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Reduction of a 4-pyrrole phenylacyl-containing peptide with trifluoroacetic acid–triisopropylsilane–phenol–H_2O during solid-phase peptide synthesis and its protein kinase C α inhibitory activity

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Abstract—Cleavage of 4-pyrrole phenylacyl TentaGel-appended peptide (**5**) containing Arg(Pbf) with trifluoroacetic acid/triisopropylsilane/phenol/H_2O (90/2/4/4) gives the 4-pyrrole phenylacyl peptide (**3**). However, cleavage of 4-pyrrole phenylacyl Rink-appended peptide (**7**) containing Arg(Pbf) using the same reagents furnishes the 4-pyrrolidine phenylacyl peptide (**8**), which contains the reduced pyrrole ring. Compound **8** displays a K_i of 2.32 μM, approximately fivefold less potent than compound **3**.

A previous paper[1] reported an extraordinarily potent and highly selective PKCα inhibitor (**1**) via the stepwise combinatorial modification of a consensus sequence scaffold. To the best of my knowledge, compound **1** is the most powerful protein binding site-directed inhibitor ever reported for a protein kinase.[2] Compound **1** displays a K_i of 800 pM versus PKCα substrate, approximately 1,000,000-fold more potent than the diacetylated control peptide **2** (Fig. 1).

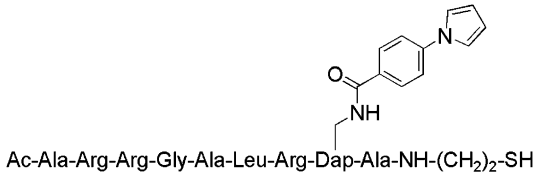
(R^1CO)Ala-Arg-Arg-Gly-Ala-Leu-Arg-Dap(COR^2)-Ala-NH-$(CH_2)_2$-SH

1 R^1 = 3,4-dichlorobenzyl, R^2 = 4-pyrrol phenyl
2 $R^1 = CH_3$, $R^2 = CH_3$

Figure 1. Chemical structure for PKCα inhibitors (**1**) and diacetylated control peptide (**2**).

Compound **1** was identified from secondary libraries derived from compound **3**, which served as one of the initial lead compounds identified from three libraries that had been prepared.

Interestingly, the best leads (**3** and **4**) from these three libraries contain the same substituent, a 4-pyrrole phenylacyl moiety (Fig. 2).



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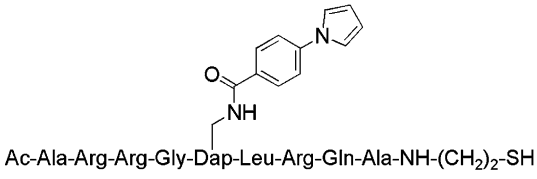

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Figure 2. Chemical structure for 4-pyrrole phenylacyl peptides (**3**, **4**).

These compounds display several orders of magnitude improvement in inhibitory efficacy relative to the diacetylated control peptide **2**.

PKCα appears to possess a binding pocket that displays a special affinity for 4-pyrrole phenylacyl substituent. Compound **3** was obtained from the

Abbreviations: Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Dap, L-2,3-diaminopropionic acid; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl; Aloc, allyloxycarbonyl.

Keywords: PKCα inhibitor; 4-Pyrrole phenylacyl peptide; 4-Pyrrolidine phenylacyl peptide; Non-competitive inhibitor kinetics.

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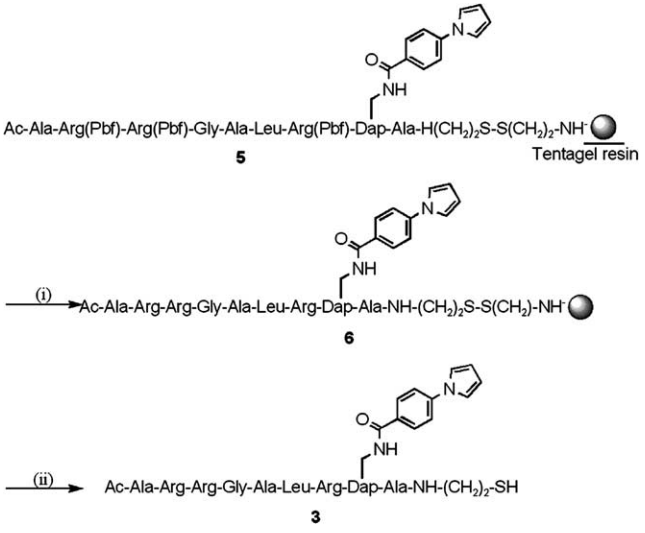
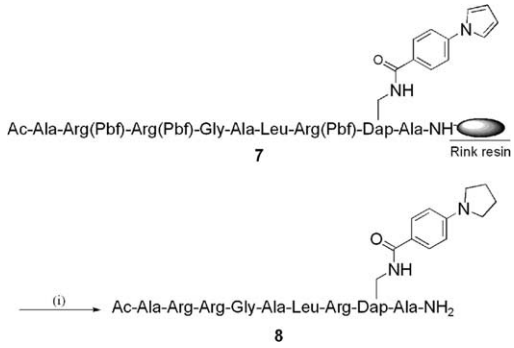
TentaGel-appended peptide **5**, which was prepared using a standard Fmoc protocol (Scheme 1). The Pbf protecting groups were removed via treatment with trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/phenol/H_2O (92/2/4/4) for 12 h at ambient temperature.[3] The deprotected peptide was subsequently released from the resin using a dithiothreitol-containing cocktail. However, the amount of peptide **3** obtained from 1 g of resin (20 mg, 66% yield) is relatively modest due to the low resin substitution level (0.025 mmol/g).



Scheme 1. Reagents and conditions: (i) TFA/TIS/phenol/H_2O (90/2/4/4), rt, 12 h; (ii) 10 mM dTT/50 mM Tris (pH 7.5), rt, 1 h.

In this communication, a side reaction is described, namely the reduction of pyrrolyl aromatic moiety, which transpires only during the deprotection and cleavage of the peptide from the Rink resin.

In order to obtain large quantities of the desired PKCα inhibitory species, the Rink resin (rather than the TentaGel resin) was employed. The Rink-appended peptide substitution was 0.7 mmol/g. Cleavage of Rink-appended peptide (**7**) using TFA/TIS/phenol/H_2O (92/2/4/4) generated the reduced 4-pyrrolidine phenylacyl peptide (**8**),[4] instead of the expected 4-pyrrole phenylacyl peptide (Scheme 2).



Scheme 2. Reagents and conditions: (i) TFA/TIS/phenol/H_2O (90/2/4/4), rt, 12 h.

During the removal of Pbf protecting group and cleavage of Rink resin with TFA/TIS/phenol/H_2O (92/2/4/4), the formation of side product four atomic mass units greater than the expected 4-pyrrole phenylacyl peptide was observed.

^1H–^{13}C NMR spectra of compound **8** confirmed the absence of the aromatic proton peaks of pyrrole ring (Fig. 3).

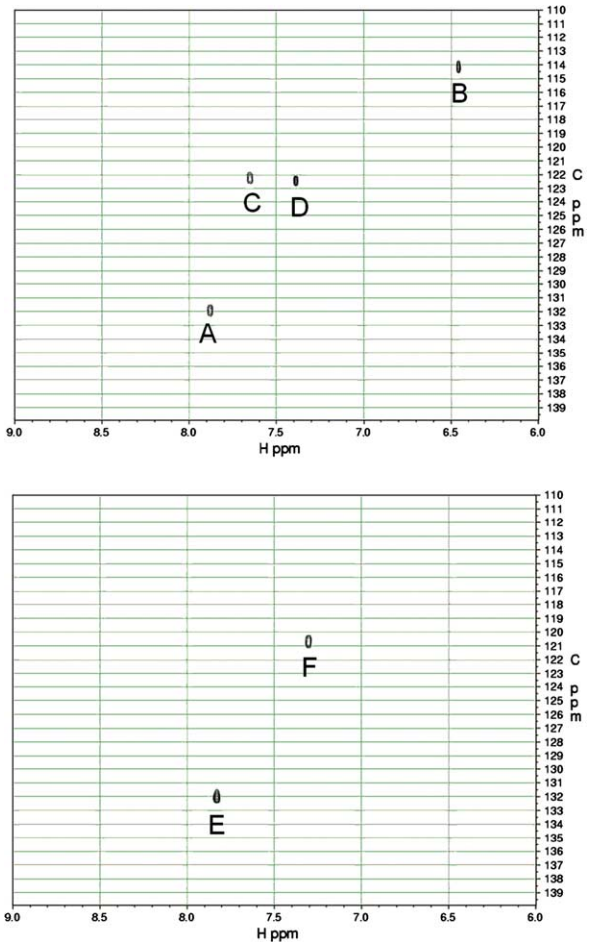


Figure 3. Expansions of 2D ^1H–^{13}C HSQC NMR (600 MHz) chemical shifts of 4-pyrrole phenylacyl (**3**) and 4-pyrrolidine phenylacyl peptide (**8**). A (7.88, 132.1) and D (7.38, 122.4): phenyl nucleus of **3**. B (6.46, 113.9) and C (7.66, 122.4): pyrrole nucleus of **3**. E (7.83, 132.1) and F (7.30, 120.5): phenyl nucleus of **8**.

These experiments confirmed that cleavage of the 4-pyrrole phenylacyl Tentagel-appended peptide containing Arg(Pbf) with TFA–TIS–phenol–H_2O gave the 4-pyrrole phenylacyl peptide (**3**). However, cleavage of 4-pyrrole phenylacyl Rink-appended peptide (**7**) containing Arg(Pbf) gave unexpected 4-pyrrolidine phenylacyl peptide (**8**). Apparently, the structural fate of the pyrrole ring is dependent upon the solid support to which the peptide is attached. Indeed TFA–TIS–phenol–H_2O does react with the 4-pyrrole phenylacyl peptide in solution and the 4-pyrrolidine phenylacyl peptide is produced. On the solid support, the pyrrole moiety is not reduced

to 4-pyrrolidine, thereby suggesting that TFA and TIS does not affect the 4-pyrrole group.

Alkylation of tryptophan[5] and sulfonation of tyrosine[6] during deprotection of Arg(Pbf) containing peptides has been reported. It is known pyrrolidine attached to phenyl is obtained from the corresponding pyrrolyl by catalytic hydrogenation in the presence of 5% rhodium on alumina in glacial acetic acid.[7] The pyrrole nucleus is rather resistant to hydrogenation and reduction of this heterocycle normally requires vigorous conditions.

Partial reduction of the Aloc double bond has been reported during removal of the Dde protecting group (2% hydrazine solution in DMF) on an Aloc and Dde protected peptide.[8] However, there are no reports for reduction of pyrrole-containing peptides with TIS–TFA on solid support.

PKCα activity was assayed using *Ac-Ser-Phe-Arg-Arg-Arg-Arg-NH*$_2$ as the substrate.[9] Compound **8** displays a K_i of 2.32 μM, approximately fivefold lower than compound **3** (Table 1).

Compound **3** is a competitive inhibitor versus variable peptide substrate but serves as an uncompetitive inhibitor with respect to ATP. Compound **8**, which contains the reduced pyrrolidine moiety, also serves as a competitive inhibitor with respect to peptide substrate.[10] By contrast, non-competitive inhibitor kinetics versus ATP are observed (Fig. 4).[11] The dramatic change in inhibition pattern versus variable ATP on conversion of the pyrrole (uncompetitive) to the pyrrolidine (non-competitive) is surprising.

Compound **8** shows no selectivity for PKCα versus its closely related conventional PKCβ1 (IC_{50} = 11.7 ± 0.9 μM) and PKCγ (IC_{50} = 11.3 ± 0.8 μM). Selectivity over the more distantly related novel PKCδ and atypical PKCζ is 7-fold (IC_{50} = 86.9 ± 9.5 μM) and 15-fold (IC_{50} = 196 ± 23 μM), respectively.[12]

In summary, the 4-pyrrolidine phenylacyl peptide (**8**) was unexpectedly obtained from 4-pyrrole phenylacyl peptide containing Arg(Pbf) with TFA/TIS/phenol/H_2O (92/2/4/4). Reduction of pyrrole to pyrrolidine resulted in at least a fivefold loss of inhibitory potency. Although the inhibitor is competitive with respect to peptide substrate, it exhibits a surprising non-competitive pattern versus ATP. This kinetic result suggests that 4-pyrrole phenylacyl is a critical determinant of the ATP-inhibitor binding pattern.

Table 1. PKCα inhibitory potencies of compound **3** and **8**

Compound	IC_{50} (μM)	K_i (μM) for PKCα substrate	K_i (μM) for ATP
3	4.70 ± 2.10	0.55 ± 0.07	68.9 ± 4.92
8	12.8 ± 1.04	2.32 ± 0.30	32.6 ± 8.05

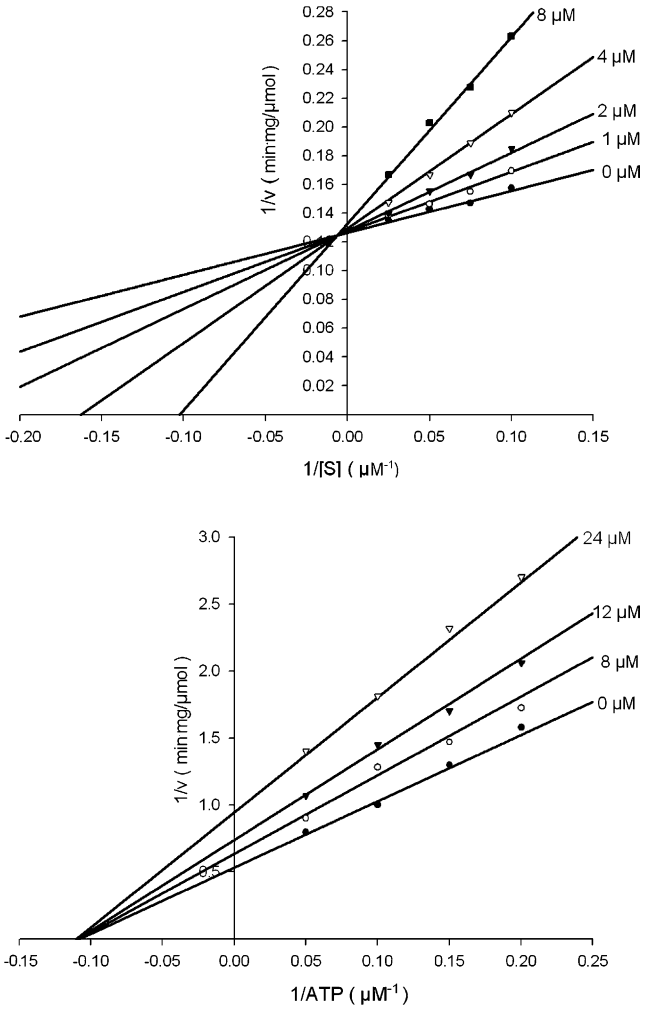


Figure 4. Inhibition pattern of compound **8** versus variable [PKCα substrate] and [ATP].

Acknowledgements

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4. *Peptide* **8**. The Rink-appended peptide **7** was prepared using a standard Fmoc protocol using Rink SS resin. The deprotected peptide was subsequently released from the resin using a TFA/TIS/phenol/H_2O cocktail (v/v 90/2/4/4) for 12 h at ambient temperature. The crude peptide was purified by HPLC (yield = 72% from 1 g resin). ^{1}H NMR (D_2O): δ 7.73–7.75 (d, *J* = 8.17 Hz, 2H), 7.23–7.20 (d, *J* = 8.84 Hz, 2H), 4.56 (m, 1H), 4.16–4.24 (m, 7H), 3.84 (s, 2H), 3.68–3.71 (d, *J* = 6.37 Hz, 2H), 3.54 (m, 4H), 3.09–3.13 (m, 6H), 1.92 (s, 3H), 1.67–1.71 (m, 8H), 1.48–1.54

(m, 7H), 1.25–1.32 (m, 13H), 0.75–0.77 (d, J = 5.67 Hz, 3H), 0.70–0.72 (d, J = 5.71 Hz, 3H). ^{13}C NMR (D_2O): δ 132.1, 120.5 (for 4-pyrrolidine phenylacyl group). ESIMS m/z calculated for $C_{51}H_{87}N_{21}O_{11}$: 1169.7, 1170.7, 1172.7, 1173.7 (MH^+), found m/z: 1169.4, 1170.3, 1172.3, 1173.4.

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9. *Protein kinase C assay*. The peptides Ac-Ser-Phe-Arg-Arg-Arg-Arg-NH_2 (PKC α, β and γ) and Ac-Pro-Arg-Lys-Arg-Glu-Gly-Ser-Val-Arg-Arg-Val-NH_2 (PKC δ and ζ) were used as substrates. The K_m value for this peptide is 15 and 6 μM, respectively. The V_{max} value is 0.526 and 1.445 μmol/min mg, respectively.
10. *Protein kinase C α assay (K_i determination for peptides **8** vs variable Ac-Ser-Phe-Arg-Arg-Arg-NH_2 substrate)*: 20 μL of peptide **8** (concentrations = 0, 1, 2, 4 and 8 μM) was added to a 20 μL assay buffer containing 62.5 mM HEPES (pH 7.5), peptide substrate (concentrations = 10, 20, 40 and 80 μM), 2.0 mM $CaCl_2{\cdot}2H_2O$, 34 mM $MgCl_2{\cdot}6H_2O$, 1.4 mM EGTA·Na, phosphatidylserine (225 μg/mL), diacylglycerol (40 μg/mL) and 313 μM cold ATP supplemented with 70–163 μCi/well [γ^{-33}P]ATP for radioactive detection. Enzyme diluted buffer (10 μL) containing 20 mM Tris (pH 7.5), PKC (0.5 ng/μL), 1 mM DTT, BSA (730 μg/mL) and 1 mM EDTA·4Na·$2H_2O$ were added to initiate the reaction. After a 10 min incubation at 30 °C, 100 μL of 6% phosphoric acid was added to each well to stop the reaction (total volume: 150 μL). Following additional 5 min incubation at ambient temperature, 75 μL from each reaction well was transferred into each well of a Unifilter (P81 cellulose phosphate paper) assay plate and washed four times with 0.1% phosphoric acid in water. Scintillation solution was added to each well and ^{33}P-incorporation measured by scintillation counting with a MicroBeta^{TM} TriLux & MicroBeta JET (Perkin–Elmer). IC_{50} values were calculated using GraFit (Erithacus Software Limited) and K_i values were calculated using Enzyme Kinetics, SigmaPlot (SPSS Inc.).
11. *Protein kinase C assay (K_i determination for peptides **8** vs variable ATP)*: 20 μL of peptide **8** (concentrations = 0, 8, 12 and 24 μM) was added to a 20 μL assay buffer containing 62.5 mM HEPES (pH 7.5), 50 μM peptide substrate, 2.0 mM $CaCl_2{\cdot}2H_2O$, 34 mM $MgCl_2{\cdot}6H_2O$, 1.4 mM EGTA·Na, phosphatidylserine (225 μg/mL), diacylglycerol (40 μg/mL) and cold ATP (concentrations = 10, 12.5, 16.5, 25 and 50 μM each) supplemented with 7–16 μCi/well [γ^{-33}P]ATP for radioactive detection. Enzyme diluted buffer (10 μL) containing 20 mM Tris (pH 7.5), PKC (0.5 ng/μL), 1 mM DTT, BSA (730 g/mL) and 1 mM EDTA·4Na·$2H_2O$ were added to initiate the reaction. Subsequent assay workup and scintillation counting were performed as described above for the PKCα assay.
12. IC_{50} determinations of compound **8** for individual PKC isoforms. *Protein kinase C α, β and γ*: 20 μL assay buffer solution, containing 62.5 mM Hepes (pH 7.5), 50 μM Ac-Ser-Phe-Arg-Arg-Arg-Arg-NH_2, $CaCl_2{\cdot}2H_2O$ (2.0 mM), $MgCl_2{\cdot}6H_2O$ (30.0 mM), EGTA·Na (1.0 mM), PS (50.0 μg/mL), DAG 10 μg/mL, cold ATP (300 μM), supplemented with 55 μCi/plate [γ-^{33}P]ATP for radioactive detection, were added to 20 μL of a solution containing inhibitor lead at various concentrations (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 μM). Enzyme buffer solution (10 μL) containing 20 mM Tris (pH 7.5), PKCα (0.5 ng/well), 0.5 mM DTT, BSA (375 μg/mL), and EDTA·4Na·$2H_2O$ (0.5 mM) was added to initiate the reaction. Reactions and their workup were carried out as described above. The IC_{50} values for pure compounds as inhibitors were calculated based on the experimental data using GraFit (Erithacus Software Limited). *Protein kinase C δ*: as described for PKCα except that the assay was performed in the absence of $CaCl_2{\cdot}2H_2O$, *Protein kinase C ζ*: as described for PKCα except that the assay was performed in the absence of $CaCl_2{\cdot}2H_2O$ and DAG.