. *J. Med. Chem.* **2012,** *55*, 10287.
23. Riofski, M. V.; John, J. P.; Zheng, M. M.; Kirshner, J.; Colby, D. A. *The Journal of Organic Chemistry* **2011,** *76*, 3676.
24. Kohn, W. D.; Zhang, L. *Tetrahedron Lett* **2001,** *42*, 4453.
25. Grimes Jr., J. H.; Zheng, W.; Kohn, W. D. *Tetrahedron Lett* **2004,** *45*, 6333.
26. Maryanoff, B. E.; Zhang, H.-C.; Cohen, J. H.; Turchi, I. J.; Maryanoff, C. A. *Chem. Rev.* **2004,** *104*, 1431.
27. Liang, J. T.; Liu, J.; Shireman, B. T.; Tran, V.; Deng, X.; Mani, N. S. *Org. Process Res. Dev.* **2010,** *14*, 380.
28. Winter, D. K.; Drouin, A.; Lessard, J.; Spino, C. *J. Org. Chem.* **2010,** *75*, 2610.