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Synthesis and biological evaluation of anthranilamide-based non-peptide mimetics of ω-conotoxin GVIA

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Abstract—Non-peptide mimetics based on an anthranilamide 'scaffold' possessing fragments that mimic Lys2, Tyr13 and Arg17 in ω-conotoxin GVIA have been prepared. Compounds were assayed for binding to the voltage-gated calcium channels $Ca_v2.2$ ('N-type') and $Ca_v2.1$ ('P/Q-type') in rat brain. The primary synthetic target, 2-(6-amino-hexanoylamino)-5-(3-guanidino-propoxy)-*N*-[4-(4-hydroxyphenoxy)-phenyl]-benzamide (**2a**), exhibited low μM binding to $Ca_v2.2$ and was more than 30-fold selective for $Ca_v2.2$ over $Ca_v2.1$.

1. Introduction

ω-Conotoxins are pharmacologically active toxins derived from the venom of cone snails.[1] ω-Conotoxin GVIA (GVIA) is produced by *Conus geographus*, while ω-conotoxin MVIIA (MVIIA) is produced by *Conus magus*. Both of these polypeptides potently block the neuronal voltage-gated N-type calcium channel ($Ca_v2.2$), which in humans is a target for the relief of neuropathic pain. Indeed, ω-conotoxin MVIIA (Prialt®, Ziconotide®, SNX-111) was approved late in 2004 for the treatment of severe chronic pain and shows efficacy in cases where morphine-based analgesics are less effective.[2]

Such therapeutic promise has driven the search for other inhibitors of $Ca_v2.2$. Another ω-conopeptide, ω-conotoxin CVID (AM336), has recently[3] been taken into phase II clinical trials by AMRAD and reportedly has a better therapeutic index than Elan's MVIIA. However, being polypeptides, these agents suffer drawbacks in that they are relatively expensive to manufacture and possess poor pharmacokinetic properties. Indeed, both MVIIA and CVID of necessity are administered intrathecally.

Pain management is one of the largest pharmaceutical markets in the world and is expected[4] to increase at a compounded annual growth rate (CAGR) of 10% to reach $29.8 billion (US) in 2008. It is therefore not surprising that intensive screening events have focused on the discovery of small molecule $Ca_v2.2$ inhibitors that might be orally active. This has culminated in NeuroMed's NMED-160, which is an orally available blocker of $Ca_v2.2$ channels that is now in Phase II clinical trials for a variety of pain conditions.[5]

Our interest is in the rational design of small molecule mimetics of peptide and protein binding epitopes. GVIA and MVIIA are structurally defined and their binding epitopes have been extensively mapped, making them attractive targets for mimetic design.[1,6]

We recently reported[7] the synthesis and biological activity of the benzothiazole derivative **1a** (Fig. 1), which was designed to mimic the side chains of K2, Y13 and R17 in GVIA and bound $Ca_v2.2$ channels with a Ki of 1.8 μM. This simple 3-residue mimetic was also selective for $Ca_v2.2$ over $Ca_v2.1$ ('P/Q-type calcium channels') and this was seen as therapeutically desirable in order to minimise off-target side effects.[8] The key to designing these mimetics is the application of interactive de novo design since no commercially available software could adequately address the challenge posed by the large, discontinuous and disparate ω-conotoxin binding epitope.

Keywords: ω-Conotoxin; Mimetic; $Ca_v2.2$ ('N-type') calcium channel blocker.
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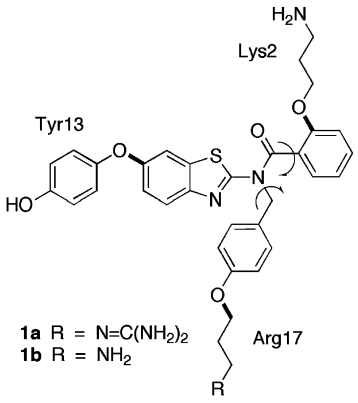


Figure 1. The structure of the type-III mimetic of ω-conotoxin GVIA based on an *N*′-benzylated benzamidobenzothiazole core (**1a**), along with the structure of an analogue (**1b**), both recently reported by us.[7] Annotations indicate the GVIA residue mimicked, and emboldened bonds are those that mimic the corresponding α–β bond vectors in GVIA.

Since the side chain termini targeted for the mimicry of GVIA are not spatially well defined in solution, our approach is to concentrate on the design of scaffolds that mimic the targeted α–β bond vectors as these are conformationally better resolved. In order to investigate whether other scaffolds could be developed besides those based on a benzothiazole template, we have designed the anthranilamide derivative **2a** (Fig. 2). A solid-state structure of the anthranilamide scaffold confirmed that it could serve as a suitable K2–Y13–R17 mimetic of GVIA. We have previously reported this crystal structure along with preliminary synthetic and functional activity details for this mimetic.[9] Herein, we characterise the full synthesis of **2a** and its analogue **2b**, using a different and more efficient synthetic route to that which we have previously outlined, and report binding data for these compounds to $Ca_v2.2$ and $Ca_v2.1$.

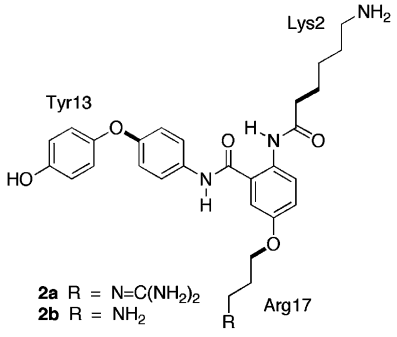


Figure 2. The structure of the type-III mimetic of ω-conotoxin GVIA based on an *N*′-aryl-*N*″-acyl anthranilamide core (**2a**), along with the structure of an analogue (**2b**), reported herein. Annotations indicate the GVIA residue mimicked, and emboldened bonds are those that mimic the corresponding α–β bond vectors in GVIA.

2. Results

2.1. Mimetic synthesis

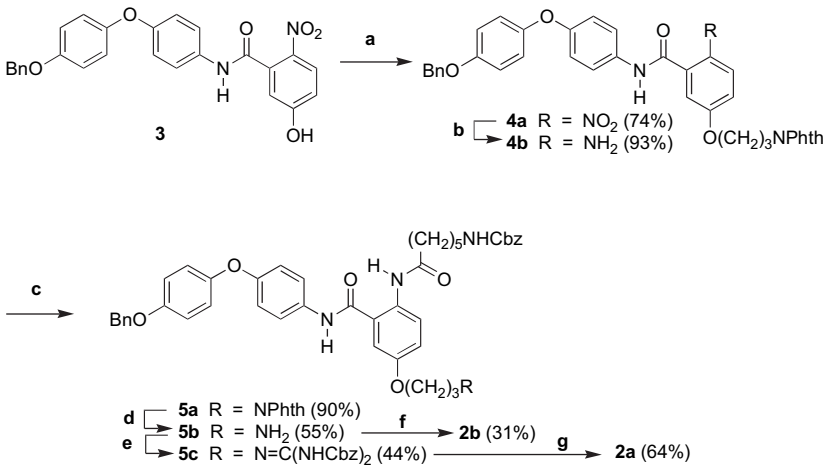
Mimetics were synthesised from key intermediate **3**, as shown in Scheme 1. Firstly, the phenolic group was alkylated with *N*-(3-bromopropyl)phthalimide and potassium carbonate in DMF at 65 °C for 4 h to give **4a** in 74% yield. The nitro group of **4a** was then reduced, after dissolution in hot 60% ethanolic THF, by portionwise addition of sodium dithionite and water at 80 °C, to give aniline **4b** in 93% yield. This aniline was then acylated with Cbz-protected 6-aminohexanoic acid using a typical HOBT/HBTU coupling protocol, to give **5a** in 90% yield. The phthalimide group was cleaved using sodium borohydride in aqueous isopropanol[10] followed by treatment with acetic acid at 60 °C for 48 h to give the free amine **5b** in 55% yield. In turn, this amine was converted into a protected guanidyl group by treatment with *N*,*N*′-bis(benzyloxycarbonyl)-1*H*-pyrazole-1-carboxamidine and triethylamine in methanolic DCM, to give **5c** in 44% yield. Finally, this was fully deprotected by catalytic hydrogenation in trifluoroethanol to afford **2a** as a colourless solid in 64% yield.

Analogue **2b** was obtained from a sample of precursor **5b** that was deprotected by catalytic hydrogenation in methanolic ethanol, to give **2b** in 31% yield.

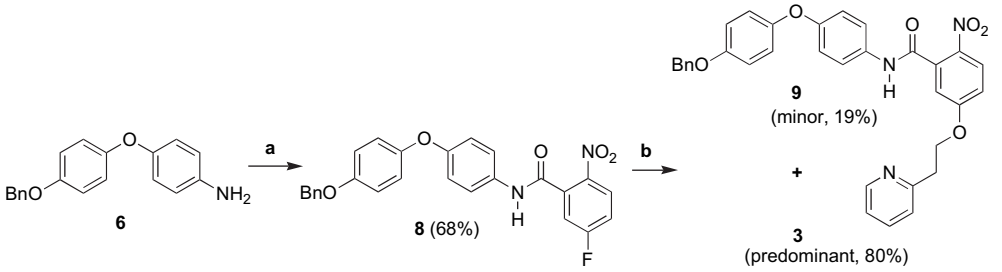
Intermediate **3** was not our initial choice of mimetic precursor. Instead, the aryl fluoride **8** in Scheme 2 was targeted on the basis that previous preliminary studies showed that the labile fluorine atom could be displaced by *N*-Boc-protected 3-aminopropanol as its alkoxide in DME. The aryl fluoride **8** was readily made by coupling our previously reported[7] aniline **6** with 5-fluoro-2-nitrobenzoic acid (**7**). In an initial side project on R17 guanidine isosteres, we investigated the use of pyridin-2-ylethanol and sodium hydride in DME to see if we could displace the labile fluoride to give a compound such as **9**. However, only occasionally did **9** result, and even then only in small yields, and the predominant product instead was repeatedly **3**. With large amounts of **3** at our disposal, we realised that alkylation of the phenolic group in **3** using readily available 3-aminopropylbromide, N-protected as the phthalimide, should provide a facile route to our other target mimetics **2a** and **2b**. As described above for Scheme 1, this indeed proved to be the case.

Although labile aryl fluoride atoms are reported to readily furnish phenols through alkaline hydrolysis,[11,12] model reactions led us to suggest that a mechanism involving base-catalysed beta-elimination of initially formed intended product **9** is involved in the production of **3**, facilitated by the acidic nature of the pyrid-2yl-methylene proton and the excellent leaving group ability of the *para*-nitrophenoxide anion, as shown in Scheme 3. The model reactions involved addition of 2,4-dinitrofluorobenzene to pyridin-2-ylethanol/NaH in DME, whereupon the only pyridine-containing species produced was 2-vinylpyridine. On the other hand, addition of the alkoxide to the model aryl fluoride, conditions under which excess strong base would not exist, gave the model product as intended. Our proposed mechanism is also supported by the published use of *S*-pyrid-2-ylethyl groups as surrogates for thiols, which are unmasked through a base-catalysed beta-elimination mechanism analogous to that postulated here.[13]

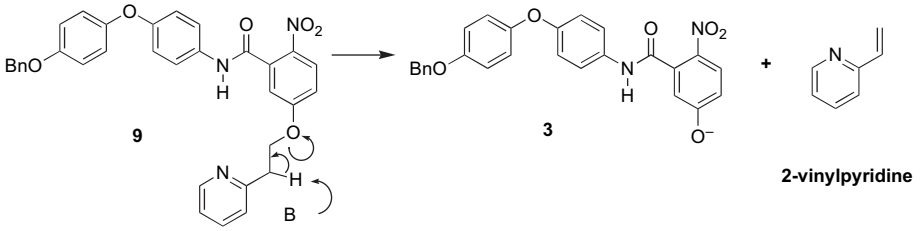
Other unexpected behaviour was encountered for this scaffold when we synthesised mimetics with a greater degree of orthogonal protection than that exhibited in **5c**. Thus,



Scheme 1. Synthesis of mimetic **2a** and its analogue **2b** from key precursor **3**. Reagents and conditions: (a) *N*′-(3-bromopropyl)phthalimide, K_2CO_3, DMF, 65 °C; (b) $Na_2S_2O_4$, EtOH, H_2O, reflux; (c) $CbzN(CH_2)_5CO_2H$, HBTU, HOBt, Et_3N, DMF, rt; (d) (i) $NaBH_4$, *i*-PrOH, H_2O, DCM, rt; (ii) CH_3CO_2H, reflux; (e) *N*,*N*′-bis(benzyloxycarbonyl)-1*H*-pyrazole-1-carboxamidine, DCM, rt; (f) H_2, Pd/C, MeOH/EtOH, rt; (g) H_2, Pd/C, trifluoroethanol, rt.



Scheme 2. Synthesis of key precursor **3** as the predominant by-product instead of the intended target **9**. Reagents and conditions: (a) 5-fluoro-2-nitrobenzoic acid (**7**), HBTU, Et_3N, DMF, rt; (b) 2-(2-hydroxyethyl)pyridine, NaH, DME, rt.

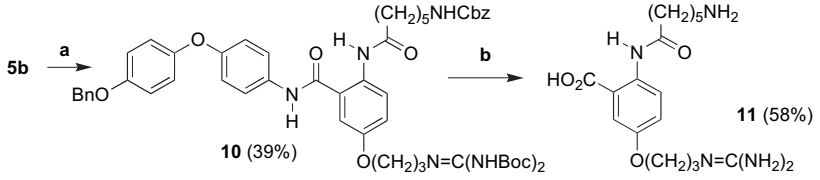


Scheme 3. Proposed base-induced (B) beta-elimination in **9** to account for the observed production of **3** under anhydrous conditions (see Scheme 2) where the intended product was **9**.

5b was readily converted to **10** by reaction with *N*,*N*′-bis(*tert*-butyloxycarbonyl)-1*H*-pyrazole-1-carboxamidine in DCM as shown in Scheme 4. However, when we attempted simultaneous bis-BOC and benzyl ether deprotection, using the TFA/thioanisole method of Kiso et al.,[14] only the cleaved compound **11** was isolated after work up and purification.

2.2. Mimetic binding affinity for $Ca_v2.2$ and $Ca_v2.1$

Mimetics **2a** and **2b** were assayed for binding to $Ca_v2.2$ using ^{125}I-GVIA as a ligand. The K2–Y13–R17 mimetic **2a** bound with a Ki of 3.5 μM while the K2–Y13–R17K mimetic **2b** bound with a Ki of 13 μM. These same mimetics were



Scheme 4. Synthesis of orthogonally protected mimetic **10** and its subsequent decomposition to **11** during attempted deprotection. Reagents and conditions: (a) *N*,*N*′-bis(*tert*-butyloxycarbonyl)-1*H*-pyrazole-1-carboxamidine, DCM, rt; (b) TFA/thioanisole, DCM, rt.

assayed for binding to $Ca_v2.1$ using ^{125}I-MVIIC as a ligand. The K2–Y13–R17 mimetic **2a** bound with a Ki of 111 μM while the K2–Y13–R17K mimetic **2b** bound with a Ki of 176 μM (Table 1).

Table 1. Binding potencies (μM) for mimetics **2a** and **2b** to $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) calcium channels (95% confidence intervals shown in parentheses)

Compound	Ki ($Ca_v2.2$)	Ki ($Ca_v2.1$)
2a	3.5 (2.5–4.8)	111 (70–180)
2b	13.1 (9.5–18.0)	176 (140–220)

3. Discussion

The relatively potent, low micromolar binding affinity for the K2–Y13–R17 mimetic **2a** to $Ca_v2.2$ (N-type calcium) channels is impressive and is made more remarkable by the selectivity of this compound for these channels over $Ca_v2.1$ (P/Q-type calcium) channels. This compound appears to be highly optimisable since the K2 and R17 mimetic side chains contain significant flexibility and are suited to conformational constraint. It is not proven that **2a** mimics the three targeted residues in GVIA exactly in binding to $Ca_v2.2$ as designed, but this is a reasonable assumption since molecular modelling has shown[9] that this scaffold is a good structural mimic of K2, Y13 and R17 in GVIA. This is more clearly shown in Figure 3, where the solid-state conformation of the scaffold[9] is superimposed on the NMR-derived solution conformation of GVIA, which is represented by its peptide backbone conformation as a yellow tube. All residues in GVIA have been removed apart from the side chains of the targeted residues. This makes it quite clear how the anthranilamide scaffold can mimic the α–β bond vectors of the targeted residues, K2, Y13 and R17, in GVIA and how, if suitably functionalised, it would be a structural K2–Y13–R17 GVIA mimetic.

We have previously reported[7] another K2–Y13–R17 mimetic using a benzothiazole-based scaffold (**1a**) and for this system, undertook more extensive testing of truncated analogues to show that each of the three mimetic side chains contributed to the low micromolar affinity for $Ca_v2.2$. For the current anthranilamide system, we have only investigated the variant **2b**, which contains a lysine-like side chain instead of an arginine-like side chain as the R17 side chain mimetic. However, the drop in potency that we see parallels the drop in potency that accompanies the analogous change in the benzothiazole-based mimetic system (**1a** to **1b**), and we propose that the mimetic side chains in **2a** all interact favourably with the ion channel as they do for **1a** and moreover, that both compounds are true GVIA mimetics.

Interactive de novo design can be the most efficient way to develop type-III mimetics of binding epitopes.[1,6,7,9,15,16] Here, synthetic and conformational knowledge is applied by the medicinal chemist interactively with a modelling package to design a suitable mimetic scaffold that is synthetically tractable, conformationally appropriate, not too

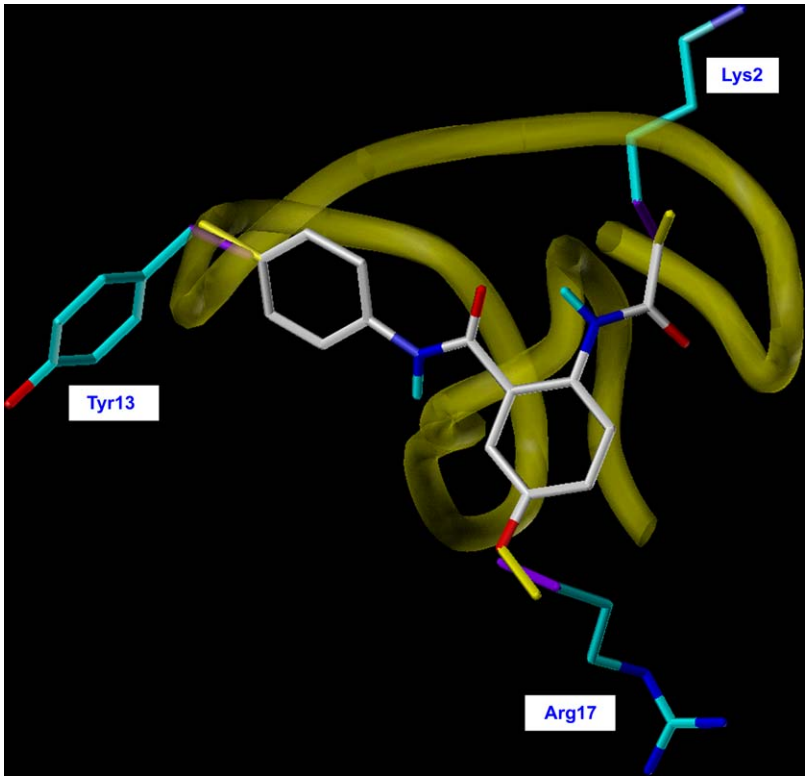


Figure 3. Three-dimensional picture of the anthranilamide scaffold 'core' superimposed on the NMR solution structure of ω-conotoxin GVIA. The peptide backbone is shown as a mustard yellow translucent tube. Only residues Lys2, Tyr13 and Arg17 of GVIA are shown, these in cyan with their respective C_α–C_β bonds being coloured purple. The scaffold is coloured by atom type with only the NH hydrogens being displayed for the sake of clarity. The mimetic C_α–C_β bonds are coloured yellow. It can be seen that the purple GVIA bonds match closely with their yellow counterparts in the mimetic. The conformation of the scaffold used is that determined by X-ray crystallographic analysis, as previously described.[9]

drug-unlike and highly analogable. Occasional structural relaxation with reference to known crystallographic substructure conformation is used in the process. Although this procedure is inherently suited to automation and computational approaches have been developed,[17] in our hands we find our approach to be the most successful and efficient. Binding epitopes targeted by interactive de novo design may vary from continuous and contiguous, to discontinuous and discontiguous[1,6,7,9,15,16] and so although we have targeted mimetics of pharmacologically active toxins, our approach may be suitable for other polypeptide–protein or protein–protein interactions. We are currently applying this technique with considerable success to the design of mimetics of the alpha-helical binding domain of pro-apoptotic BH3-only proteins in their complex with anti-apoptotic Bcl-2 homologues.[18] In the current work, we target a pharmacophore that is both discontinuous and discontiguous. In a sense, the disparate nature of the pharmacophore allows for more choice in the scaffold construction, and we have now shown how two, quite distinct scaffolds can act as GVIA mimetics in targeting the same three residues. This gives some additional versatility to the process where one scaffold may be interchanged for another with better ADMET properties.

One important factor in the success of our approach is the choice of neither more nor less than three residues to target. Less than three is unlikely to register activity, more than three is likely to render initial design and synthesis overly complex. In this regard, we found it quite surprising that mimetic **2a** is as potent as it is. Rudimentary thermodynamic considerations suggest that the side chains in this type of mimetic might contribute significantly more to the binding process than they do in GVIA itself.[1,7,15]

4. Conclusion

Non-peptide mimetics of ω-conotoxin GVIA have been prepared based on an anthranilamide scaffold that projects side chain mimetics of lysine, tyrosine and arginine in a similar respective manner to the projection of K2, Y13 and R17 in GVIA. In so doing, mimetic **2a** exhibits a Ki to $Ca_v2.2$ of 3.5 μM and is over 30-fold more selective for this channel than $Ca_v2.1$ channels. We have selected **2a** for further optimisation and assessment of functional antagonism of the $Ca_v2.2$ channel.

5. Experimental

All commercially obtained chemicals and reagents were used as received. Melting points were recorded on a Reichert hot stage melting point apparatus. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury 300 MHz spectrometer using the solvents specified; exchangeable NH and OH protons are only assigned where specified. ^{19}F-decoupled ^1H and ^{13}C NMR spectra were run on Bruker DPX 300 MHz spectrometer FTIR were run on Perkin–Elmer 1600 Series FTIR. ATR IR spectra were run on a Bruker IFS 55 FTIR Specac single reflection ATR system fitted with a single bounce diamond top plate. HRMS of compounds were recorded on a Bruker BioApex 47e Fourier Transform mass spectrometer. Low Resolution MS was recorded on a Micromass Platform II mass spectrometer. The compounds analysed were dissolved in organic solvent and ionised using an electrospray ionisation source. MS data are recorded as positive electrospray ions unless indicated by (ESI-) where negative ions are reported. Elemental analyses were conducted by CMAS Chemical and Micro Analytical Services Pty. Ltd. (P.O. Box 248, Belmont, Victoria 3216, Australia).

5.1. 2-(6-Aminohexanoylamino)-5-(3-guanidinopropoxy)-*N*-[4-(4-hydroxyphenoxy)-phenyl]-benzamide (2a)

The fully protected compound (**5c**) (17.5 mg, 0.017 mmol) was dissolved in trifluoroethanol (6 mL) and 10% Pd/C was added. The reaction mixture was stirred under H_2 at atmospheric pressure for 4 h. The catalyst was then removed by filtration and washed with MeOH (2×3 mL) and the combined filtrates and washings concentrated to dryness under reduced pressure. The solid was then triturated with DCM (2×3 mL), dissolved in water and centrifuged. Freeze-drying of the supernatant afforded **2a** as a white solid (5.8 mg, 64%). Mp 134–136 °C; ^1H NMR (300 MHz, methanol-d_4) δ ppm: 1.44 (m, 4H), 1.70 (p, J=7.2 Hz, 2H), 2.10 (p, J=7.2 Hz, 2H), 2.40 (t, J=6.0 Hz, 2H), 2.60 (t, J=6.9 Hz, 2H), 3.44 (t, J=6.8 Hz, 2H), 4.16 (t, J=5.9 Hz, 2H), 6.76–6.94 (m, 6H), 7.14 (dd, J=3.0, 9.0 Hz, 1H), 7.32 (d, J=2.7 Hz, 1H), 7.58 (dd, J=2.1, 9.0 Hz, 2H), 7.89 (d, J=8.7 Hz, 1H); ^{13}C NMR-APT (75 MHz, methanol-d_4; 1 unassigned ArC) δ ppm: 27.5 (CH_2), 28.3 (CH_2), 30.5 (CH_2), 34.3 (CH_2), 38.9 (CH_2), 40.4 (CH_2), 43.2 (CH_2), 67.4 (CH_2), 116.0 (CH), 118.6 (CH), 119.1 (CH), 119.3 (CH), 122.7 (CH), 124.7 (CH), 127.2 (CH), 130.4 (C), 132.1 (C), 134.7 (C), 150.5 (C), 157.7 (C), 158.3 (C), 159.6 (C), 169.5 (C), 175.5 (C); ATR (neat) cm^{-1}: 3278 (O–H), 2932, 2847 (aliphatic C–H), 1652, 1600 (C═O), 1497 (C═N); HRMS calcd for $C_{29}H_{36}N_6O_5$ (M+H$^+$) 549.28254, found 549.2828.

5.2. 2-(6-Aminohexanoylamino)-5-(3-aminopropoxy)-*N*-[4-(4-hydroxyphenoxy)-phenyl]-benzamide (2b)

The protected compound (**5b**) (26 mg, 0.036 mmol) was dissolved in absolute EtOH (5 mL) and MeOH (2 mL) and stirred under H_2 with 10% Pd/C for 3 h. The mixture was filtered and the filtrate evaporated to dryness. The residue was triturated with hexanes (4×8 mL) and ether (3×8 mL) and then dissolved in water and centrifuged. The supernatant was removed and freeze-dried to give **2b** as a green solid (4.8 mg, 31%). Mp 146–148 °C; ^1H NMR-COSY (300 MHz, methanol-d_4) δ ppm: 1.46 (p, J=7.5 Hz, 2H), 1.71 (m, J=8.2, 7.2 Hz, 4H), 2.20 (p, J=6.6 Hz, 2H), 2.43 (t, J=7.4 Hz, 2H), 2.91 (t, J=7.4 Hz, 2H), 3.19 (t, J=7.2 Hz, 2H), 4.22 (t, J=5.9 Hz, 2H), 6.81–6.96 (m, 6H), 7.14–7.18 (dd, J=3.0, 9.0 Hz, 1H), 7.37 (d, J=2.7 Hz, 1H), 7.52 (d, J=9.0 Hz, 2H), 7.93 (d, J=9.0 Hz, 1H); ^{13}C NMR (75 MHz, methanol-d_4) δ ppm: 26.9 (CH_2), 27.7 (CH_2), 29.1 (CH_2), 29.4 (CH_2), 38.6 (CH_2), 39.5 (CH_2), 41.4 (CH_2), 67.7 (CH_2), 112.5 (CH), 116.0 (CH), 116.1 (2CH), 117.9 (2CH), 118.2 (CH), 119.3 (2CH), 119.5 (2CH), 130.0 (C), 132.4 (C), 134.9 (C), 151.5 (C), 155.8 (C), 157.4 (C), 158.1 (C), 169.6 (C), 175.0 (C); ATR (neat) cm^{-1}: 3237 (O–H), 2927, 2849 (aliphatic C–H), 1647, 1596 (weak), 1496 (C═O); MS (ESI) (M+H$^+$) 507.3; HRMS calcd for $C_{28}H_{34}N_4O_5$ (M+H$^+$) 507.26075, found 507.2606.

5.3. *N*-[4-(4-Benzyloxyphenoxy)-phenyl]-5-hydroxy-2-nitrobenzamide (3)

2-(2-Hydroxyethyl)pyridine (0.1 mL, 0.89 mmol) was added to a slurry of NaH (60% in mineral oil, 21.6 mg, 0.54 mmol) in anhydrous DME (0.25 mL) under N_2. After 20 min, a solution of **8** (36 mg, 0.079 mmol) dissolved in anhydrous DMF (3 mL) was added dropwise over 25 min at room temperature. The reaction mixture changed from an orange-yellow colour to a brown colour overnight (24 h). DME was removed under reduced pressure and the resulting residue dissolved in EtOAc (30 mL). The EtOAc solution was washed with 2 M HCl (2×40 mL). Washings with 2 M NaOH (2×40 mL) were carried out until the aqueous solution became colourless. The organic layer was then washed with saturated brine (2×30 mL), dried ($MgSO_4$) and filtered. The organic solvent was removed under reduced pressure. Purification by radial chromatography using 100% EtOAc afforded **3** (28.7 mg, 80%) as a yellow solid after removal of solvent (a small amount of **9** (8.6 mg, 19%) was also furnished as a brown amorphous solid). ^{1}H NMR (300 MHz, acetone-d_6) δ ppm: 5.08 (s, 2H), 6.88–7.05 (m, 8H), 7.35–7.47 (m, 5), 7.62 (d, J=9.3 Hz, 2H), 8.09 (d, J=9.3 Hz, 1H), 10.50 (s, 1H, N*H*); ^{13}C NMR (75 MHz, acetone-d_6; 1 ArCH remains unassigned) δ ppm: 70.30 (CH2), 115.52 (CH), 116.13 (2CH), 118.40 (2CH), 120.31 (2CH), 121.50 (2CH), 127.41 (CH), 127.76 (2CH), 127.94 (CH), 128.59 (2CH), 134.48 (C), 136.49 (C), 137.68 (C), 138.21 (C), 151.05 (C), 154.60 (C), 155.20 (C), 163.08 (C), 164.43 (C); ATR (neat) cm^{-1}: 3217 s, 3047 m, 2875 w, 1580 m, 1494 s, 1444 m, 1386 w, 1323 s, 1263 m, 1209 s, 1102 w, 1063 m, 878 m, 819 m, 742 m, 695 m cm^{-1}. MS (ESI) *m/z* 457.3 [M+H]$^+$. HRMS: Found 456.1303 (requires 456.1321 for $C_{26}H_{20}N_2O_6$). Microanalysis: Found (%) C 68.51 H 4.38 N 6.19 (requires (%) C 68.42 H 4.42 N 6.14 for $C_{26}H_{20}N_2O_6$).

5.4. *N*-[4-(4-Benzyloxyphenoxy)-phenyl]-5-[3-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-propoxy]-2-nitrobenzamide (4a)

Phenol (**3**) (0.223 g, 0.489 μmol) and *N*-(3-bromopropyl)-phthalimide (0.131 g, 0.489 μmol) were dissolved in DMF (10 mL) with K_2CO_3 (0.27 g, 0.19 mmol) and the reaction mixture heated under nitrogen at 65 °C for 4 h. After allowing to cool to room temperature, the mixture was poured into iced 2 M HCl (60 mL) with stirring. The beige flocculant was filtered off and the residue washed with water. This precipitate was then dissolved in 40:60 EtOAc/THF, dried ($MgSO_4$), filtered and the solvent removed under reduced pressure. The crude residue was re-crystallised from EtOAc to give **4a** as a beige coloured powder (0.232 g, 74%). Mp 186–188 °C; ^{1}H NMR (300 MHz, acetone-d_6) δ ppm: 2.28 (p, J=6.2 Hz, 2H), 3.94 (t, J=6.5 Hz, 2H), 4.35 (t, J=5.9 Hz, 2H), 5.16 (s, 2H), 6.99–7.15 (m, 9H), 7.34–7.57(m, 5H), 7.75–7.91 (m, 5H+NH), 8.12–8.16 (m, 1H); ^{13}C NMR-APT (75 MHz, acetone-d_6) δ ppm: 27.9 (CH_2), 35.2 (CH_2), 66.7 (CH_2), 70.5 (CH_2), 114.1 (CH), 115.1 (CH), 115.8 (2CH), 116.2 (2CH), 120.1 (2CH), 121.9 (2CH), 123.2 (2CH), 126.9 (CH), 127.8 (CH), 127.3 (2CH), 128.4 (2CH), 131.7 (C), 132.6 (C), 134.1 (2CH), 135.4 (C), 136.7 (C), 138.6 (2C), 150.5 (C), 154.7 (C), 154.8 (C), 162.6 (2C), 164.5 (C), 168.4 (C); ATR (neat) cm^{-1}: 3274 (aromatic C–H), 2940, 2875 (aliphatic C–H), 1705 (C═O) 1510, 1208 (NO_2); HRMS calcd for $C_{37}H_{29}N_3O_8$ (M+Na$^+$) 666.1852, found 666.1846.

5.5. 2-Amino-*N*-[4-(4-benzyloxyphenoxy)-phenyl]-5-[3-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-propoxy]-benzamide (4b)

Nitrobenzene (**4a**) (0.288 g, 0.448 mmol) was dissolved in 95% EtOH (20 mL) and THF (15 mL) and heated to 80 °C. Upon dissolution of the solid, $Na_2S_2O_4$ (0.312 g, 1.8 mmol) was added, followed by H_2O (4 mL). The temperature was maintained at 80 °C for a further 2 h. The solvent was then removed under reduced pressure. EtOAc (40 mL) and 2 M HCl (40 mL) were added to the residue and the aqueous layer extracted with EtOAc (2×30 mL). The combined organic extracts were then washed with saturated $NaHCO_3$ (2×40 mL), saturated brine (2×30 mL) and dried ($MgSO_4$), filtered and evaporated to dryness to give **4b** as a light yellow solid (0.255 g, 93%). Mp 140–143 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 2.03 (p, J=6.4 Hz, 2H), 3.87 (t, J=6.9 Hz, 2H), 3.94 (t, J=5.7 Hz, 2H), 4.98 (s, 2H), 6.57–6.92, 7.08–7.09, 7.26–7.38, 7.52–7.55 (m, 16H), 7.60–7.76 (m, 4H); ^{13}C NMR-APT (75 MHz, $CDCl_3$; 1 ArCH and 11 ArC unassigned) δ ppm: 28.1 (CH_2), 35.3 (CH_2), 67.7 (CH_2), 70.5 (CH_2), 115.4 (CH), 115.8 (2CH), 118.4 (2CH), 119.0 (CH), 120.2 (2CH), 122.3 (2CH), 123.2 (2CH), 127.4 (2CH), 127.9 (CH), 128.5 (2CH), 131.9 (C), 132.9 (C), 133.9 (2CH); ATR (neat) cm^{-1}: 3461, 3372 (N–H), 3271 (aromatic C–H), 2951, 2862 (aliphatic C–H), 1811 (C═O), 1639 (phthalimide C═O); HRMS calcd for $C_{37}H_{31}N_3O_6$ (M+H$^+$) 614.2291, found 614.2291.

5.6. (5-{2-[4-(4-Benzyloxyphenoxy)-phenyl-carbomoyl]-4-[3-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-propoxy]-phenylcarbomyl}-pentyl)carbamic acid benzyl ester (5a)

Aniline (**4b**) (0.180 g, 0.29 mmol) and 6-benzyloxy-carbonyl aminohexanoic acid (0.156 g, 0.58 mmol) were dissolved in DMF (10 mL). Triethylamine (0.16 mL, 0.11 mol) was added, followed by HOBt (0.080 g, 0.5 mmol) and HBTU (0.223 g, 0.5 mmol). The reaction mixture was stirred at room temperature overnight then poured into 2 M HCl (50 mL). This aqueous solution was extracted with DCM (3×30 mL). The combined DCM extracts were washed with 2 M HCl (30 mL), then with saturated $NaHCO_3$ (2×30 mL) followed by saturated brine (2×30 mL). After drying ($MgSO_4$) and filtering, the DCM was removed under reduced pressure to give the crude product as a white precipitate. This precipitate was re-crystallised from EtOAc to afford **5a** as a white solid (0.228 g, 90%). Mp 157–159 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1.39 (p, J=6.6 Hz, 2H), 1.52 (p, J=7.5 Hz, 2H), 1.71 (p, J=7.5 Hz, 2H), 2.11 (p, J=6.2 Hz, 2H), 2.35 (t, J=7.4 Hz, 2H), 3.17 (q, J=6.4 Hz, 2H), 3.92 (t, J=6.8 Hz, 2H), 4.05 (t, J=5.6 Hz, 2H), 4.84 (s, 2H, 2NH), 5.06 (s, 2H), 5.07 (s, 2H), 6.90–7.04 (m, 8H), 7.15–7.16 (m, 2H), 7.28–7.49 (m, 9H), 7.56–7.59 (m, 2 H), 7.67–7.81 (m, 4H); ^{13}C NMR-APT (75 MHz, $CDCl_3$) δ ppm: 25.1 (CH_2), 26.3 (CH_2), 28.1 (CH_2), 29.7 (CH_2), 35.3 (CH_2), 37.7 (CH_2), 40.9 (CH_2), 66.6 (CH_2), 70.5 (CH_2), 77.0 (CH_2), 114.9 (CH), 115.9 (2CH), 118.2 (2CH), 119.1 (CH), 120.5 (2CH), 122.4 (2C), 122.5 (2CH), 123.2 (2CH), 123.4 (CH), 127.3 (4CH), 127.4 (CH), 127.9 (CH), 128.4 (2CH), 128.5 (2CH), 131.9

(C), 132.0 (2C), 133.1 (C), 134.0 (2CH), 136.5 (C), 136.8 (C), 150.3 (C), 153.6 (C), 155.0 (C), 155.4 (C), 166.8 (C), 168.4 (2C), 171.4 (C); ATR (neat) cm^{-1}: 3270, 3058 (amide N–H), 2936, 2869 (aliphatic C–H), 1711, 1502 (C═O); HRMS calcd for $C_{51}H_{48}N_4O_9$ ($M+Na^+$) 883.3319, found 883.3306.

5.7. (5-{4-(3-Aminopropoxy)-[4-(4-benzyloxyphenoxy)-phenylcarbonoyl]-phenylcarbamoyl}-pentyl)-carbamic acid benzyl ester (5b)

The phthalimide protected precursor (**5a**) (85 mg, 99 μmol) was dissolved in a solution of 6:1 *i*-PrOH/H_2O (10 mL), and DCM was added until a homogeneous mixture was obtained. To this was added $NaBH_4$ (0.019 g, 0.5 mmol) was added and the reaction mixture stirred at room temperature for 18 h. Acetic acid was added to adjust the pH to 2 and the reaction heated at 60 °C for 48 h. The solvent was then evaporated from the reaction mixture. Saturated $NaHCO_3$ (20 mL) was added and the resulting mixture was extracted with EtOAc (3×20 mL). The combined EtOAc extracts were washed with water (20 mL), saturated brine (2×20 mL), dried ($MgSO_4$) and filtered. After the removal of solvent from the filtrate, the residue was purified by radial chromatography using a 2:1 $CHCl_3$/MeOH eluent, or by first flushing through an 80:20 EtOAc/hexanes eluent, then eluting the product **5b** (40 mg, 55%) with MeOH. ^1H NMR (300 MHz, methanol-d_4) δ ppm: 1.39 (p, J=6.6 Hz, 2H), 1.50 (p, J=6.6 Hz, 2H), 1.69 (p, J=6.6 Hz, 2H), 2.19 (p, J=6.6 Hz, 2H), 2.39 (t, J=7.2 Hz, 2H), 3.07 (t, J=6.9 Hz, 2H), 3.18 (t, J=7.2 Hz, 2H), 4.21 (t, J=5.9 Hz, 2H), 5.06 (s, 2H), 5.09 (s, 2H), 6.94–7.05 (m, 6H), 7.13–7.6 (m, 15H); ATR (neat) cm^{-1}: 3463, 3367 (N–H), 3272, 3049 (aromatic C–H), 2946, 2877 (aliphatic C–H), 1710 (C═O); HRMS calcd for $C_{43}H_{46}N_4O_7$ ($M+H^+$) 731.3445, found 731.3444.

5.8. (5-{4-(3-[*N*,*N*′-Bis(benzyloxycarbonyl)-guanidino-propoxy])-[4-(4-benzyloxyphenoxy)-phenylcarbonoyl]-phenylcarbamoyl}-pentyl)-carbamic acid benzyl ester (5c)

Crude amine (**5b**) (0.106 g, 0.12 mmol) was dissolved in MeOH (4 mL) with DCM (2 mL) added to aid dissolution. Triethylamine was then added (0.05 mL, 0.51 mmol), followed by *N*,*N*′-bis(benzyloxycarbonyl)-1*H*-pyrazole-1-caboxamidine (38.3 mg, 0.10 mmol). The reaction mixture was stirred at room temperature for 8 h during which a white precipitate formed. The precipitate was filtered off and the filtrate concentrated to afford more precipitate. The combined precipitate was dissolved in DCM (15 mL) and washed with 2 M HCl (3×10 mL) followed by water (20 mL) and saturated brine (2×10 mL). The product was purified by radial chromatography using a 1:4 EtOAc/DCM solvent system to give **5c** as a white amorphous solid (56 mg, 44%) which solidified only after an extended period under vacuum (25 °C, 0.1 mmHg). Mp 114–116 °C; ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 1.36 (p, J=8.4 Hz, 2H), 1.51 (p, J=7.5 Hz, 2H), 1.70 (p, J=7.5 Hz, 2H), 2.05 (p, J=6.3 Hz, 2H), 2.35 (p, J=7.5 Hz, 2H), 3.16 (q, J=6.4 Hz, 2H), 3.66 (q, J=6.0 Hz, 2H), 4.06 (t, J=5.6 Hz, 2H), 5.05 (s, 2H), 5.06 (s, 2H), 5.06 (s, 2H), 5.09 (s, 2H), 6.90–6.93 (m, 6H), 7.20–7.45 (m, 25H); ^{13}C NMR-APT (75 MHz, $CDCl_3$) δ ppm: 25.0 (CH_2), 26.2 (CH_2), 28.4 (CH_2), 29.6 (CH_2), 37.9 (CH_2), 39.4 (CH_2), 40.8 (CH_2), 66.5 (CH_2), 67.0 (CH_2), 67.3 (CH_2), 38.2 (CH_2), 70.5 (CH_2), 114.1 (CH), 115.9 (2CH), 117.2 (CH), 118.2 (2CH), 120.5 (2CH), 122.8 (2CH), 123.3 (CH), 127.5 (4CH), 127.9 (2CH), 127.9 (2CH), 128.0 (2CH), 128.1 (2CH), 128.3 (2CH), 128.4 (2CH), 128.5 (CH), 128.6 (CH), 128.7 (CH), 128.8 (CH), 132.0 (C), 132.7 (C), 134.3 (C), 136.5 (C), 136.7 (C), 136.9 (2C), 150.3 (C), 153.7 (C), 153.9 (C), 155.1 (C), 155.6 (C), 155.8 (C), 156.4 (C), 163.4 (C), 167.1 (C), 171.6 (C); ATR (neat) cm^{-1}: 3228 (N–H), 3078, 3036 (aromatic C–H), 3743, 3871 (aliphatic C–H), 1729, 1679 (C═O), 1500 (C═N); HRMS calcd for $C_{60}H_{60}N_6O_{11}$ ($M+Na^+$) 1063.4218, found 1063.4220.

5.9. 5-Fluoro-2-nitrobenzoic acid (7)

2-Fluorobenzoic acid (1.807 g, 12.9 mmol) was dissolved in concentrated H_2SO_4 (100 mL) after which P_2O_5 (~8 g) was added. The mixture was then cooled to 0 °C and concentrated HNO_3 (15 mL) was added. After stirring for 3 h at 0 °C, the reaction mixture was poured into iced water (500 mL) and filtered. The precipitate was dissolved in DCM (30 mL) and the filtrate was extracted with DCM (3×30 mL). The combined DCM extracts were washed with water (2×30 mL) and saturated brine (2×40 mL). After drying ($MgSO_4$) and filtering, DCM was removed under reduced pressure to give **7** as a white solid (2.280 g, 95%). Mp 136–138 °C (lit.[19] 138–139 °C); ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 7.37 (m, 1H), 7.53 (dd, J=2.7, 7.8 Hz, 1H), 8.00 (dd, J=4.5, 9.0 Hz, 1H); ^1H (^{19}F-decoupled) NMR (300 MHz, $CDCl_3$) δ ppm: 7.37 (dd, J=1.8, 8.9 Hz, 1H), 7.52 (d, J=2.7 Hz, 1H), 8.00 (d, J=9.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 117.5 (d, J=25.4 Hz), 119.2 (d, J=23.1 Hz), 126.7 (d, J=9.4 Hz), 129.0 (d, J=8.6 Hz), 144.3 (s), 164 (d, J=256.7 Hz), 168.4 (s).

5.10. *N*-[4-(4-Benzyloxyphenoxy)-phenyl]-5-fluoro-2-nitrobenzamide (8)

4-(4-Benzyloxyphenoxy)-phenylamine (**6**) (0.889 g, 3 mmol) and 5-fluoro-2-nitrobenzoic acid (**7**) (0.558 g, 3 mmol) were dissolved in DMF (15 mL). Triethylamine (1.2 mL, 12 mmol) was added followed by HBTU (1.14 g, 3 mmol). The reaction mixture was stirred for 5 h at room temperature then poured into 2 M HCl (40 mL). The acidic mixture was then extracted with EtOAc (3×30 mL). The combined organic extracts were washed with water (2×25 mL), saturated brine (2×20 mL), dried ($MgSO_4$) and filtered. The EtOAc solution was then concentrated to one-third volume and cooled in ice to afford the coupled product as a beige coloured precipitate which was filtered off (0.940 g, 68%). Mp 177–179 °C; ^1H (^{19}F-decoupled) NMR (300 MHz, $CDCl_3$) δ ppm: 5.07 (s, 2H), 6.98 (s, 4H), 7.00 (s, 1H), 7.28–7.53 (m, 10H), 8.22 (d, J=9.0 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm: 70.52 (CH_2), 116.8 (2CH), 117.5 (d, J=25.7 Hz, CH), 118.5 (d, J=23.3 Hz, CH), 119.0 (2CH), 120.8 (2CH), 122.2 (2CH), 128.4 (CH), 128.5 (2CH), 128.6 (CH), 129.2 (2CH), 134.5 (C), 136.4 (C), 137.9 (C), 143.5 (d, J=3.1 Hz, C), 151.0 (C), 154.6 (C), 155.2 (C), 163.2 (C), 164.9 (d, J=253.6 Hz, C); ATR (neat) cm^{-1}: 3250 m, 3070 m, 2940 w, 2870 w, 1650 s, 1550 s, 1490 s, 1340 s, 1210 s, 1010 m, 820 m, 740 w; MS (ESI): *m/z* 459.2 $[M+H]^+$. HRMS: Found 481.1177 (requires 481.1176 for $[C_{26}H_{19}FN_2O_5]Na^+$). Microanalysis: Found

(%) C 68.07 H 4.12 N 6.08 (requires (%) C 68.12 H 4.18 N 6.11 for $C_{26}H_{19}FN_2O_5$).

5.11. *N*-[4-(4-Benzyloxyphenoxy)-phenyl]-2-nitro-5-(2-pyridin-2-yl-ethoxy)-benzamide (9)

This was obtained only in low yields from the reaction of 2-(2-hydroxyethyl)pyridine with **8**, as described earlier for the synthesis of **3**. Mp 99–102 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 3.31 (t, J=6. 5 Hz, 2H), 4.52 (t, J=6. 5 Hz, 2H), 5.05 (s, 2H), 6.95–7.05 (m, 9H), 7.19–7.52 (m, 8H), 7.67 (dt, J= 1.8, 7.8 Hz, 1H), 8.14 (d, J=8.7 Hz, 1H), 8.54 (dd, J=0.9, 5.7 Hz, 1H); ^{13}C NMR-APT (75 MHz, $CDCl_3$) δ ppm: 37.4 (CH_2), 68.0 (CH_2), 70.5 (CH_2), 114.2 (CH), 115.3 (CH), 115.8 (CH), 118.2 (CH), 120.2 (CH), 121.9 (CH), 122.2 (CH), 123.8 (CH), 127.0 (CH), 127.4 (CH), 127.9 (CH), 128.4 (CH), 132.1 (C), 135.1 (C), 136.6 (CH), 136.8 (C), 138.3 (C), 149.1 (CH), 150.4 (C), 154.8 (C), 155.1 (C), 157.1 (C), 162.8 (C), 164.4 (C); ATR (neat) cm^{-1}: 3283, 3065 (aromatic C–H), 2997, 2941 (aliphatic C–H), 1620 (C═O), 1582 (C═N); MS (ESI) (M+H$^+$) 562.5; HRMS calcd for $C_{33}H_{27}N_3O_6$ (M+Na$^+$) 548.17975, found 584.1793.

5.12. (5-{4-(3-[*N*,*N*′-Bis(*tert*-butoxycarbonyl)-guanidino-propoxy])-[4-(4-benzyloxyphenoxy)-phenylcarbonoyl]-phenylcarbamoyl}-pentyl)-carbamic acid benzyl ester (10)

The protected anthranilamide derivative (**5b**) (55 mg, 0.064 mmol) was stirred with 1-*H*-pyrazole-1-(*N*,*N*′-bis-(*tert*-butoxycarbonyl)]-caboxamidine (20 mg, 0.064 mmol) in DCM (5 mL) overnight. The DCM was then removed under reduced pressure. Excess guanidylating agent was removed by triturating with hexanes (3×3 mL). After purification of the residue by radial chromatography using a 50:50 EtOAc/hexanes solvent system, **10** was obtained as a white amorphous solid (24 mg, 39%). Mp 56–59 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1.43, 1.45 (s, 18H), 1.34 (p, J=7.2 Hz, 2H), 1.49 (p, J=7.2 Hz, 2H), 1.65 (p, J=7.2 Hz, 2H), 2.02 (p, J=7.2 Hz, 2H), 2.31 (t, J=7.5 Hz, 2H), 3.14 (t, J=6.4 Hz, 2H), 3.58 (t, J=6.0 Hz, 2H), 4.03 (t, J= 5.4 Hz, 2H), 5.29 (s, 2H), 5.31 (s, 2H), 6.94–6.97 (m, 6H), 7.10–7.54 (m, 15H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 25.1 (CH_2), 26.3 (CH_2), 28.1 ($3CH_3$), 28.3 ($3CH_3$), 28.7 (CH_2), 29.7 (CH_2), 37.9 (CH_2), 39.1 (CH_2), 40.9 (CH_2), 66.5 (CH_2), 67.0 (CH_2), 70.5 (CH_2), 79.4 (C), 83.1 (C), 113.7 (CH), 115.8 (2CH), 117.3 (CH), 118.2 (2CH), 120.4 (2CH), 122.6 (2CH), 122.9 (CH), 123.2 (CH), 127.4 (2CH), 127.7 (2CH), 128.4 (2CH), 128.5 (3CH), 132.1 (C), 132.3 (C), 136.5 (C), 136.8 (C), 150.3 (C), 152.9 (C), 153.9 (2C), 154.9 (C), 155.3 (C), 156.0 (C), 156.2 (C), 163.3 (C), 167.0 (C), 171.4 (C); ATR (neat) cm^{-1}: 3314 (N–H), 2925, 2856 (aliphatic C–H), 1718, 1607 (C═O), 1494 (C═N); HRMS calcd for $C_{54}H_{64}N_6O_{11}$ (M+H$^+$) 973.4711, found 973.4713.

5.13. 2-(6-Aminohexanoylamino)-5-(3-guanidino-propoxy)-benzoic acid (11)

To the bis-Boc protected compound (**10**) (25.6 mg, 26 μmol) was added thioanisole (0.15 mL, 1.3 mmol) and TFA (1 mL). The clear, colourless solution turned red upon addition of TFA. The resulting mixture was stirred at room temperature for 8 h. The solvent was then removed under reduced pressure and the residue triturated with hexanes (6×5 mL) followed by ether (4×5 mL) to remove any residual thioanisole. The crude compound was dissolved in DCM and purified by flushing through a silica plug with a 50:50 EtOAc/hexanes eluant. The most polar band was eluted with MeOH to give **11** (5.6 mg, 58%) as a brown solid. Mp 99–102 °C; ^{1}H NMR (300 MHz, methanol-d_4) δ ppm: 1.51 (p, J=6.3 Hz, 2H), 1.78 (m, 4H), 2.09 (q, J=6.3 Hz, 2H), 2.47 (t, J=7.4 Hz, 2H), 2.97 (t, J=7.4 Hz, 2H), 3.44 (t, J=6.8 Hz, 2H), 4.11 (t, J=5.7 Hz, 2H), 7.09 (d, J=9.0 Hz, 1H), 7.64 (s, 1H), 8.43 (d, J=9.0 Hz, 1H); ^{13}C NMR (75 MHz, methanol-d_4; 2 ArC remain unassigned) δ ppm: 26.9 (CH_2), 27.69 (CH_2), 29.09 (CH_2), 30.59 (CH_2), 39.59 (CH_2), 40.59 (CH_2), 41.39 (CH_2), 67.29 (CH_2), 118.3 (CH), 119.7 (C), 120.3 (CH), 123.6 (CH), 131.8 (C), 156.2 (C), 159.7 (C); ATR (neat) cm^{-1}: 3440 (O–H), 3166, 3063 (aromatic C–H), 2952, 2923, 2868 (aliphatic C–H), 1660, 1589 (C═O), 1449 (C═N); HRMS calcd for $C_{17}H_{27}N_5O_4$ (M+H$^+$) 366.21423, found 366.2153.

6. Biological methods

Peptide synthesis, radiolabelling of the peptides with ^{125}I and rat brain preparation were conducted following previously described procedures.[20–22] Radioligand binding assays were run in triplicate in 96-well plates at room temperature. Each well of the 96-well plate (Polystyrene, Round bottom, Nunc™, Denmark) contained compound **2a** or **2b** (first dilution 0.6 μM of compound total of seven dilutions, 1:10), 5–10 fmol of radiolabelled peptide (^{125}I-GVIA) and 8 μg of crude rat membrane (added last). All dilutions were made in assay buffer (20 mM HEPES, 75 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 μM Leupeptin, 2 μL apoprotinin (to 30 mL assay buffer), 0.1% BSA, pH 7.4) and the final volume in each well was 150 μL. The plate was left on a shaker for 1 h at room temperature before being filtered. Incubation was terminated by washing the plate with wash buffer (20 mM HEPES, 125 mM NaCl, pH 7.4) and filtered under vacuum (Tomtec). The glass fibre filter used (90×120 mm, double thickness, Wallac, Finland) was soaked in 0.6% polyethyleneimine immediately prior to filtering to reduce non-specific binding. The filter was put in a Sample Bag (Wallac, Finland) containing 8 mL BetaPlate Scint (Wallac, Finland) and the radioactivity bound to the filter was counted using a 1450 MicroBeta Wallac Jet (Wallac, Finland). The data were analysed using GraphPad Prism 2.0 (GraphPad Software, Inc, San Diego, USA).

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