Preparation of novel alkylated arginine derivatives suitable for click-cycloaddition chemistry and their incorporation into pseudosubstrate- and bisubstrate-based kinase inhibitors†

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Efficient strategies for the introduction of arginine residues featuring acetylene or azide moieties in their side chains are described. The substituents are introduced in a way that maintains the basicity of the guanidine moiety. The methodology can be used *e.g.* for non-invasive labeling of arginine-containing peptides. Its applicability is demonstrated by the introduction of 'click' handles into a Protein Kinase C (PKC) pseudosubstrate peptide, and the subsequent preparation and evaluation of a novel bisubstrate-based inhibitor based on such a peptide.

Introduction

Because of its permanent positive charge at physiological pH, the amino acid arginine often plays a crucial role in the interactions of peptides and proteins, *e.g.* in kinase substrates,[1] carbohydrate binding domains,[2] nucleic acid binding domains[3] and cationic antimicrobial peptides.[4]

There is a growing need for methods to selectively and efficiently attach auxiliary moieties to peptides, *e.g.* for the introduction of probes used in proteomics protein 'fishing' experiments or for the attachment of radioactive ligands.[5] Obviously, it is particularly advantageous if such moieties can be introduced into unprotected peptides with minimal change to the peptide's structure and properties.

Here, we present a strategy to introduce substituted arginine residues suitable for the well-known chemoselective acetylene–azide 'click' reaction.[6] This reaction can be carried out on unprotected peptides virtually irrespective of the sequence.[7] The modified arginine residues described here bear azido or acetylene groups on the guanidine side chain that are introduced by alkylation, and therefore the often crucial basicity of the guanidine side chain is maintained. In addition, the azido-containing building blocks have ethyleneglycol spacers of variable length, which allows fine tuning of the structural requirements of a modified peptide for biological activity. Finally, this methodology is compatible with routine Fmoc–*t*Bu solid phase peptide synthesis. In contrast, most methods currently described for the preparation of alkylated arginine residues,[7] do not offer a general, convenient strategy for monomers with suitable protection for solid phase peptide synthesis.[8]

Recently, our group described a strategy for the preparation of several different functionalised arginine building blocks suitable for incorporation into peptides.[9] In that synthesis, an alternative guanylation procedure was employed using amines. In the method described here, alcohols are used for the required modified arginine residues, thus widening the scope for preparation of such building blocks.

As an interesting application, the preparation of functionalised kinase substrate peptides is described, which contain arginine residues close to the phosphorylation site. These 'clickable' peptides can then be chemoselectively ligated to ATP binding site inhibitors, yielding so-called bisubstrate-based inhibitors.[10,11] Such inhibitors feature both high affinity, due to the ATP-competitive inihibitor part, as well as high selectivity as a result of the peptide part, which can bind to the high selectivity but low affinity peptide binding groove of kinases.

As an example of the applicability of the novel arginine derivatives, a series of modified peptides will be described here based on a sequence we have recently[12] identified as a suitable peptidic component for the preparation of isozyme-selective Protein Kinase C (PKC) bisubstrate-based inhibitors. Then, one of these modified peptides is attached to an ATP-competitive inhibitor using 'click' chemistry to afford a novel bisubstrate-based inhibitor. The PKC family is involved in diseases such as cancer and diabetes, and have attracted attention as potential drug targets for a long time.[13] Homology between the twelve isoforms of the PKC family is very high, in particular for their catalytic domains, and selective inhibition is a difficult issue, making preparation of selective inhibitors a challenging but rewarding prospect.

Results

Chemistry

The synthesis of several Fmoc-protected arginine derivatives is outlined in Scheme 1 (routes A and B). As can be seen, we decided on employing pyrazole guanylation[14] to convert a suitably protected ornithine derivative into the corresponding arginine

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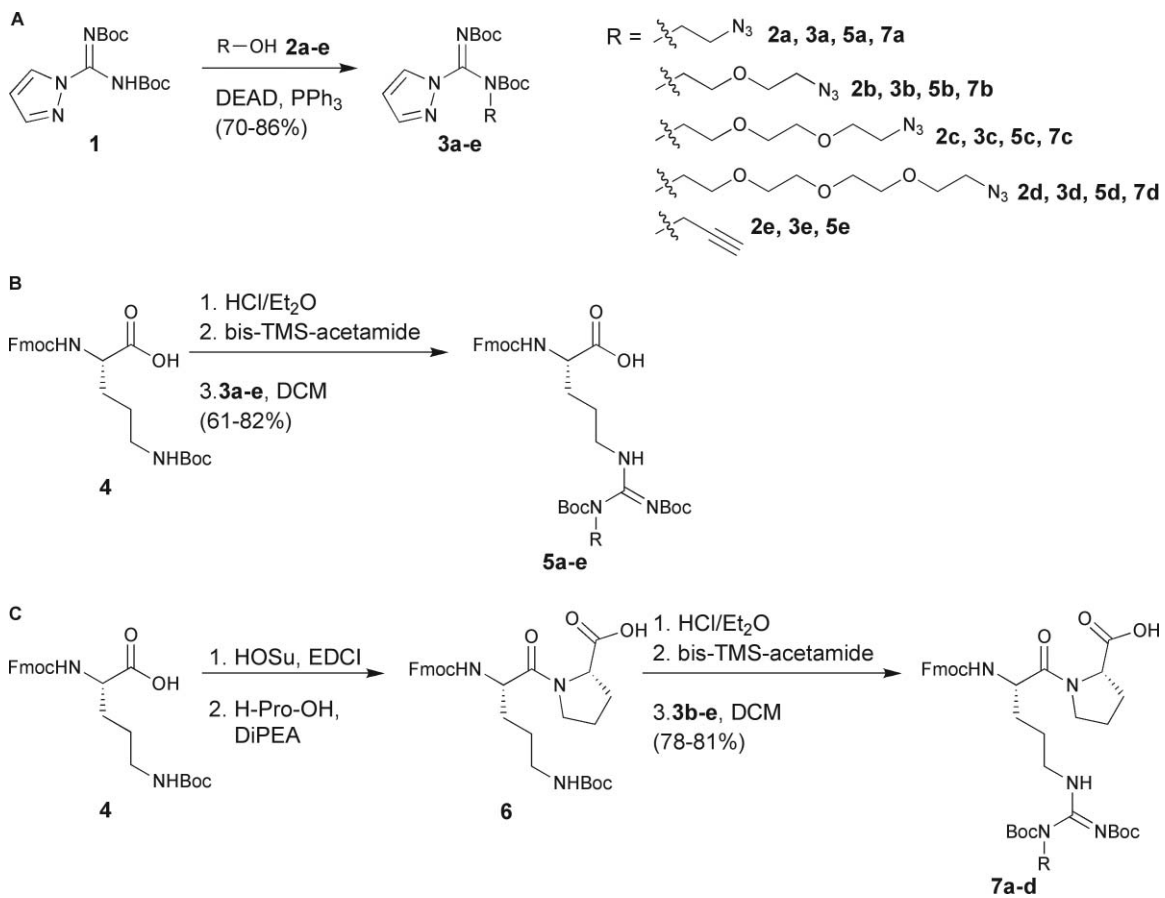
building blocks. The pyrazole derivatives used in this methodology usually react cleanly and can be conveniently furnished with a side-chain using a Mitsunobu reaction.[15]

The required pyrazole derivatives **3a–e** were prepared by treating commercially available *N*,*N*-bis-Boc-guanylpyrazole **1** with either propargyl alcohol **2a**, or known azido oligoethyleneglycols **2b–e**,[16] in the presence of DEAD and triphenylphosphine (Scheme 1, route A). Although these Mitsunobu alkylations proceeded slowly, clean product formation was observed in all cases, and reagents **3a–e** were obtained in 70–86% yield after purification by column chromatography.

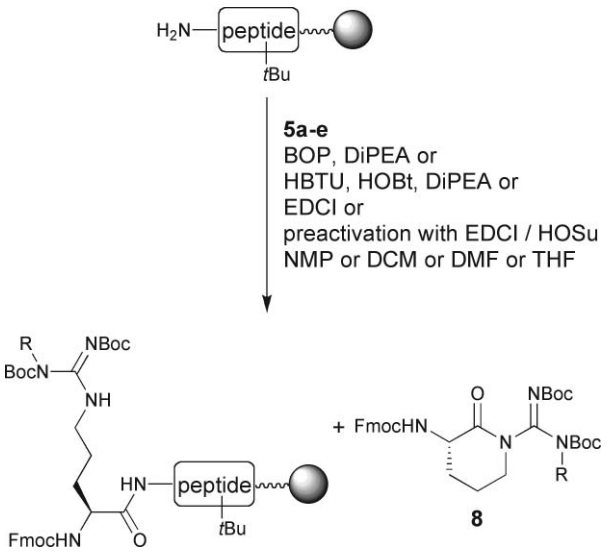
For the preparation of building blocks suitable for Fmoc–*t*Bu solid phase peptide synthesis, arginine derivatives **5a–e** were envisaged. These could be conveniently prepared from commercial Fmoc-Orn(Boc)-OH **4** (Scheme 1, route B). First, the Boc group was removed using a saturated solution of HCl in diethylether. TFA could also be used but was tedious to remove completely, which is deleterious for the subsequent step, generally leading to lower overall yields. After deprotection, the carboxylic acid was protected *in situ* as a trimethylsilyl ester by treatment with *N*,*O*-bis(trimethylsilyl)acetamide in the presence of DiPEA. These conditions were inspired by a paper on guanylation with trifluoromethyl sulfonamides.[17] The resulting precipitates went into solution virtually instantaneously, after which guanylating agents **3a–e** were added. Although considerable conversion was observed on TLC after 3 h, complete consumption of starting material only occurred after stirring for 24 h at room temperature. During subsequent acidic work-up, the trimethylsilyl group was cleaved, after which the alkylated arginine building blocks **5a–e** could be obtained in good yields (61–82%).

Next, we evaluated the applicability of these substituted arginine building blocks in solid phase peptide synthesis. Initial attempts to incorporate building block **5a–e** into several peptide sequences on the solid phase led to low coupling efficiency in many cases, depending on coupling conditions (coupling reagents, added equivalents and solvent) and on the peptide sequence. Since the corresponding non-alkylated arginine building block has been coupled successfully in the past,[18] the difficulties observed here were surprising.

When the coupling was carried out in DCM, the solvent could be evaporated and the reaction mixture analysed. This led to the isolation of side product **8** in near-quantitative yield (Scheme 2). This product can be explained by nucleophilic attack of the guanidine NH on the intermediate activated ester, leading to a favourable six-membered ring, and can be considered as a 6-membered ring derivative of the Freidinger lactam.[19] Because this cyclisation was clearly very efficient under the conditions where side product analysis was possible, it can be assumed to be the cause of low coupling yield, since the coupling reagent is present in all cases. As stated above, the efficiency of the cyclisation in this case was surprising, since the corresponding Boc-protected non-alkylated arginine may be coupled without problems. In all



Scheme 1 Synthesis of the "Arg-Pro" building block.



Scheme 2 Side product formation.

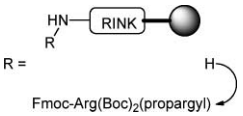
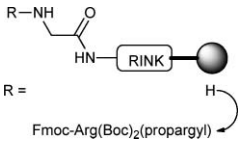
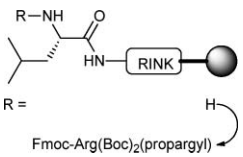
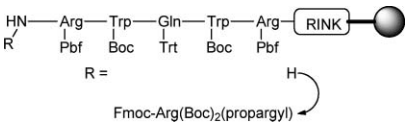
probability, the guanidine protecting group selected is important, and we have indeed found that Pbf-protected alkylated arginine residues may be coupled efficiently.[9]

On evaluation of coupling conditions (Table 1), we found that good efficiency (>90% as judged by subsequent Fmoc-determination) could be achieved by treating the resin with a solution of 5 equivalents of **5a–e** in DCM followed by addition of 5 equivalents of DIC. As expected, coupling conditions where a base was present in the reaction mixture enhanced the efficiency of the side reaction, leading to lower yields (conditions **A** and **B**). Therefore, neutral coupling conditions using DIC are clearly preferred. However, even without base, the presence of merely the coupling reagent appeared sufficient to promote significant side product formation (conditions **C** and **D**). Even using the preferred procedure (condition **E**), there was competition between coupling and cyclisation, and an excess of monomer was required. However, since in this case there was no pre-activation time, the probability of reaction by coupling was increased, leading to an optimal coupling efficiency. This procedure worked well for coupling the monomers to bare Rink-resin[20] and to glycine or leucine modified resin, and to the peptide sequence Arg-Trp-Gln-Trp-Arg derived from anti-microbial peptide Lactoferricin B[21] leading to H-Arg(R)-Arg-Trp-Gln-Trp-Arg-NH_2.

However, when the building blocks were coupled to a proline residue, poor conversion was observed even with these optimised conditions, presumably due to the increased steric demand of the proline nucleophile, which allows more time for the side reaction to occur.

In order to devise a generally applicable alternative methodology for the incorporation of these modified arginine building blocks, two alternative strategies were envisaged based on either incorporation of dipeptide building blocks or orthogonal deprotection of a complete peptide followed by guanylation. Both methods were evaluated as part of the preparation of a series of modified peptides based on a sequence which we have recently described as a suitable component of bisubstrate-based inhibitors of individual PKC isozymes.[12] The peptide sequence of this inhibitor was identified from a microarray containing hundreds of

Table 1 Solid phase evaluation of building block **5a**

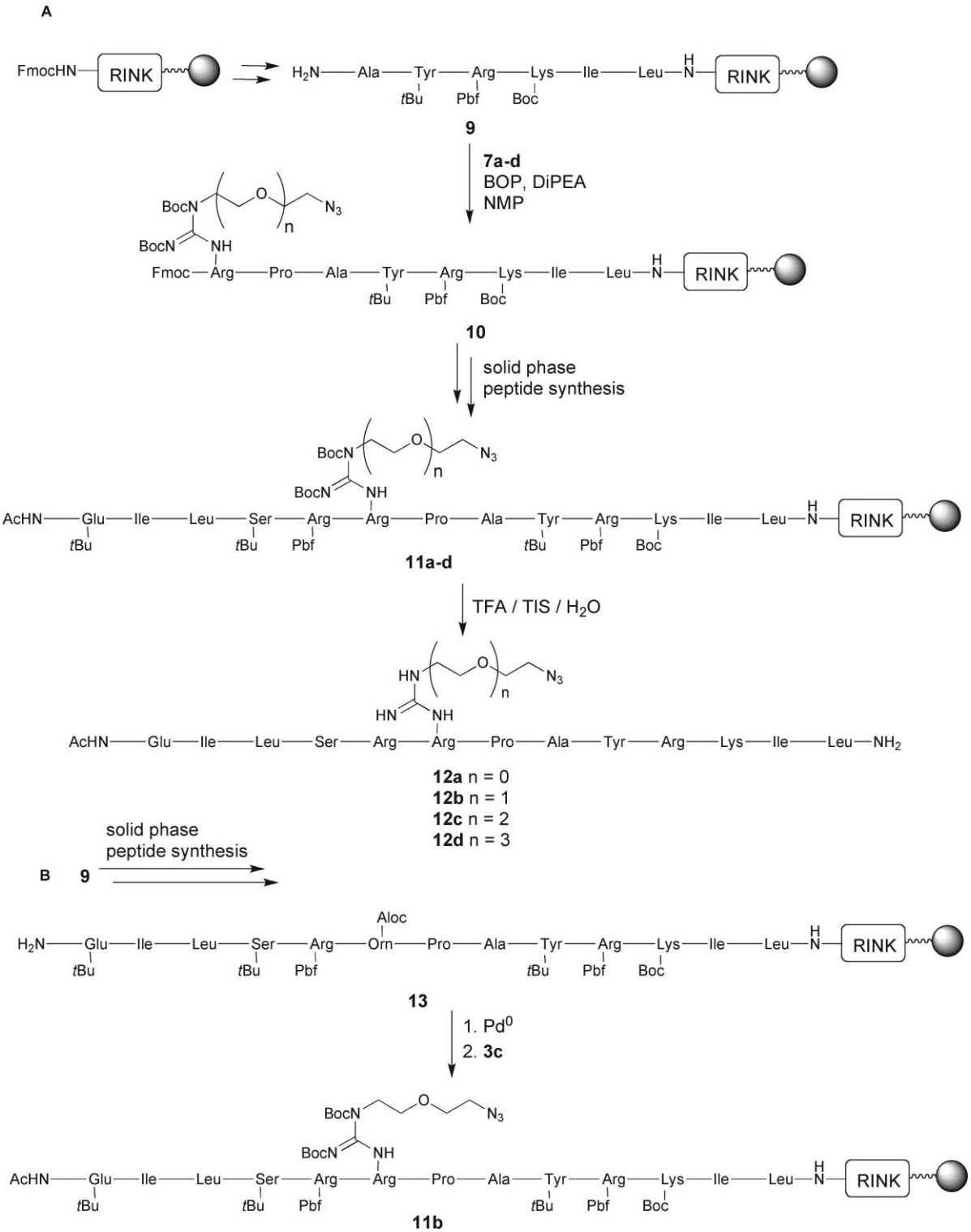
Resin/product	Equiv. **5a**	Cond.[a]	Yield (%)[b]
	2	A	<10
	5	A	15
	2	B	<10
	5	B	41
	5	C	<10
	5	D	28
	5	E	>95
	5	E	>95
	5	E	>95
	2	A	<10
	5	A	<10
	5	B	34
	5	E	>95

[a] Conditions: **A** **5a**/BOP/DiPEA in NMP added as a solution. **B** **5a**/HOAt/HATU/DiPEA in NMP added as a solution. **C** **5a**/DIC in NMP added as a solution. **D** **5a**/DIC in DCM added as a solution. **E** **5a** in DCM added as a solution, followed by DIC. In all cases the amount of coupling reagent added corresponded to the number of equivalents of **5a**. Where a base was used, its amount corresponded to two times the number of equivalents of **5a**. [b] Yields are determined through spectrophotometric measurement of the amount of Fmoc cleavage product after treatment of a known quantity of resin with 20% piperidine in NMP.

kinase (peptide) substrates by investigating the phosphorylating activity of three highly homologous PKC isozymes. It was shown that this peptide sequence was phosphorylated more strongly by PKCζ compared to PKCα and PKCθ.

The first synthetic strategy used dipeptide building blocks containing a modified arginine residue. These building blocks were prepared as shown in Scheme 1 (route C). Dipeptides **7a–d** were prepared first by the coupling of unprotected proline, which is present at the *C*-terminus of the modifed arginine in the inhibitor peptide sequence, to the succinimidyl ester of commercially available Fmoc-Orn(Boc)-OH **4**. Then, after acidolysis of the Boc-group in the resulting dipeptide **6**, a guanylation was performed using azido-reagents **3a–d** in a similar procedure as the preparation of arginine building blocks **5a–e** following route B. The reaction proceeded cleanly in all cases and after column chromatography, dipeptides **7a–d** were obtained in 78–81% yield.

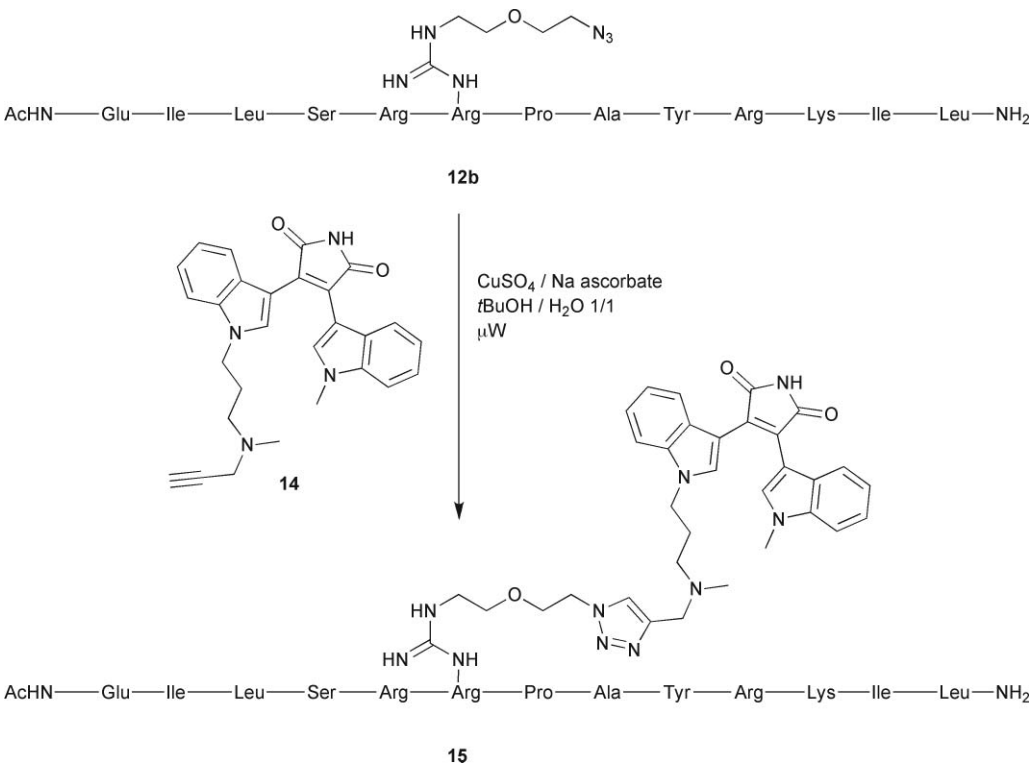
In order to prepare the desired inhibitor peptides, sequence **9** was first prepared using solid phase peptide synthesis (Scheme 3, route A). Then, dipeptide building blocks **7a–d** were coupled under normal coupling conditions with excellent efficiency within 45 min as judged by the Kaiser test[22] and Fmoc-determination. Synthesis of the remainder of the peptide proceeded uneventfully and after cleavage from the resin, a single main product was observed on RP-HPLC which could be purified by preparative HPLC, after



Scheme 3 Solid phase synthesis of pseudosubstrates **12a–d**.

which the identity of peptides **12a–d** was confirmed by mass spectrometry.

In an alternative approach (Scheme 3, route B), peptide **13** was prepared containing an Aloc-protected ornithine residue. This protecting group was selectively removed on the solid phase using [$Pd(PPh_3)_4$] and a scavenger. After this deprotection, the peptide was treated on the solid phase with a solution of pyrazole derivative **3c** and triethylamine in THF. Reaction overnight at room temperature resulted in a negative Kaiser test, indicating successful guanylation to the alkylated arginine derivative in **11b**. The peptide was then cleaved and purified using preparative HPLC, affording peptide **12b**.



Scheme 4 Synthesis of bisubstrate based inhibitor **15**.

Biological activity

We decided to evaluate the biological activity of the peptides containing a modified arginine residue by converting one of them into a potential bisubstrate-based inhibitor for PKC. Thus, peptide **12b** was treated with ATP-competitive inhibitor **14** in the presence of copper sulfate and sodium ascorbate under microwave irradiation. This led to clean conversion into the desired bisubstrate-based inhibitor **15** in 62% yield after purification by preparative HPLC (Scheme 4).

Inhibitor **15** was applied in a dynamic peptide microarray-based kinase inhibition assay using three highly homologous PKC isozymes (PKCα, ζ and θ). PKCα is a subject of cancer research because of its ability to regulate phospholipase D,[23] PKCζ is involved in the immune system[24] and long-term memory,[25] and PKCθ has recently emerged as a target in T cell leukaemias.[11] The Pamchip® microarray setup used employs chips made of a highly porous material onto which several hundreds of peptides derived from endogenous kinase substrates are immobilised in discrete spots.[12,26] The porosity of the surface allows mixtures containing a kinase, co-factors and fluorescent antibodies against phosphorylated amino acid residues for detection, to be pumped through the solid support of the microarray, so that fluorescence pictures may be taken from the top-side of the chip while the analyte droplet with bulk background fluorescence is underneath the surface. By taking pictures at discrete time intervals, the development of fluorescence, and therefore the progress of phosphorylation, can be monitored in real-time, and kinetic data on the kinase reaction may be obtained.

The inhibition data for **15** derived from initial velocities (v_{ini}) measured in the presence of different inhibitor concentrations is summarised in Table 2. As can be seen, attachment of the peptide containing the modified arginine residue has a marked effect on both the affinity and selectivity of the resulting bisubstrate-based inhibitor. The IC_{50} values obtained for **15** were significantly better than those of ATP-competitive inhibitor **14**. It should be noted that for the inhibition experiments, a high concentration of ATP (100 μM) was used to obtain strong signals throughout the experiments, and K_i values can be expected to be in the low nanomolar range. We previously demonstrated that structures such as bisubstrate-based inhibitor **15** are competitive inhibitors both with respect to the ATP- and peptide-binding site.[12] Therefore the K_i may be estimated using the Cheng–Prusoff equation[27]

Table 2 Inhibition data of compounds **12b**, **14** and **15**

		IC_{50}/μM[a]		
Enzyme	Substrate[b]	**14**	**12b**	**15**
PKCα	CREB1	1.8 ± 0.6	—	—
	KPCB	2.9 ± 0.9	—	—
	MARCS	1.9 ± 0.5	—	—
PKCζ	CREB1	—	—	0.33 ± 0.029
	KPCB	—	—	0.23 ± 0.016
	MARCS	—	—	0.59 ± 0.026
PKCθ	CREB1	2.9 ± 0.3	—	0.81 ± 0.019
	KPCB	2.3 ± 0.3	—	0.45 ± 0.012
	MARCS	2.9 ± 0.4	—	0.17 ± 0.029

[a] If no value is given, no inhibition was observed up to 10 μM. Errors are given as standard error of the mean over six experiments. [b] CREB1 (cAMP response element binding protein 126-138, EILSRRPSYRKIL), KPCB (PKC splice isoform β-II 19-31, RFARKGSLRQKNV), MARCS (myristoylated alanine-rich C-kinase substrate 152-164, KKKKKRFSKKSF) and NCF1 (neutrophil cytosol factor 1 296-308, RGAPPRRSSIRNA).

($K_i = IC_{50}/(1+[S]/K_m)$). Employing a $K_{m,ATP}$ of 5 μM previously determined for the assay described here,[12] this leads to a K_i of 8–39 nM. The peptide part (**12b**) itself was unable to significantly inhibit any of the PKC isozymes at the concentrations tested (10 nM–10 μM).

With respect to the selectivity, ATP-competitive inhibitor **14** only inhibits PKCα and PKCθ, whereas bisubstrate-based inhibitor **15** does not inhibit PKCα any more, and instead has affinity for PKCζ and PKCθ. Gratifyingly, this matched the selectivity profile originally observed for this particular peptide sequence as noted above. This clearly demonstrates the influence of the peptide part on the inhibitory properties of **15**. It is interesting to note that the previously studied[12] bisubstrate-based inhibitors, which contained a neutral amino acid residue instead of the positively charged modified arginine monomer in **15**, did not show any inhibitory activity against PKCζ, and instead were selective towards PKCθ.

Both the improved affinity and changed selectivity profile demonstrate the power of the novel bisubstrate-based inhibitors such as **15**, and therefore the usefulness of the alkylated charged arginine residues described in this paper.

Conclusions

We have successfully prepared a series of guanylating reagents and building blocks for the introduction of arginine derivatives functionalised with 'click' handles on variable spacers. The monomeric building blocks may be used in certain sequences under carefully controlled coupling conditions, however, with demanding sequences, coupling efficiency may be low. Alternatively, dipeptide building blocks are easily accessible and can be incorporated without problems. Furthermore, it is also possible to employ the guanylating reagents described in this paper to directly derivatise a complete peptide by incorporating orthogonally protected ornithine residues at the desired locations, followed by treatment with one of the pyrazole derivatives described here.

The resulting modified peptides may be used to attach different cargos to arginine-containing peptides in an orthogonal way using click-cycloaddition chemistry. This was illustrated by the incorporation of an ATP-competitive inhibitor, leading to bisubstrate-based kinase inhibitor **15**, and can be easily extended to the introduction of, for example, fluorescent labels and radionuclide chelators.

The thus developed bisubstrate-based kinase inhibitor was tested for affinity and selectivity towards three highly homologous PKC isozymes. The resulting compound showed improved affinity and a highly interesting shift in selectivity.

Experimental

General remarks

All reactions were carried out at ambient temperature unless stated otherwise. All reagents were used as supplied from commercial sources unless stated otherwise. THF was distilled from $LiAlH_4$ prior to use. DCM, NMP and DiPEA were stored on molecular sieves (4 Å) prior to use. R_f values were determined by thin layer chromatography (TLC) on Merck precoated silicagel $60F_{254}$ plates. Spots were visualized by UV-quenching, ninhydrin or Hanessian's reagent. Column chromatography was carried out using Silicycle UltraPure silicagel (40–63 μm). For optical rotations $[\alpha]_D$ values are expressed as 10^{-1} deg cm^2 g^{-1}. FTIR spectra were recorded on a PerkinElmer Spectrum™ 100 system using the ATR accessory. ^{1}H NMR spectra were recorded on a Varian G-300 (300.1 MHz) spectrometer and chemical shifts are given in ppm relative to TMS (0.00 ppm). ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence on a Varian G-300 (75.5 MHz) spectrometer and chemical shifts are given in ppm relative to the solvent signal. Electrospray ionisation mass spectrometry was performed on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer in positive ionisation mode. MALDI-TOF analysis was performed on a Kratos Axima CFR apparatus with human ACTH(18 – 39) (monoisotopic M + H$^+$ 2465.198), bovine insulin oxidized B chain (monoisotopic M + H$^+$ 3494.651), or bovine insulin (monoisotopic M + H$^+$ 5730.609) as external references and α-cyano-4-hydroxycinnamic acid as the matrix. Preparative HPLC was carried out on a Gilson preparative HPLC system equipped with a reverse phase C8 column (Alltech Altima XL C8, 100 Å, 10 μm, 250 × 22 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O–MeCN 95 : 5 v/v) to 100% buffer B (0.1% TFA in H_2O–MeCN 5 : 95 v/v) in 40 min at a flow rate of 11.5 mL min^{-1}. Analytical HPLC was carried out on a Shimadzu HPLC workstation using a reverse phase C8 column (Alltech Altima XL C8, 90 Å, 5 μm, 250 × 4.6 mm) using a linear gradient of the same buffers as above in 20 min at a flow rate of 1.0 mL min^{-1}. Microwave assisted reactions were conducted in closed reaction vessels using a Biotage Initiator microwave reactor equipped with a temperature and pressure sensor for monitoring reaction conditions.

General procedure A

N,*N*-Bis-Boc-1-guanylpyrazole **1** (620 mg, 2.0 mmol), alcohol **2a–e** (2.0 mmol) and triphenylphosphine (786 mg, 3.0 mmol) were dissolved in THF (15 mL). At 0 °C diethyl azodicarboxylate (471 μL, 3.0 mmol) was added dropwise over a period of 30 min. The reaction mixture was stirred at room temperature for 16 h. EtOAc (30 mL) and water were added and the layers separated. After washing with brine, the organic layer was dried with Na_2SO_4 and concentrated under reduced pressure. The resulting crude product was purified by column chromatography.

General procedure B

Fmoc-Orn(Boc)-OH **4** (454 mg, 1.0 mmol) was dissolved in a mixture of DCM (4.0 mL) and a saturated solution of HCl in diethylether (4.0 mL). After stirring at room temperature for 20 min, a white precipitate had formed and the reaction mixture was evaporated to dryness. The resulting crude HCl salt was suspended in DCM (5.0 mL) and DiPEA (348 μL, 2.0 mmol) was added. After addition of *N*,*O*-bis(trimethylsilyl)acetamide (489 μL, 2.0 mmol), all precipitates went into solution instantly. After stirring at room temperature for 15 min, the guanylating agent **3a–e** (1.0 mmol) was added and the reaction mixture was stirred for 24 h at room temperature. After addition of DCM (20 mL), washing with 1 M $KHSO_4$ (20 mL) and brine (20 mL), and drying on Na_2SO_4, the solvents were evaporated. The resulting crude product was purified by column chromatography.

General procedure C

Fmoc-Orn(Boc)-Pro-OH **6** (552 mg, 1.0 mmol) was dissolved in a mixture of DCM (5.0 mL) and sat. HCl in Et_2O (5.0 mL), and stirred for 15 min, after which the solvents were evaporated. The resulting white solid was suspended in dry THF (10 mL) after which DiPEA (523 μL, 3.0 mmol) and *N*,*O*-bis(trimethylsilyl)acetamide (734 μL, 3.0 mmol) were added. This mixture was stirred for 10 min at room temperature, at which point a clear solution was obtained. Guanylating agent **3a–d** (1.0 mmol) was added and the reaction mixture stirred for 24 h at room temperature, after which the solvent was evaporated. The residue was suspended in EtOAc (10 mL), washed with 1 M HCl (10 mL) and dried on Na_2SO_4. The resulting crude product was purified by column chromatography.

General procedure D

The peptide was prepared on Tentagel® S RAM resin on a 0.080 mmol scale using Fmoc–*t*Bu solid phase chemistry. Agitation was achieved by bubbling with nitrogen. *Fmoc-deprotection*: The resin was treated with 20% piperidine in DMF (2.0 mL, 3 × 8 min), followed by washing with DMF (2.0 mL, 3 × 2 min) and DCM (2.0 mL, 3 × 2 min). A positive Kaiser test[22] indicated successful deprotection. *Coupling*: The resin was treated with a solution of Fmoc-Xxx-OH (0.32 mmol), HBTU (0.32 mmol), HOBt (0.32 mmol) and DiPEA (0.64 mmol) in DMF (2.0 mL) for 30 min. The resin was washed with DMF (2.0 mL, 3 × 2 min) and DCM (2.0 mL, 3 × 2 min). A negative Kaiser test indicated successful coupling. *Capping*: The resin was treated with a solution of Ac_2O (0.5 M), DiPEA (0.125 M) and HOBt·H_2O (0.015 M) in DMF (2.0 mL, 2 × 10 min). The resin was washed with DMF (2.0 mL, 3 × 2 min) and DCM (2.0 mL, 3 × 2 min). A negative Kaiser test indicated successful capping. *Cleavage and deprotection*: The resin was stirred with a mixture of TFA (9.5 mL), TIS (0.25 mL) and H_2O (0.25 mL) for 4 h at room temperature, after which it was removed by filtration and rinsed with TFA (3.0 mL). The crude peptide was precipitated from the combined filtrate by dropwise addition to a cold (–20 °C) mixture of MTBE and hexanes (40 mL, 1 : 1 v/v). After centrifugation (3000 rpm, 5 min) and decantation, the pellet was washed with ether (3 × 40 mL). The resulting crude peptide was dissolved in water and lyophilized.

N-(2-Azidoetyl)-*N*,*N*′-bis-Boc-1-guanylpyrazole 3a

This compound was prepared according to General procedure A. The crude product was purified using column chromatography (EtOAc–hexanes, 1 : 9 v/v) yielding **3b** (610 mg, 86%) as a clear, colorless oil. (Found C, 50.9; H, 6.5; N, 25.6. $C_{16}H_{25}N_7O_4$ requires C, 50.65; H, 6.6; N, 25.8); R_f 0.76 (EtOAc–hexanes, 1 : 2 v/v); ν_{max}/cm^{-1} 2105 (N_3), 1724 (CO), 1660 (CN); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.26 (9 H, s, $C(CH_3)_3$), 1.48 (9 H, s, $C(CH_3)_3$), 3.63 (2 H, t, *J* 6.1, CH_2N_3), 3.84 (2 H, t, *J* 6.1, CH_2NBoc), 6.41 (1 H, m, ArH), 7.67 (1 H, t, *J* 0.7, ArH), 7.95 (1H, s, ArH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 27.6 and 27.8 ($C(CH_3)_3$), 47.4, 49.6 (CH_2), 82.6, 83.2 ($C(CH_3)_3$), 109.0, 130.0, 143.2 (ArC), 152.1 (C=N), 157.3 (C=O); *m*/*z* (ESI) (M + H^+. $C_{16}H_{26}N_7O_4$ requires 380.2), 379.8.

N-(2-(2-Azidoethoxy)ethyl)-*N*,*N*′-bis-Boc-1-guanylpyrazole 3b

This compound was prepared according to General procedure A. The crude product was purified using column chromatography (EtOAc–hexanes, 1 : 4 v/v) yielding **3c** (706 mg, 84%) as a clear, colorless oil. (Found C, 51.2; H, 6.7; N, 23.1. $C_{18}H_{29}N_7O_5$ requires C, 51.05; H, 6.9; N, 23.15); R_f 0.74 (EtOAc–hexanes, 1 : 1 v/v); ν_{max}/cm^{-1} 2101 (N_3), 1726 (CO), 1664 (CN); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.12 (9 H, s, $C(CH_3)_3$), 1.34 (9 H, s, $C(CH_3)_3$), 3.10 (2 H, t, *J* 4.9, CH_2), 3.42 (2 H, t, *J* 5.0, CH_2), 3.62 (2 H, t, *J* 5.4, CH_2), 3.77 (2 H, t, *J* 5.0, CH_2), 6.26 (1 H, m, ArH), 7.52 (1 H, t, *J* 0.8, ArH), 7.82 (1 H, s, ArH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 27.4, 27.6 ($C(CH_3)_3$), 47.4, 50.4, 68.4, 69.0 (CH_2), 81.9, 82.5 ($C(CH_3)_3$), 108.5, 129.7, 142.7 (ArC), 152.1 (C=N), 157.2 (C=O); *m*/*z* (ESI) (M + H^+. $C_{18}H_{30}N_7O_5$ requires 424.2), 424.5.

N-(2-(2-(2-Azidoethoxy)ethoxy)ethyl)-*N*,*N*′-bis-Boc-1-guanylpyrazole 3c

This compound was prepared according to General procedure A. The crude product was purified using column chromatography (EtOAc–hexanes, 1 : 4 v/v) yielding **3d** (660 mg, 70%) as a clear, colorless oil. (Found C, 51.3; H, 7.3; N, 21.1. $C_{20}H_{33}N_7O_6$ requires C, 51.4; H, 7.1; N, 21.0); R_f 0.66 (EtOAc–hexanes, 1 : 1 v/v); ν_{max}/cm^{-1} 2102 (N_3), 1726 (CO), 1665 (CN); δ_H (300 MHz; $CDCl_3$ Me_4Si) 1.25 (9 H, s, $C(CH_3)_3$), 1.49 (9 H, s, $C(CH_3)_3$), 3.32 (2 H, t, *J* 5.1, CH_2), 3.55 (6 H, m, 3 × CH_2), 3.75 (2 H, t, *J* 5.6, CH_2), 3.90 (2 H, t, *J* 5.2, CH_2), 6.38 (1 H, dd, *J* 1.1 and 1.6, ArH), 7.66 (1 H, dd, *J* 0.5 and 1.1, ArH), 7.95 (1 H, s,ArH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 27.9, 28.2 ($C(CH_3)_3$), 48.1, 50.9 (NCH_2), 60.0, 70.2, 70.4, 70.7 (OCH_2), 82.4, 83.0 ($C(CH_3)_3$), 108.8, 130.4, 143.2 (ArC), 152.6 (C=N), 157.7 (C=O); *m*/*z* (ESI) (M + H^+. $C_{20}H_{34}N_7O_6$ requires 468.3), 468.4.

N-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl)-*N*,*N*′-bis-Boc-1-guanylpyrazole 3d

This compound was prepared according to General procedure A. The crude product was purified using column chromatography (MeOH–DCM, 0.5 : 99.5 v/v) yielding **3e** (800 mg, 78%) as a clear, colorless oil. (Found C, 51.6; H, 7.3; N, 19.3. $C_{22}H_{37}N_7O_7$ requires C, 51.65; H, 7.3; N, 19.2); R_f 0.66 (EtOAc–hexanes, 1 : 1 v/v); ν_{max}/cm^{-1} 2102 (N_3), 1727 (CO), 1664 (CN); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.27 (9 H, s, $C(CH_3)_3$), 1.50 (9 H, s, $C(CH_3)_3$), 3.37 (2 H, t, *J* 5.1, CH_2), 3.59 (10 H, m, 5 × CH_2), 3.75 (2 H, t, *J* 5.6, CH_2), 3.91 (2 H, t, *J* 5.2, CH_2), 6.40 (1H, dd, *J* 1.1 and 1.6, ArH), 7.68 (1 H, d, *J* 1.1, ArH), 7.98 (1 H, s, ArH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 27.4, 27.6 ($C(CH_3)_3$), 47.6, 60.4 (NCH_2), 68.3, 69.7, 69.9, 70.3 (OCH_2), 81.9, 82.4 ($C(CH_3)_3$), 108.4, 130.0, 142.7 (ArC), 152.1 (C=N), 157.2 (C=O); *m*/*z* (ESI) (M + H^+. $C_{22}H_{38}N_7O_7$ requires 512.3), 512.1.

N,*N*′-Bis-Boc-*N*-propargyl-1-guanylpyrazole 3e

This compound was prepared according to General procedure A. The crude product was purified using column chromatography (EtOAc–hexanes, 1 : 4 v/v) yielding **3a** (550 mg, 79%) as a white solid. (Found C, 58.9; H, 6.8; N, 16.3. $C_{17}H_{24}N_4O_4$ requires C, 58.6; H, 6.9; N, 16.1); R_f 0.80 (EtOAc–hexanes, 1 : 1 v/v); ν_{max}/cm^{-1} 1737 and 1709 (CO), 1653 (CN); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.30

(9 H, s, $C(CH_3)_3$), 1.50 (9 H, s, $C(CH_3)_3$), 2.27 (1 H, s, ≡CH), 4.48 (2 H, s, CH_2C≡), 6.41 (1 H, s, ArH), 7.70 (1 H, s, ArH), 7.95 (1 H, s, ArH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 27.5, 27.7 ($C(CH_3)_3$), 37.9 (CH_2), 72.8 (≡CH), 77.4 (*C*≡CH), 82.3, 83.4 ($C(CH_3)_3$), 109.0, 129.8, 143.2 (ArC), 151.4 (C=N), 156.9 (C=O); *m/z* (ESI) (M + H^+. $C_{17}H_{25}N_4O_4$ requires 349.2), 349.2.

Fmoc-N_ω-(2-azidoethyl)-N_ω,$N_{\omega'}$-bis-Boc-L-arginine 5a

This compound was prepared according to General procedure B. After column chromatography (MeOH–DCM, 4 : 96 v/v), **5b** (411 mg, 62%) was obtained as a white foam. (Found C, 59.7; H, 6.4; N, 15.0. $C_{33}H_{43}N_7O_8$ requires C, 59.5; H, 6.5; N, 14.7); R_f 0.35 (MeOH–DCM, 1 : 9 v/v); $[\alpha]_D^{20}$ +8.3 (*c* 0.28 in MeOH); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.46 (9 H, s, $C(CH_3)_3$), 1.48 (9 H, s, $C(CH_3)_3$), 1.77 (4 H, br m, $C^\beta H_2$ and $C^\gamma H_2$), 3.33 (2H, s, $C^\delta H_2$), 3.51 (2 H, s, CH_2), 3.69 (2 H, s, CH_2), 4.19 (1 H, m, $C^\alpha H$), 4.33 (3 H, m, Fmoc-CH and Fmoc-CH_2), 5.96 (1 H, br s, Fmoc-NH), 7.29 (4 H, m, ArH), 7.60 (2 H, m, ArH), 7.73 (2 H, d, *J* 7.1, ArH), 11.7 (2 H, br s, NH and OH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 24.5 (C^β), 27.8 ($C(CH_3)_3$), 29.9 (C^γ), 47.0 (Fmoc-CH), 49.9 (CH_2), 53.3 (C^δ), 55.9 (C^α), 66.7 (Fmoc-CH_2), 83.6 ($C(CH_3)_3$), 119.7, 125.0, 126.8, 127.5, 141.0, 143.7 (ArC), 143.8 (C=N), 141.8, 155.9, 176.1 (C=O); *m/z* (ESI) (M + H^+. $C_{33}H_{44}N_7O_8$ requires 666.3), 666.2.

Fmoc-N_ω-(2-(2-azidoethoxy)-ethyl)-N_ω,$N_{\omega'}$-bis-Boc-L-arginine 5b

This compound was prepared according to General procedure B. After purification by column chromatography (MeOH–DCM, 4 : 96 v/v), **5c** (451 mg, 64%) was obtained as a white foam. (Found C, 59.2; H, 6.5; N, 13.5. $C_{35}H_{47}N_7O_9$ requires C, 59.2; H, 6.7; N, 13.8); R_f 0.31 (MeOH–DCM, 1 : 9 v/v); $[\alpha]_D^{20}$ +7.2 (*c* 0.20 in MeOH); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.47 (9 H, s, $C(CH_3)_3$), 1.48 (9 H, s, $C(CH_3)_3$), 1.81 (4 H, m, $C^\beta H_2$ and $C^\gamma H_2$), 3.30-3.71 (10 H, br m, $C^\delta H_2$ and 4 × CH_2), 4.19 (1 H, t, *J* 7.0, $C^\alpha H$), 4.34 (3 H, m, Fmoc-CH and Fmoc-CH_2), 6.02 (1 H, br s, Fmoc-NH), 7.30 (4 H, m, ArH), 7.60 (2 H, m, ArH), 7.73 (2 H, d, *J* 7.3, ArH), 12.2 (2 H, br s, NH and OH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 24.2 (C^β), 27.9 ($C(CH_3)_3$), 30.0 (C^γ), 47.0 (Fmoc-CH), 48.3 (C^δ), 50.4 (CH_2), 54.1 (C^α), 66.7, 69.0, 69.4 (Fmoc-CH_2 and CH_2), 83.8 ($C(CH_3)_3$), 119.8, 125.0, 126.9, 127.5, 141.1, 143.7 (ArC), 143.9 (C=N), 151.9, 155.8, 176.2 (C=O); *m/z* (ESI) (M + H^+. $C_{35}H_{48}N_7O_9$ requires 710.4), 710.8.

Fmoc-N_ω-(2-(2-(2-azidoethoxy)ethoxy)-ethyl)-N_ω,$N_{\omega'}$-bis-Boc-L-arginine 5c

This compound was prepared according to General procedure B. After purification by column chromatography (MeOH–DCM, 8 : 92 v/v), **5d** (460 mg, 61%) was obtained as a white foam. (Found C, 58.8; H, 6.8; N, 13.1. $C_{37}H_{51}N_7O_{10}$ requires C, 58.95; H, 6.8; N, 13.0); R_f 0.41 (MeOH–DCM, 1 : 9 v/v); $[\alpha]_D^{20}$ +7.3 (*c* 0.24 in MeOH); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.45 (9 H, s, $C(CH_3)_3$), 1.47 (9 H, s, $C(CH_3)_3$), 1.79 (4 H, m, $C^\beta H_2$ and $C^\gamma H_2$), 3.29 (4 H, m, $C^\delta H_2$ and CH_2), 3.61 (10 H, m, 5 × CH_2), 4.19 (1 H, m, $C^\alpha H$), 4.32 (3 H, br m, Fmoc-CH and Fmoc-CH_2), 6.07 (1 H, s, Fmoc-NH), 7.28 (4 H, m, ArH), 7.59 (2 H, m, ArH), 7.59 (2 H, m, ArH), 11.0 (2 H, br s, NH and OH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 24.4 (C^β), 27.9 ($C(CH_3)_3$), 30.0 (C^γ), 47.0 (Fmoc-CH), 48.3 (C^δ), 50.4 (CH_2), 54.5 (C^α), 66.6, 69.1,69.8, 70.0, 70.3 (Fmoc-CH_2 and OCH_2), 82.9 ($C(CH_3)_3$), 119.8, 125.0, 126.9, 127.5, 128.1, 141.1, 143.7 (ArC), 143.9 (C=N), 151.4, 155.9, 176.8 (C=O); *m/z* (ESI) (M + H^+. $C_{37}H_{52}N_7O_{10}$ requires 754.4), 753.9.

Fmoc-N_ω-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-ethyl)-N_ω,$N_{\omega'}$-bis-Boc-L-arginine 5d

This compound was prepared according to General procedure B. After purification by column chromatography (MeOH–DCM, 4 : 96 v/v), **5e** (503 mg, 63%) was obtained as a white foam. (Found C, 58.8; H, 6.8; N, 12.55. $C_{39}H_{55}N_7O_{11}$ requires C, 58.7; H, 6.95; N, 12.3); R_f 0.51 (MeOH–DCM, 1 : 9 v/v); $[\alpha]_D^{20}$ +6.0 (*c* 0.21 in MeOH); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.46 (9 H, s, $C(CH_3)_3$), 1.48 (9 H, s, $C(CH_3)_3$), 1.72 (4 H, br m, $C^\beta H_2$ and $C^\gamma H_2$), 3.32 (4 H, m, $C^\delta H_2$ and CH_2), 3.57 (14H, m, 7 × CH_2), 4.19 (1 H, t, *J* 6.9, $C^\alpha H$), 4.36 (3 H, m, Fmoc-CH and Fmoc-CH_2), 6.00 (1 H, br s, Fmoc-NH), 7.29 (4 H, m, ArH), 7.61 (2 H, m, ArH), 7.34 (2 H, d, *J* 7.8, ArH), 11.6 (2 H, br s, OH and NH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 24.4 (C^β), 27.9 ($C(CH_3)_3$), 29.9 (C^γ), 47.1 (Fmoc-CH), 48.3 (C^δ), 50.4 (CH_2), 54.1 (C^α), 66.6, 68.9, 69.7, 69.9, 70.3, 70.4 (Fmoc-CH_2 and CH_2), 82.2 ($C(CH_3)_3$), 119.8, 125.0, 126.9, 127.5, 141.1, 143.7 (ArC), 143.9 (C=N), 151.3, 155.8, 176.0 (C=O); *m/z* (ESI) (M + H^+. $C_{39}H_{56}N_7O_{11}$ requires 798.4), 798.9.

Fmoc-N_ω,$N_{\omega'}$-bis-Boc-N_ω-propargyl-L-arginine 5e

This compound was prepared according to General procedure B. After column chromatography (MeOH–DCM, 5 : 95 v/v), **5a** (518 mg, 82%) was obtained as a white foam. (Found C, 64.5; H, 6.6; N, 8.7. $C_{34}H_{42}N_4O_8$ requires C, 64.3; H, 6.7; N, 8.8); R_f 0.45 (MeOH–DCM, 1 : 9 v/v); $[\alpha]_D^{20}$ +4.5 (*c* 0.27 in MeOH); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.34 (9 H, s, $C(CH_3)_3$), 1.35 (9 H, s, $C(CH_3)_3$), 1.64 (4 H, m, $C^\beta H_2$ and $C^\gamma H_2$), 2.21 (1 H, br s, ≡CH), 3.21 (2 H, br s, CH_2C≡), 4.05 (1 H, m, $C^\alpha H$), 4.21 (3 H, m, Fmoc-CH and Fmoc-CH_2), 5.78 (1 H br d, *J* 5.2, Fmoc-NH), 7.18 (4 H, m, ArH), 7.44 (2 H, m, ArH), 7.60 (2 H, d, *J* 7.4, ArH), 11.6 (2H, br s, NH and OH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 25.3 (C^β), 27.9 ($C(CH_3)_3$), 30.9 (C^γ), 36.8 (≡CH), 41.4 (C^δ), 45.0 (CH_2C≡), 47.0 (Fmoc-CH), 54.2 (C^α), 66.5 (Fmoc-CH_2), 67.7 (*C*≡CH), 82.8 ($C(CH_3)_3$), 119.7, 124.9, 126.8, 127.4, 141.0, 143.6 (ArC), 143.8 (C=N), 152.9, 155.8, 156.2, 175.8 (C=O); *m/z* (ESI) (M + H^+. $C_{34}H_{43}N_4O_8$ requires 635.3), 635.5.

Fmoc-Orn(Boc)-Pro-OH 6

Fmoc-Orn(Boc)-OH **4** (909 mg, 2.0 mmol) and *N*-hydroxysuccinimide (230 mg, 2.0 mmol) were dissolved in dry DCM (10 mL). After cooling to 0 °C, EDCI·HCl (383 mg, 2.0 mmol) was added and the resulting mixture stirred for 3 h at room temperature. After washing with 1 M HCl (10 mL) and brine (10 mL), and drying on Na_2SO_4, the solvent was evaporated and the crude activated ester redissolved in DMF (10 mL). H–Pro–OH (253 mg, 2.2 mmol) and DiPEA (383 µL, 2.2 mmol) were added and the reaction mixture was stirred overnight at room temperature. After evaporation to dryness, the residue was redissolved in EtOAc (10 mL), washed with 1 M HCl (10 mL) and dried on Na_2SO_4. Column chromatography (MeOH–DCM, 5 : 95 v/v) yielded **6** (625 mg, 57%) as a white foam. (Found C, 65.6; H, 6.8; N, 7.3. $C_{30}H_{37}N_3O_7$ requires C, 65.3; H, 6.8; N, 7.6);

R_f 0.23 (MeOH–DCM, 1 : 9 v/v); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.42 (9 H, s, $C(CH_3)_3$), 1.40-2.21 (8 H, br m, Pro-$C^\beta H_2$, Pro-$C^\gamma H_2$, Orn-$C^\beta H_2$ and Orn-$C^\gamma H_2$), 3.07 (2 H, m, Orn-$C^\delta H_2$), 3.68 (2 H, m, Pro-$C^\delta H_2$), 4.08 (3 H, m, Fmoc-CH and Fmoc-CH_2), 4.55 (2 H, m, Pro-$C^\alpha H$ and Orn-$C^\alpha H$), 5.06 (1 H, br s, Boc-NH), 6.79 (1 H, br s, Fmoc-NH), 7.26 (4 H, m, ArH), 7.58 (2 H, m, ArH), 7.73 (2 H, d, *J* 7.4, ArH), 7.80 (1 H, br s, OH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 24.9, 25.7 (Orn-C^β and Pro-C^β), 28.3 ($C(CH_3)_3$), 28.7, 29.0 (Pro-C^γ and Orn-C^γ), 37.0 (Fmoc-CH), 40.1, 47.1 (Pro-C^δ and Orn-C^δ), 52.3, 59.1 (Pro-C^α and Orn-C^α), 66.9 (Fmoc-CH_2), 79.3 ($C(CH_3)_3$), 119.7, 125.2, 126.9, 127.5, 141.1, 143.7, 143.9 (ArC), 156.4, 171.6, 174.2 (C=O); *m/z* (ESI) (M + H^+. $C_{30}H_{38}N_3O_7$ requires 552.3), 551.8.

Fmoc-N_ω-(2-azidoethyl)-N_ω,$N_{\omega'}$-bis-Boc-Arg-Pro-OH 7a

This compound was prepared according to General procedure C. Column chromatography (MeOH–DCM, 5 : 95 v/v) yielded **7a** (615 mg, 81%) as a white foam. (Found C, 60.0; H, 6.5; N, 14.8. $C_{38}H_{50}N_8O_9$ requires C, 59.8; H, 6.6; N, 14.7); R_f 0.36 (MeOH–DCM, 9 : 1 v/v); $[\alpha]_D^{20}$ −25.6 (*c* 0.19 in MeOH); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.47 (9 H, s, $C(CH_3)_3$), 1.48 (9 H, s, $C(CH_3)_3$), 1.66-2.25 (8 H, m, Pro-$C^\beta H_2$, Pro-$C^\gamma H_2$, Arg-$C^\beta H_2$ and Arg-$C^\gamma H_2$), 3.20-3.80 (8 H, br m, 2 × CH_2, Pro-$C^\delta H_2$ and Arg-$C^\delta H_2$), 4.18 (1 H, m, Fmoc-CH), 4.28 (2 H, d, *J* 6.1, Fmoc-CH_2), 4.55 (2 H, m, Pro-$C^\alpha H$, Arg-$C^\alpha H$), 6.61 (1 H, br s, Fmoc-NH), 7.32 (4 H, m, ArH), 7.60 (2 H, m, ArH), 7.73 (2 H, d, *J* 7.5, ArH), 9.58 (2 H, br s, OH and NH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 24.9 (Pro-C^β and Arg-C^β), 28.0 ($C(CH_3)_3$), 26.2, 28.6 (Pro-C^γ and Arg-C^γ), 43.5 (Pro-C^δ and Arg-C^δ), 46.9 (Fmoc-CH), 46.7, 50.0 (CH_2), 52.0, 59.2 (Pro-C^α and Arg-C^α), 66.9 (Fmoc-CH_2), 80.1, 83.2 ($C(CH_3)_3$), 119.7, 125.1, 126.9, 127.5, 141.1, 143.6 (ArC), 143.9 (C=N), 156.3, 170.9, 174.1 (C=O); *m/z* (ESI) (M + H^+. $C_{38}H_{51}N_8O_9$ requires 763.4), 763.3.

Fmoc-N_ω-(2-(2-azidoethoxy)-ethyl)-N_ω,$N_{\omega'}$-bis-Boc-Arg-Pro-OH 7b

This compound was prepared according to General procedure C. Column chromatography (MeOH–DCM, 8 : 92 v/v) yielded **7b** (637 mg, 79%) as a white foam. (Found C, 59.7; H, 6.6; N, 14.1. $C_{40}H_{54}N_8O_{10}$ requires C, 59.5; H, 6.75; N, 13.9); R_f 0.35 (MeOH–DCM, 1 : 9 v/v); $[\alpha]_D^{20}$ −22.7 (*c* 0.10 in MeOH); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.46 (9 H, s, $C(CH_3)_3$), 1.48 (9 H, s, $C(CH_3)_3$), 1.63-2.19 (8 H, br m, Pro-$C^\beta H_2$, Pro-$C^\gamma H_2$, Arg-$C^\beta H_2$ and Arg-$C^\gamma H_2$), 3.23-3.80 (12 H, br m, 4 × CH_2, Pro-$C^\delta H_2$ and Arg-$C^\delta H_2$), 4.25 (3 H, m, Fmoc-CH and Fmoc-CH_2), 4.56 (2 H, m, Pro-$C^\alpha H$ and Arg-$C^\alpha H$), 6.98 (0.5 H, d, *J* 7.9, Fmoc-NH^a), 7.14 (0.5 H, d, *J* 7.1, Fmoc-NH^b), 7.23 (4 H, m, ArH), 7.59 (2 H, m, ArH), 7.72 (2 H, d, *J* 7.4, ArH), 10.5 (2 H, br s, OH and NH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 27.8 ($C(CH_3)_3$), 24.7, 24.9, 28.6, 29.0 (Pro-C^β, Pro-C^γ, Arg-C^β and Arg-C^γ), 40.4 (Pro-C^δ and Arg-C^δ), 50.3 (CH_2), 46.8, 52.1 (Pro-C^α and Arg-C^α), 59.1 (Fmoc-CH), 66.6, 68.9, 69.0 (Fmoc-CH_2 and CH_2), 79.2, 82.4, 83.0 ($C(CH_3)_3$), 119.5, 125.0, 125.1, 126.8, 127.3, 127.9, 128.7, 140.9, 143.5 (ArC), 143.7 (C=N), 151.8, 152.8, 156.0, 156.2, 162.7, 170.8, 170.9, 174.0, 174.2 (C=O); *m/z* (ESI) (M + H^+. $C_{40}H_{55}N_8O_{10}$ requires 807.4), 807.1.

Fmoc-N_ω-(2-(2-(2-azidoethoxy)ethoxy)-ethyl)-N_ω,$N_{\omega'}$-bis-Boc-Arg-Pro-OH 7c

This compound was prepared according to General Procedure C. Column chromatography (MeOH–DCM, 5 : 95 v/v) yielded **7c** (687 mg, 81%) as a white foam. (Found C, 59.15; H, 6.5; N, 13.2. $C_{42}H_{58}N_8O_{11}$ requires C, 59.3; H, 6.9; N, 13.2); R_f 0.35 (MeOH–DCM, 1 : 9 v/v); $[\alpha]_D^{20}$ −32.8 (*c* 0.10 in MeOH); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.46 (9 H, s, $C(CH_3)_3$), 1.48 (9 H, s, $C(CH_3)_3$), 1.58-2.23 (8 H, br m, Pro-$C^\beta H_2$, Pro-$C^\gamma H_2$, Arg-$C^\beta H_2$ and Arg-$C^\gamma H_2$), 3.20-3.80 (16 H, br m, 6 × CH_2, Pro-$C^\delta H_2$ and Arg-$C^\delta H_2$), 4.16 (1 H, m, Fmoc-CH), 4.28 (2 H, d, *J* 7.2, Fmoc-CH_2), 4.55 (2 H, m, Pro-$C^\alpha H$ and Arg-$C^\alpha H$), 6.72 (1 H, br s, Fmoc-NH), 7.32 (4 H, m, ArH), 7.61 (2 H, m, ArH), 7.74 (2 H, d, *J* 7.1, ArH), 9.60 (2 H, br s, OH and NH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 28.0 ($C(CH_3)_3$), 24.5, 24.9, 28.7, 29.2 (Pro-C^β, Pro-C^γ, Arg-C^β and Arg-C^γ), 39.7 (Pro-C^δ and Arg-C^δ), 50.4 (CH_2), 47.0, 52.3 (Pro-C^α and Arg-C^α), 59.3 (Fmoc-CH), 66.8, 69.2, 69.7, 70.0 (Fmoc-CH_2 and CH_2), 82.5 ($C(CH_3)_3$), 119.7, 125.1, 127.0, 127.5, 141.1, 143.6 (ArC), 143.9 (C=N), 151.9, 156.2, 170.9, 173.6 (C=O); *m/z* (ESI) (M + H^+. $C_{42}H_{59}N_8O_{11}$ requires 851.4), 851.6.

Fmoc-N_ω-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-ethyl)-N_ω,$N_{\omega'}$-bis-Boc-Arg-Pro-OH 7d

This compound was prepared according to General Procedure C. Column chromatography (MeOH–DCM, 5 : 95 v/v) yielded **7d** (701 mg, 78%) as a white foam. (Found C, 58.9; H, 7.15; N, 12.8. $C_{44}H_{62}N_8O_{12}$ requires C, 59.05; H, 7.0; N, 12.5); R_f 0.28 (MeOH–DCM, 1 : 9 v/v); $[\alpha]_D^{20}$ +4.6 (*c* 2.86 in MeOH); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.46 (9 H, s, $C(CH_3)_3$), 1.48 (9 H, s, $C(CH_3)_3$), 1.57-2.22 (8 H, br m, Pro-$C^\beta H_2$, Pro-$C^\gamma H_2$, Arg-$C^\beta H_2$ and Arg-$C^\gamma H_2$), 3.33 (4 H, m, 2 × CH_2), 3.61 (16 H, m, 6 × CH_2, Pro-$C^\delta H_2$ and Arg-$C^\delta H_2$), 4.17 (1 H, t, *J* 6.9, Fmoc-CH), 4.30 (2 H, d, *J* 6.9, Fmoc-CH_2), 4.56 (2 H, m, Pro-$C^\alpha H$ and Arg-$C^\alpha H$), 6.43 (1 H, br s, Fmoc-NH), 7.34 (4 H, m, ArH), 7.59 (2 H, m, ArH), 7.74 (2 H, d, *J* 7.4, ArH), 8.13 (2 H, br s, NH and OH); δ_C (75.5 MHz; $CDCl_3$; Me_4Si) 28.1 ($C(CH_3)_3$), 24.9, 28.6, 29.4, 29.5 (Pro-C^β, Arg-C^β, Pro-C^γ and Arg-C^γ), 47.0, 52.1 (Pro-C^α and Arg-C^α), 50.5 (Pro-C^δ and Arg-C^δ), 59.2 (Fmoc-CH), 66.8, 69.1, 69.9, 70.2, 70.5 (Fmoc-CH_2 and CH_2), 79.9, 82.3 ($C(CH_3)_3$), 119.8, 125.1, 127.0, 127.6, 141,1, 143.7 (ArC), 143.9 (C=N), 151.9, 156.0, 170.7, 173.3 (C=O); *m/z* (ESI) (M + H^+. $C_{44}H_{63}N_8O_{12}$ requires 895.5), 895.3.

Monoethyleneglycol peptide 12a

This compound was prepared according to General procedure D. Preparative RP-HPLC yielded **12a** (27.6 mg, 20%, 94% *per* step) as a white solid *m/z* (ESI) (M + $2H^{2+}$. $C_{77}H_{135}N_{27}O_{18}$ requires 863.0), 863.1; R_t = 17.6 min.

Diethyleneglycol peptide 12b

Method 1. This compound was prepared according to General procedure D. Preparative RP-HPLC yielded **12b** (39.6 mg, 28%, 95% *per* step) as a white solid.

Method 2. This compound was prepared according to General procedure D, incorporating Fmoc-Orn(Aloc)-OH at the guanylation site. The Aloc-group was removed by treatment of the resin with a solution of $PhSiH_3$ (1.6 mmol) and $[Pd(PPh_3)_4]$ (8 μmol) in

NMP (2.0 mL) for 45 min while bubbling through Ar. The resin was then washed with NMP (2.0 mL), diethyldithiocarbamate trihydrate (2.0 mL), a 20% solution of DiPEA in NMP (2.0 mL) and DCM (2.0 mL). The deprotection was repeated three times. Guanylation was achieved by treatment with a solution of *N*,*O*-(bistrimethylsilyl)acetamide (0.32 mmol), DiPEA (0.32 mmol) and **3e** (0.32 mmol) in DCM (2.0 mL) for 48 h. The resin was then washed and the peptide cleaved according to the protocols described in General procedure D.

m/*z* (ESI) (M + $2H^{2+}$. $C_{79}H_{139}N_{27}O_{19}$ requires 885.0), 885.4.

Triethyleneglycol peptide 12c

This compounds was prepared according to General procedure D. Preparative RP-HPLC yielded **12c** (29.6 mg, 20%, 94% *per* step) as a white solid *m*/*z* (ESI) (M + $2H^{2+}$. $C_{81}H_{143}N_{27}O_{20}$ requires 907.0), 907.2; R_t = 17.9 min.

Tetraethyleneglycol peptide 12d

This compound was prepared according to General procedure D. Preparative RP-HPLC yielded **12d** (49.0 mg, 41%, 96% *per* step) as a white solid *m*/*z* (ESI) (M + $2H^{2+}$. $C_{83}H_{147}N_{27}O_{21}$ requires 929.1), 929.3; R_t = 17.9 min.

Bisubstrate-based inhibitor 15

Peptide **12b** (10.0 mg, 5.6 μmol), $CuSO_4 \cdot 5H_2O$ (1.4 mg, 5.6 μmol), staurosporine mimic **14**[12] (2.5 mg, 5.6 μmol) and sodium ascorbate (1.1 mg, 5.6 μmol) were dissolved in a 1 : 1 v/v *tert*-BuOH : H_2O (5.0 mL). The resulting reaction mixture was heated under microwave irradiation to 80 °C for 20 min.[28] After lyophilization, the peptide was purified by preparative HPLC. Lyophilization of pure fractions afforded **15** (7.7 mg, 62%) as a red powder *m*/*z* (MALDI) (M + H^+. $C_{107}H_{164}N_{31}O_{21}$ requires 2219.3), 2219.6; R_t = 18.6 min.

Inhibitor evaluation

One 96 well plate was used for each PKC isozyme, which was fully blocked by treatment with 2% BSA (aq) for 15 min prior to use. The inhibitor of interest was dissolved in DMSO, after which serial dilutions were made in 20% aqueous DMSO at 10 times the final concentrations. Then, to all 96 wells were added a mixture of water (6.25 μL), a 0.5 mg mL^{-1} solution of phosphatidylserine (Fluka BioChimika) in water (10 μL), Abl buffer (New England Biolabs) (2.5 μL), a 0.1 mg mL^{-1} solution of BSA (New England Biolabs) in water (0.25 μL), a solution of anti-PKA antibody #2261 (Cell Signalling Technologies) in buffer (0.25 μL), a 4 μg mL^{-1} solution of FITC labelled Goat-anti-Rabbit antibody (Santa Cruz Biotechnology) in buffer (0.25 μL), and 20 ng kinase (Invitrogen) in 0.5 μL buffer. The inhibitor solution, or 20% DMSO (aq) for the positive controls, (2.5 μL) were added, as well as a 1 mM solution of ATP in water, or water for the negative controls (2.5 μL). All inhibitor concentrations were tested in triplicate for each isozyme. Samples were placed on the PamChip® 96 STK-array plate (Pamgene, 's-Hertogenbosch, The Netherlands) and during 60 min incubation at 30 °C, real time images were taken automatically every 2.5 min. All images were analysed by BioNavigator software (Pamgene, 's-Hertogenbosch, The Netherlands).

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