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Design and physicochemical properties of new fluorescent ligands of protein kinase C isozymes focused on CH/π interaction

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Abstract—Phorbol ester-type tumor promoters such as indolactam-V (IL-V, **1**) bind to the C1 domains of protein kinase C (PKC) isozymes. A more convenient method to investigate the interaction between each tumor promoter and PKC C1 domain is needed. Focusing on our recent finding that the indole ring of IL-V is involved in the CH/π interaction with Pro-11 of the PKCδ-C1B domain, we developed new fluorescent probes (**2**–**4**) from IL-V by forming a pyrroloindazole ring. Compound **2** without a substituent at the pyrroloindazole ring bound most strongly to PKC C1 domains with a potency similar to IL-V, but its fluorescent intensity was the weakest of any of the probes. Although the binding affinity of **3** with a methyl group was significantly weaker than that of IL-V, **4** with a trifluoromethyl group showed moderate affinity and the most potent fluorescence intensity. The fluorescence intensity and emission maxima of **4** changed significantly when bound to the PKCδ-C1B peptide in both the presence and absence of phosphatidylserine. These results suggest that **4** could be a useful probe for analyzing the interaction of tumor promoters with PKC C1 domains.
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1. Introduction

The members of a protein kinase C (PKC) family of serine/threonine kinases play critical roles in complex signal transduction networks that regulate a diverse range of cellular processes, such as cell proliferation, differentiation, and apoptosis.[1,2] They are activated by phorbol ester-type tumor promoters[3] as well as the endogenous second messenger *sn*-1,2-diacylglycerol (DAG).[4,5] Recent investigations revealed that PKC isozymes (PKCs) are important therapeutic targets because they are involved in various diseases such as diabetes, nerve pain, AIDS, and tumor promotion.[6–10]

The PKCs comprise classical PKCs (α, βI, βII, γ) that are calcium-dependent, novel PKCs (δ, ε, η, θ) that are calcium-independent, and atypical PKCs (ζ, ι/λ) that are not activated by DAG or tumor promoters (Fig. 1).[4,5] Each of the classical and novel PKCs contains two highly conserved C1 domains (C1A and C1B),[11] zinc finger motifs with cysteine-rich sequences that act as the binding sites for DAG and tumor promoters.[12,13] When DAG or tumor promoters bind to the C1 domains of PKCs, they translocate to the membrane to be activated.[14] Since the translocation and activation of PKCs are characteristic of each isoform and activator,[15,16] a precise and intimate analysis of the binding between each tumor promoter and C1 domain is needed to understand the mechanism of PKCs activation both in vivo and in vitro.

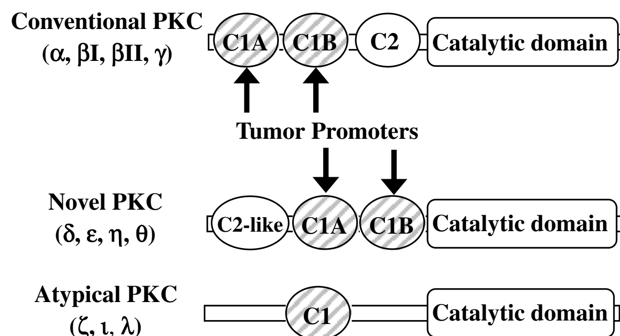

Figure 1. Structure of PKC isozymes.

Keywords: CH/π interaction; Fluorescent probe; Indolactam-V; Protein kinase C; Pyrroloindazole ring.

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The most reliable method of analyzing the binding of a ligand to PKC C1 domains is the competitive assay using [^{3}H]phorbol 12,13-dibutyrate (PDBu) established by Sharkey and Blumberg.[17] As a more convenient way to assess the ability of a ligand to bind PKCs, a modified binding assay using synthetic peptides of each PKC C1 domain was developed in our laboratory.[18,19] However, the use of radioisotopes is troublesome, and the assay using [^{3}H]PDBu cannot estimate weak binding, especially in the absence of phosphatidylserine, because of difficulty in separating the bound [^{3}H]PDBu from free [^{3}H]PDBu. A fluorescent PKC ligand has the potential to circumvent these problems. For example, sapintoxin-D (SAPD), a fluorescent phorbol ester, is used to estimate binding affinity to PKC C1 domains.[20] Resonance energy transfer between the tryptophan residue in each PKC C1 domain and the SAPD fluorophore allows direct determination of SAPD binding without separating the bound SAPD from free SAPD, as is required in the assay using [^{3}H]PDBu. However, this assay is not valid when using C1 domains without a tryptophan residue such as the C1B domains of classical PKCs. A new fluorescent probe with a fluorophore that can interact directly with any residue of the PKC C1 domains is thus strongly desired.

To develop the new fluorescent probe, we selected (−)-indolactam-V[21] (IL-V) as a lead compound which is the basic structure of tumor-promoting teleocidins[3] and was isolated from *Streptomyces blastmyceticum*.[22] Because of its stability and relatively simpler structure, many IL-V derivatives have been synthesized for structure–activity studies.[23–27] Since the indole ring of IL-V has recently been shown to be involved in the CH/π interaction with Pro-11 of the PKCδ-C1B domain (Fig. 2),[28] transformation of the indole ring to a fluorofore is preferable. Although we previously introduced various substituents into the indole ring of IL-V to find that 2,7-diacylindolactam-Vs fluoresced strongly, their binding affinity for PKCs was extremely low.[29]

In this study, we transformed the indole moiety of IL-V to a pyrroloindazole ring to give three fluorescent probes (**2**, **3**, and **4**). Compound **4** with a trifluoromethyl group showed moderate binding affinity to PKC C1 domains and the most potent fluorescence intensity. The fluorescence intensity and emission maxima of **4** changed significantly when bound to the PKCδ-C1B peptide in both the presence and absence of phosphatidylserine. The results suggest that **4** could be a useful tool for analyzing the interaction of ligands with PKC C1 domains.

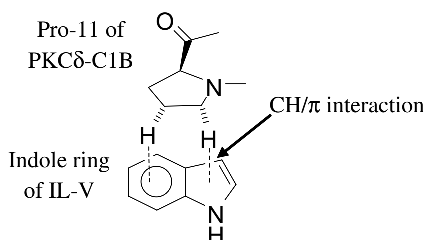


Figure 2. CH/π interaction between IL-V and Pro-11 of the PKCδ-C1B domain.

2. Results and discussion

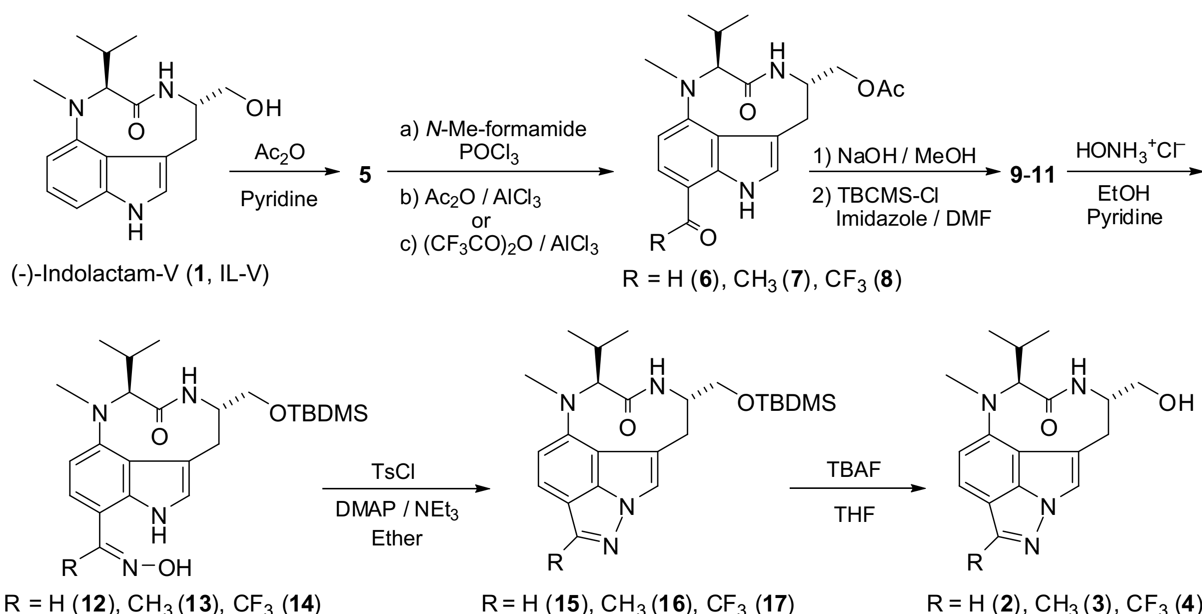
2.1. Synthesis of new fluorescent derivatives of IL-V (2–4)

It is quite difficult to anticipate whether a designed compound will fluoresce or not. However, elongation of the conjugation is an effective way to make it fluoresce. Previous structure–activity studies on IL-V revealed that a hydrogen atom at position 1 is not necessary for binding to PKC C1 domains, and hydrophobic substituents at position 7 enhanced the binding.[23,24,30,31] We introduced an additional ring between positions 1 and 7 to elongate the conjugation. A pyrroindazole ring was selected because of ease of synthetic accessibility. In order to tune the PKC binding ability and fluorescence intensity, three kinds of compounds with distinct substituents (**2**–**4**) were synthesized (Scheme 1).

A strategy for synthesizing these derivatives is to introduce each acyl chain at position 7 of IL-V and then to form a pyrroloindazole ring. After the protection of the hydroxyl group of IL-V with an acetyl group (**5**, 91%), acyl substituents were introduced at position 7 of **5**. For the introduction of a formyl group, the Vilsmeier–Haack reaction of **5** with *N*-methylformamide gave **6** (54%). For the introduction of an acetyl or trifluoroacetyl group, the Friedel–Crafts acylation of **2** with acetic anhydride and trifluoroacetic anhydride gave **7** and **8**, respectively (21% and 40%). Resultant compounds (**6**, **7**, and **8**) were deprotected and treated with *t*-butyldimethylsilyl chloride (TBDMS-Cl) for reprotection to afford **9**, **10**, and **11**, respectively (79%, 76%, and 82%). The carbonyl groups of **9**, **10**, and **11** were converted to the corresponding oximes (**12**, **13**, and **14**) with hydroxyammonium chrolide (67%, 64%, and 51%, respectively) and were tosylated in the presence of dimethylaminopyridine (DMAP) and triethylamine (TEA). Resultant tosylated compounds were very labile and a ring-closing reaction at position 1 proceeded successively to yield **15**, **16**, and **17** (35%, 31%, and 48%, respectively). A nitrogen atom at position 1 would have attacked nucleophilically the nitrogen atom bonded with the tosyl group as a good leaving group. Final deprotection of the hydroxyl groups with tetrabutylammonium fluoride (TBAF) gave **2**, **3**, and **4** (85%, 87%, and 88%, respectively). The ^{1}H NMR spectra showed that **2**, **3**, and **4** existed only as the twist conformer with a *cis* amide[32] at room temperature. All proton and carbon atoms in **2**, **3**, and **4** were assigned in $CDCl_3$ or CD_3OD using ^{1}H–^{1}H COSY and scalar heteronuclear experiments (HMBC and HMQC). Compounds **2**, **3**, and **4** showed strong fluorescence on TLC plates under light at 365 nm. Black et al.[33] reported a synthesis of the pyrroloindazole ring that shows fluorescence.

2.2. Fluorescent properties of the IL-V derivatives with a pyrroloindazole ring (2–4)

The data on the fluorescence of these derivatives (**2**–**4**) are summarized in Table 1. The fluorescent intensity of all derivatives was considerably higher in EtOH than in Tris–maleate buffer. Since the fluorescence intensity of **4** both in EtOH and in Tris–maleate buffer was high-



Scheme 1. Synthesis of fluorescent IL-V derivatives (**2**–**4**).

Table 1. Effects of solvents on fluorescent IL-V derivatives (**2**–**4**)[a]

Compound	Solvent	λ_{max} (excitation)	λ_{max} (emission)	Relative fluorescence intensity[b]
2	EtOH	340	379	4.22
	Buffer	343	390	1.00
3	EtOH	362	422	3.81
	Buffer	361	432	1.64
4	EtOH	376	422	9.18
	Buffer	378	437	3.47

[a] Measurements were carried out at 25 °C at a concentration of 10^{-6} M. Each sample was excited at the wavelength of its excitation maximum.

[b] Compound **2** in buffer was chosen as the standard for the relative fluorescence intensity.

est, the excitation and emission spectra of **4** are shown in Figure 3 as representative. Compound **4** with an electron-withdrawing group showed the strongest fluorescence, and the fluorescence intensity of **2** was similar to that of **3**. These results suggest that the electron-withdrawing group in the pyrroloindazole ring increases the intensity. In addition, the fluorescence intensity of these probes greatly increased when 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (PS), a co-activator of PKC, was added (data not shown). This reflects the strong fluorescence of these probes in EtOH compared that to in Tris–maleate buffer.

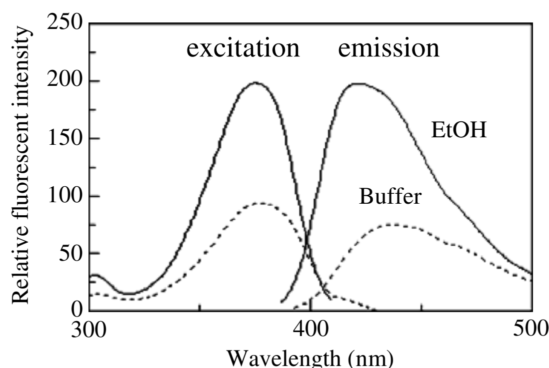


Figure 3. Fluorescence spectra of **4**. Fluorescent excitation and emission spectra of **4** (10^{-6} M) are shown with solid lines (in EtOH) and dotted lines (in 50 mM Tris–maleate buffer).

2.3. Binding affinity of the fluorescent IL-V derivatives (2–4) for PKC C1 domains

The affinity of **2**, **3**, and **4** for the PKC C1 peptides was evaluated by inhibition of the specific binding of [^{3}H]phorbol 12,13-dibutyrate (PDBu) to these peptides with a method reported previously.[17–19] Table 2 shows the inhibition constant (K_i) values of **2**, **3**, and **4** for the PKC C1 peptides. The binding affinity of **2** was similar to that of IL-V, suggesting that steric hindrance does not exist at positions 1 and 7 of IL-V as previously reported.[23,24,30,31] However, the affinity of **3** and **4** was significantly lower than that of IL-V and **2**. This is an unexpected result since the introduction of a hydrophobic substituent at position 7 of IL-V enhanced the biological activities related to PKCs activation.[24] The pyrroloindazole ring with a substituent is more rigid than the indole ring with a substituent at position 7 of IL-V. This might produce some steric hindrance to reduce the binding of these derivatives.

However, an electronic factor is also deduced to play a critical role because the nitrogen atoms on the pyorroloindazole ring may have some polarity. Since the substituents at positions 1 and 7 of IL-V are involved in the hydrophobic interaction with phosphatidylserine when bound to PKC C1 domains,[23,24,27] the polar nitrogens would decrease the binding ability. Electron-donating

Table 2. K_i values for the inhibition of the specific binding of [^{3}H]PDBu by **3**, **4**, and **5**

PKC C1 peptide	K_i (nM)			
	2 (R = H)	**3** (R = CH_3)	**4** (R = CF_3)	IL-V
α-C1A (72-mer)[a]	85.5 (14)[b]	1090 (180)	112 (13)	21
α-C1B	5960 (730)	>10,000	9380 (32)	4000
β-C1A (72-mer)	88.0 (6.4)	1100 (650)	127 (31)	19
β-C1B	357 (4.3)	2620 (350)	721 (110)	140
γ-C1A	61.3 (3.2)	1510 (220)	234 (3.7)	140
γ-C1B	225 (6.4)	2580 (20)	1100 (65)	210
δ-C1B	17.7 (3.0)	128 (5.4)	52.1 (1.7)	11
ε-C1B	22.5 (1.9)	407 (34)	80.1 (9.6)	7.7
η-C1B	9.9 (1.2)	223 (4.5)	29.7 (7.3)	5.5
θ-C1B	18.6 (0.1)	149 (24)	51.1 (15)	8.7

[a] Ten residues from both the N- and C-terminal of α-C1A and β-C1A were elongated because the solubility of the original 52-mer peptides was extremely low.[16]

[b] Standard deviation of at least two separate experiments.

methyl group might make the nitrogens more polar to reduce the binding affinity while electron-withdrawing trifluoromethyl group might delocalize the electrons on the nitrogen to increase the binding affinity. The fact that the binding affinity of **4** was lower than **2** could be explained by the polality of the trifluoromethyl group itself.

These results suggest that **2**, **3**, and **4** have potential as fluorescent probes of PKCs since they showed significant ability to bind most PKC C1 peptides.

2.4. Fluorescence spectra of 4 when bound to PKCδ-C1B

The above data indicate that **4** would be the most suitable fluorescent ligand among the three compounds because it showed the strongest fluorescence both in EtOH and in Tris–maleate buffer and a moderate affinity for most PKC C1 peptides. To examine the potential of **4** as a new fluorescent probe for PKCs, the fluorescence spectra of **4** in the presence of a PKC C1 peptide with or without phosphatidylserine were recorded. Figure 4 shows the emission spectrum of **4** when bound to PKCδ-C1B. In the presence of PKCδ-C1B, the fluorescence intensity of **4** increased and the emission maximum moved to a lower wavelength. Since the indole ring of IL-V is involved in the CH/π interaction,[28] binding to PKCδ-C1B would have changed the electron density of the pyrroloindazole ring to vary the fluorescence property of **4**. This result corresponds with the fact that the fluorescence intensity of **4** increased in EtOH compared to that in Tris–maleate buffer since the binding pocket of PKCδ-C1B was thought to have a hydrophobic nature.

On the other hand, the fluorescence intensity of **4** decreased when bound to PKCδ-C1B in the presence of phosphatidylserine (Fig. 4b). This would be because the nature inside the micelle of phosphatidylserine vesicle is more hydrophobic than that in the binding pocket of PKCδ-C1B. To examine whether these changes in the fluorescence reflect the specific binding of **4** to PKCδ-C1B, the competition assay using PDBu was carried out. Some part of the changed fluorescence intensity of **4** recovered when added excess amount of PDBu both in the presence and absence of phosphatidylserine

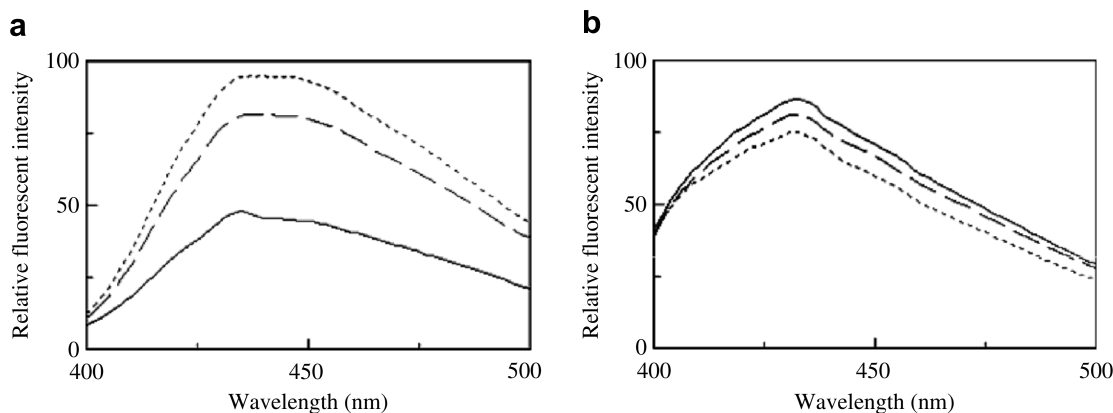


Figure 4. The change in the fluorescent property of **4** when bound to PKCδ-C1B peptide in 50 mM Tris–maleate buffer. Fluorescence emission spectra of **4** in the absence of PKCδ-C1B peptide are shown with solid lines, those in the presence of PKCδ-C1B peptide with dotted lines, and those in the presence of both PKCδ-C1B peptide and excess amount (10 μM) of PDBu with dashed lines. (a) In the absence and (b) in the presence of phosphatidylserine. Excitation wavelength was 377 nm. The concentrations of **4** were $10^{-6.5}$ M (a) and 10^{-7} M (b), respectively. In both experiments, 200 nM of PKCδ-C1B was employed since the B_{max} value in the Scatchard analysis was 20–30% as reported previously.[19]

(Fig. 4a and b, dashed lines), suggesting that at least some part of the change in the fluorescence intensity of **4** could reflect the specific binding. These results suggest that **4** could be used to estimate the ability of a ligand to bind PKC C1 domains both in the presence and in the absence of phosphatidylserine.

3. Conclusion

In summary, new fluorescent probes (**2**, **3**, and **4**) with a pyrroroindazole group as a fluorophore were designed from IL-V, to focus on the CH/π interaction of the aromatic ring with PKC C1 domains. These probes bound significantly to most of the PKC C1 peptides. Since the fluorophore of these probes can directly interact with Pro-11 of the PKC C1 domains, the probes could be useful for analyzing the direct interaction between each PKC ligand and C1 domain. Notably, **4** could be most useful since **4** with moderate binding affinity for PKC C1 peptides fluoresced most strongly among them. A combination of **4** and PKC C1 peptides would be useful for estimating the binding ability of various PKC ligands.

4. Experimental

4.1. General remarks

The following spectroscopic and analytical instruments were used: UV, Shimadzu UV-2200A; $[\alpha]_D$, Jasco DIP-1000; ^{1}H NMR, Bruker AVANCE 400 (reference to TMS); HPLC, Waters Model 600E with Model 2487 UV Detector; (HR) EIMS, JEOL JMS-600H; fluorescence spectra, JASCO FP-6500.

HPLC was carried out on a YMC-packed SH-342-5 (ODS, 20 mm id × 150 mm) column and YMC-packed SH-042-5 (SIL, 20 mm id × 150 mm) column (Yamamura Chemical Laboratory). Wakogel C-200 (silica gel, Wako Pure Chemical Industries) and YMC A60-350/250 gel (ODS, Yamamura Chemical Laboratory) were used for column chromatography. [^{3}H]PDBu (15.6 Ci/mmol) was purchased from Perkin-Elmer Life Science. 1,2-Dioleoyl-*sn*-glycero-3-phospho-L-serine and γ-globulin were purchased from Sigma. All the other chemicals and reagents were purchased from Wako and Aldrich, and used without further purification.

4.2. Synthesis of the fluorescent IL-V derivatives (2–4)

4.2.1. (−)-14-*O*-Acetylindolactam-V (5).[26] (−)-Indolactam-V (**1**, 213 mg, 0.71 mmol) was treated as reported to give **5** (211 mg, 0.65 mmol, 91%).

4.2.2. (−)-14-*O*-Acetyl-7-formylindolactam-V (6).[26] Compound **5** (48.5 mg, 0.14 mmol) was treated as reported to give **6** (27.8 mg, 0.075 mmol, 54%).

4.2.3. (−)-14-*O*-Acetyl-7-acetylindolactam-V (7).[26] Compound **5** (219.2 mg, 0.64 mmol) was treated as reported to give **7** (51.8 mg, 0.134 mmol, 21%).

4.2.4. (−)-14-*O*-Acetyl-7-trifluoroacetylindolactam-V (8). To a mixture of **5** (77.2 mg, 0.23 mmol) and trifluoroacetic anhydride (150 μl) in nitrobenzene (1 ml) was added $AlCl_3$ (33.6 mg) at room temperature. The reaction mixture was stirred for 7 h at room temperature and partitioned between EtOAc and H_2O. The collected EtOAc layer was washed with brine, dried over Na_2SO_4, and concentrated. The residue was purified by column chromatography on Wakogel C-200 using hexane and increasing amounts of EtOAc, followed by HPLC on YMC SH-342-5 using 60%, 70%, and 100% MeOH to give **8** (39.8 mg, 0.091 mmol, 40%).

Compound **8**: $[\alpha]_D$ −340° (*c* = 0.80, MeOH, 23.8 °C); UV λ_{max} (MeOH) nm (ε): 339 (22,000), 300 (2800), 266 (9200); ^{1}H NMR δ (500 MHz, 0.043 M, $CDCl_3$, 295 K) ppm: 0.59 (3H, d, *J* = 6.8 Hz), 0.96 (3H, d, *J* = 6.4 Hz), 2.11 (3H, s), 2.62 (1H, m), 3.04 (3H, s), 3.19 (1H, dd, *J* = 17.5, 3.5 Hz), 3.15 (1H, d, *J* = 17.5 Hz), 4.00 (1H, dd, *J* = 11.3, 8.4 Hz), 4.17 (1H, m), 4.22 (1H, dd, *J* = 11.3, 3.6 Hz), 4.58 (1H, d, *J* = 10.2 Hz), 6.18 (1H, s), 6.56 (1H, d, *J* = 8.8 Hz), 7.06 (1H, d, *J* = 1.6 Hz), 7.84 (1H, d, *J* = 8.8 Hz), 10.7 (1H, br s); ^{13}C NMR δ (125 MHz, 0.043 M, $CDCl_3$, 295 K) ppm: 18.9, 20.8, 21.0, 28.6, 33.1, 34.6, 52.7, 65.7, 71.3, 106.0, 106.4, 114.5, 116.4, 117.5 (q, *J* = 290 Hz), 122.8, 129.9, 140.3, 155.4, 171.0, 171.2, 177.9 (q, *J* = 34 Hz); HR-EI-MS *m*/*z*: 439.1734 (M^+, calcd for $C_{21}H_{24}F_3N_3O_4$, 439.1719).

4.2.5. (−)-14-*O*-TBDMS-7-formyl-indolactam-V (9). To a solution of **6** (27.8 mg, 0.075 mmol) in MeOH (1 ml) was added 2 M NaOH (100 μl). The reaction mixture was stirred for 30 min at room temperature and partitioned between EtOAc and saturated aqueous $NaHCO_3$. The collected EtOAc layer was washed with brine, dried over Na_2SO_4, and concentrated to give crude (−)-7-formyl-indolactam-V. To this compound dissolved in dry DMF (0.1 ml) were added imidazole (10.5 mg) and TBDMS-Cl (22.4 mg) under cooling with a water bath. The reaction mixture was stirred for 40 min at room temperature and partitioned between EtOAc and H_2O. The collected EtOAc layer was washed with brine, dried over Na_2SO_4, and concentrated. The residue was purified by column chromatography on Wakogel C-200 using hexane and increasing amounts of EtOAc to give **9** (26.0 mg, 0.059 mmol, 79% for two steps).

Compound **9**: $[\alpha]_D$ −560° (*c* = 1.15, MeOH, 20.0 °C); UV λ_{max} (MeOH) nm (ε): 371 (25,000), 261 (16,100); ^{1}H NMR δ (400 MHz, 0.047 M, $CDCl_3$, 298 K) ppm: 0.03 (3H, s), 0.06 (3H, s), 0.57 (3H, d, *J* = 6.8 Hz), 0.88 (9H, s), 0.94 (3H, d, *J* = 6.3 Hz), 2.62 (1H, m), 2.97 (1H, dd, *J* = 17.7, 3.2 Hz), 3.00 (3H, s), 3.15 (1H, d, *J* = 17.7 Hz), 3.49 (1H, dd, *J* = 10.1, 9.7 Hz). 3.65 (1H, dd, *J* = 10.1, 4.4 Hz), 3.97 (1H, m), 4.56 (1H, d, *J* = 10.2 Hz), 6.23 (1H, s), 6.56 (1H, d, *J* = 8.3 Hz), 6.99 (1H, d, *J* = 1.6 Hz), 7.51 (1H, d, *J* = 8.3 Hz), 9.83 (1H, s), 10.7 (1H, br s); ^{13}C NMR δ (100 MHz, 0.047 M, $CDCl_3$, 298 K) ppm: −5.4, −å5.3, 18.3, 19.0, 21.1, 25.8 (3C), 28.6, 32.9, 34.0, 54.8, 65.3, 71.2, 105.4, 113.9, 115.1, 114.6, 116.2, 122.4, 132.2, 138.1, 154.1, 171.6, 190.4; HR-EI-MS *m*/*z*: 443.2604 (M^+, calcd for $C_{24}H_{37}N_3O_3Si$, 443.2604).

4.2.6. (−)-14-*O*-TBDMS-7-acetyl-indolactam-V (10). Compound **7** (47.7 mg, 0.12 mmol) was treated similarly as described for the synthesis of **9** to give **10** (41.7 mg, 0.091 mmol, 76% for two steps).

Compound **10**: $[\alpha]_D$ −480° (c = 0.92, MeOH, 24.7 °C); UV λ_{max} (MeOH) nm (ε): 370 (19,500), 257 (14,500); ^{1}H NMR δ (400 MHz, 0.040 M, $CDCl_3$, 297 K) ppm: 0.02 (3H, s), 0.05 (3H, s), 0.57 (3H, d, J = 6.8 Hz), 0.87 (9H, s), 0.94 (3H, d, J = 6.4 Hz), 2.61 (1H, m), 2.61 (3H, s), 2.95 (1H, dd, J = 17.6, 3.2 Hz), 2.99 (3H, s), 3.15 (1H, d, J = 17.6 Hz), 3.48 (1H, dd, J = 10.2, 9.7 Hz). 3.64 (1H, dd, J = 10.2, 4.4 Hz), 4.01 (1H, m), 4.53 (1H, d, J = 10.2 Hz), 6.21 (1H, s), 6.48 (1H, d, J = 8.5 Hz), 6.98 (1H, s), 7.70 (1H, d, J = 8.5 Hz), 10.8 (1H, br s); ^{13}C NMR δ (100 MHz, 0.040 M, $CDCl_3$, 297 K) ppm: −5.4, −5.3, 18.2, 19.1, 21.1, 25.8 (3C), 28.6, 32.9, 34.1, 54.8, 65.3, 71.2, 104.8, 113.2, 114.1, 116.7, 122.4, 128.3, 139.1, 153.4, 171.9, 198.0; HR-EI-MS m/z: 454.2774 (M^+, calcd for $C_{25}H_{39}N_3O_3Si$, 454.2761).

4.2.7. (−)-14-*O*-TBDMS-7-trifluoroacetyl-indolactam-V (11). Compound **8** (312.2 mg, 0.71 mmol) was treated similarly as described for the synthesis of **9** to give **11** (296.6 mg, 0.580 mmol, 82% for two steps).

Compound **11**: $[\alpha]_D$ −620° (c = 1.28, MeOH, 24.5 °C); UV λ_{max} (MeOH) nm (ε): 399 (22,000), 300 (3200), 267 (9800); ^{1}H NMR δ (500 MHz, 0.065 M, $CDCl_3$, 298 K) ppm: 0.03 (3H, s), 0.05 (3H, s), 0.57 (3H, d, J = 6.8 Hz), 0.87 (9H, s), 0.95 (3H, d, J = 6.4 Hz), 2.61 (1H, m), 2.98 (1H, dd, J = 17.5, 3.1 Hz), 3.03 (3H, s), 3.15 (1H, d, J = 17.5 Hz), 3.49 (1H, dd, J = 9.9, 9.7 Hz). 3.64 (1H, dd, J = 9.9, 4.5 Hz), 3.90 (1H, m), 4.60 (1H, d, J = 10.2 Hz), 6.25 (1H, s), 6.54 (1H, d, J = 8.8 Hz), 7.02 (1H, s), 7.82 (1H, d, J = 8.8 Hz), 10.7 (1H, br s); ^{13}C NMR δ (125 MHz, 0.065 M, $CDCl_3$, 298 K) ppm: −5.4, −5.3, 18.3, 18.9, 20.9, 25.8 (3C), 28.6, 33.0, 34.1, 54.5, 65.3, 71.3, 105.9, 106.2, 115.1, 115.9, 117.6 (q, J = 289 Hz), 122.6, 130.0, 140.3, 155.6, 171.1, 177.7 (q, J = 33.4 Hz); HR-EI-MS m/z: 511.2479 (M^+, calcd for $C_{25}H_{36}F_3N_3O_3Si$, 511.2478).

4.2.8. Compound 12. To a solution of **9** (33.7 mg, 0.076 mmol) in dry pyridine (0.3 ml) were added hydroxyammonium chloride (31.7 mg) and EtOH (0.1 ml). The reaction mixture was stirred for 3 h at 70 °C and partitioned between EtOAc and H_2O. The collected EtOAc layer was washed with brine, dried over Na_2SO_4, and concentrated. The residue was purified by HPLC on YMC SH-342-5 using 85% MeOH, then on YMC SH-042-5 (hexane/$CHCl_3$/*i*-PrOH = 90:8:2) to give **12** (23.5 mg, 0.051 mmol, 67%).

Compound **12**: $[\alpha]_D$ −400° (c = 2.7, MeOH, 20.0 °C); UV λ_{max} (MeOH) nm (ε): 343 (27,600), 255 (17,300); ^{1}H NMR δ (400 MHz, 0.12 M, $CDCl_3$, 298 K) ppm: 0.01 (3H, s), 0.04 (3H, s), 0.60 (3H, d, J = 6.7 Hz), 0.87 (9H, s), 0.94 (3H, d, J = 6.3 Hz), 2.64 (1H, m), 2.95 (1H, dd, J = 17.5, 3.4 Hz), 2.95 (3H, s), 3.16 (1H, d, J = 17.5 Hz), 3.50 (1H, dd, J = 10.1, 9.6 Hz), 3.65 (1H, dd, J = 10.1, 4.4 Hz), 4.13 (1H, m), 4.46 (1H, d, J = 10.2 Hz), 6.28 (1H, s), 6.50 (1H, d, J = 8.1 Hz), 6.95 (1H, s), 7.08 (1H, d, J = 8.1 Hz), 7.85 (1H, br s), 8.28 (1H, s), 9.96 (1H, br s); ^{13}C NMR δ (100 MHz, 0.12 M, $CDCl_3$, 298 K) ppm: −5.4, −5.3, 14.1, 18.2, 19.3, 21.4, 25.8 (3C), 28.6, 32.9, 34.0, 55.0, 65.3, 71.2, 105.6, 108.1, 114.5, 117.2, 121.9, 126.5, 136.2, 149.9, 151.4, 172.8; HR-EI-MS m/z: 458.2714 (M^+, calcd for $C_{24}H_{38}N_4O_3Si$, 458.2713).

4.2.9. Compound 13. Compound **10** (41.7 mg, 0.091 mmol) was treated similarly as described for the synthesis of **12** to give **13** (27.4 mg, 0.058 mmol, 64%).

Compound **13**: $[\alpha]_D$ −320° (c = 1.5, MeOH, 26.6 °C); UV λ_{max} (MeOH) nm (ε): 339 (21,200), 250 (15,800); ^{1}H NMR δ (400 MHz, 0.11 M, $CDCl_3$, 298 K) ppm: 0.01 (3H, s), 0.03 (3H, s), 0.61 (3H, d, J = 6.7 Hz), 0.86 (9H, s), 0.94 (3H, d, J = 6.3 Hz), 2.39 (3H, s), 2.68 (1H, m), 2.94 (1H, dd, J = 17.3, 3.4 Hz), 2.95 (3H, s), 3.17 (1H, d, J = 17.3 Hz), 3.47 (1H, dd, J = 10.2, 9.7 Hz). 3.63 (1H, dd, J = 10.2, 4.4 Hz), 4.15 (1H, m), 4.45 (1H, d, J = 10.2 Hz), 6.25 (1H, s), 6.51 (1H, d, J = 8.3 Hz), 6.92 (1H, s), 7.33 (1H, d, J = 8.3 Hz), 7.64 (1H, s), 10.5 (1H, br s); ^{13}C NMR δ (100 MHz, 0.11 M, $CDCl_3$, 298 K) ppm: −5.4, −5.3, 18.2, 19.1, 21.1, 25.8 (3C), 28.6, 32.9, 34.1, 54.8, 65.3, 71.2, 104.8, 113.2, 114.1, 116.7, 122.4, 128.3, 139.1, 153.4, 171.9, 198.0; HR-EI-MS m/z: 472.2850 (M^+, calcd for $C_{25}H_{40}N_4O_3Si$, 472.2870).

4.2.10. Compound 14. Compound **11** (99.6 mg, 0.195 mmol) was treated similarly as described for the synthesis of **12** to give **14** (52.8 mg, 0.100 mmol, 51%).

Compound **14**: $[\alpha]_D$ −230° (c = 0.90, MeOH, 26.1 °C); UV λ_{max} (MeOH) nm (ε): 333 (9800), 245 (13,000); ^{1}H NMR δ (500 MHz, 0.032 M, $CDCl_3$, 300 K) ppm: 0.04 (3H, s), 0.05 (3H, s), 0.62 (3H, d, J = 6.8 Hz), 0.87 (9H, s), 0.94 (3H, d, J = 6.3 Hz), 2.63 (1H, m), 2.95 (3H, s), 2.99 (1H, dd, J = 17.6, 3.3 Hz), 3.16 (3H, d, J = 17.6 Hz), 3.51 (1H, dd, J = 9.8, 9.4 Hz). 3.66 (1H, dd, J = 9.8, 4.6 Hz), 4.16 (1H, m), 4.46 (1H, d, J = 10.2 Hz), 6.39 (1H, s), 6.57 (1H, d, J = 8.4 Hz), 6.93 (1H, s), 7.26 (1H, d, J = 8.4 Hz), 8.24 (1H, s), 10.6 (1H, br s); ^{13}C NMR δ (125 MHz, 0.032 M, $CDCl_3$, 300 K) ppm: −5.5, −5.3, 18.2, 19.3, 21.4, 25.8 (3C), 28.6, 32.9, 34.0, 54.9, 65.3, 71.3, 102.3, 106.0, 114.7, 117.8, 121.3 (q, J = 274 Hz), 122.0, 123.8, 136.9, 146.8 (q, J = 32 Hz), 150.6, 173.0; HR-EI-MS m/z: 526.2576 (M^+, calcd for $C_{25}H_{37}F_3N_4O_3Si$, 526.2587).

4.2.11. Compound 15. To a mixture of **12** (23.5 mg, 0.051 mmol), TEA (18 µl), and DMAP (310 µg) in diethyl ether (1 ml) was added *p*-toluenesulfonyl chloride (11.4 mg) at 0 °C. The reaction mixture was stirred at 0 °C for 10 min and then at room temperature for 58 h. Diethyl ether was removed by an Ar atmosphere and the residue was partitioned between EtOAc and H_2O. The collected EtOAc layer was washed with brine, dried over Na_2SO_4, and concentrated. The residue was purified by HPLC on YMC SH-342-5 using 85% MeOH to give **15** (8.0 mg, 0.018 mmol, 35%).

Compound **15**: $[\alpha]_D$ −630° (c = 1.6, MeOH, 20.0 °C); UV λ_{max} (MeOH) nm (ε): 340 (31,200), 244 (43,100); ^{1}H NMR δ (400 MHz, 0.036 M, $CDCl_3$, 297 K) ppm: 0.03 (3H, s), 0.06 (3H, s), 0.56 (3H, d, J = 6.7 Hz), 0.88 (9H, s), 0.93 (3H, d, J = 6.3 Hz), 2.61 (1H, m), 2.94 (3H, s), 2.95 (1H, dd, J = 17.6, 3.3 Hz), 3.14 (1H, d, J = 17.6 Hz), 3.50 (1H, dd, J = 10.2, 9.6 Hz). 3.64 (1H, dd, J = 10.2, 4.4 Hz), 4.00 (1H, m), 4.43 (1H, d, J = 10.2 Hz), 6.20 (1H, s), 6.49 (1H, d, J = 8.3 Hz), 6.99 (1H, s), 7.40 (1H, d, J = 8.3 Hz), 8.69 (1H, s); ^{13}C NMR δ (100 MHz, 0.036 M, $CDCl_3$, 297 K) ppm: −5.4, −5.3, 18.2, 19.1, 21.2, 25.8 (3C), 28.6, 32.9, 34.0, 54.7, 65.3, 71.3, 85.7, 106.0, 115.7, 117.0, 118.5, 122.3, 128.6, 139.7, 152.2, 171.7; HR-EI-MS m/z: 440.2607 (M^+, calcd for $C_{24}H_{36}N_4O_2Si$, 440.2608).

4.2.12. Compound 16. Compound **13** (22.6 mg, 0.048 mmol) was treated similarly as described for the synthesis of **15** to give **16** (6.8 mg, 0.015 mmol, 31%).

Compound **16**: $[\alpha]_D$ −230° (c = 0.84, MeOH, 25.2 °C); UV λ_{max} (MeOH) nm (ε): 362 (15,800), 300 (4100), 248 (11,300); ^{1}H NMR δ (400 MHz, 0.030 M, $CDCl_3$, 298 K) ppm: 0.01 (3H, s), 0.05 (3H, s), 0.72 (3H, d, J = 6.7 Hz), 0.87 (9H, s), 0.99 (3H, d, J = 6.4 Hz), 2.63 (1H, m), 2.66 (3H, s), 2.92 (1H, dd, J = 17.0, 3.5 Hz), 3.04 (3H, s), 3.19 (1H, d, J = 17.0 Hz), 3.50 (1H, dd, J = 10.0, 9.0 Hz), 3.63 (1H, dd, J = 10.0, 4.3 Hz), 3.71 (1H, m), 4.59 (1H, d, J = 10.4 Hz), 6.15 (1H, s), 6.68 (1H, d, J = 8.0 Hz), 7.32 (1H, d, J = 1.6 Hz), 7.59 (1H, d, J = 8.0 Hz); ^{13}C NMR δ (100 MHz, 0.030 M, $CDCl_3$, 298 K) ppm: −5.5, −5.4, 13.9,18.3, 19.0, 20.5, 25.8 (3C), 28.6, 32.0, 33.5, 55.4, 65.3, 73.0, 107.7, 109.6, 112.8, 120.0, 122.3, 122.9, 145.6, 151.9, 153.0, 172.2; HR-EI-MS m/z: 454.2772 (M^+, calcd for $C_{25}H_{38}N_4O_2Si$, 454.2764).

4.2.13. Compound 17. Compound **14** (34.7 mg, 0.066 mmol) was treated similarly as described for the synthesis of **15** to give **17** (16.2 mg, 0.032 mmol, 48%).

Compound **17**: $[\alpha]_D$ −284° (c = 0.135, MeOH, 26.9 °C); UV λ_{max} (MeOH) nm (ε): 376 (20,000), 301 (5200), 250 (15,000); ^{1}H NMR δ (500 MHz, 0.032 M, $CDCl_3$, 300 K) ppm: 0.03 (3H, s), 0.06 (3H, s), 0.73 (3H, d, J = 6.7 Hz), 0.88 (9H, s), 1.00 (3H, d, J = 6.4 Hz), 2.64 (1H, m), 2.98 (1H, dd, J = 16.9, 3.2 Hz), 3.10 (3H, s), 3.22 (3H, d, J = 16.9 Hz), 3.54 (1H, dd, J = 10.7, 10.5 Hz). 3.65 (1H, dd, J = 10.7, 4.3 Hz), 3.65 (1H, m), 4.54 (1H, d, J = 10.4 Hz), 6.16 (1H, s), 6.87 (1H, d, J = 8.3 Hz), 7.42 (1H, d, J = 1.3Hz), 7.73 (1H, d, J = 8.3 Hz); ^{13}C NMR δ (125 MHz, 0.032 M, $CDCl_3$, 300 K) ppm: −5.5, −5.3, 18.3, 18.9 20.2, 25.8 (3C), 28.6, 32.1, 33.4, 55.2, 65.4, 72.7, 105.7, 107.7, 113.1, 119.9, 123.5 (q, J = 244 Hz), 124.0, 125.6, 142.3, 142.4 (q, J = 35 Hz), 144.0, 152.8, 171.7; HR-EI-MS m/z: 508.2487 (M^+, calcd for $C_{25}H_{35}F_3N_4O_2Si$, 508.2481).

4.2.14. Compound 2. Compound **15** (5.9 mg, 0.013 mmol) was dissolved in THF (0.1 ml). To the solution, 90 μl of a solution of TBAF·$3H_2O$ (13.5 mg) in THF (0.1 ml) was added, and the mixture was stirred at 0 °C for 40 min and then partitioned between EtOAc and 5% $KHSO_4$. The collected EtOAc layer was washed with brine, dried over Na_2SO_4, and concentrated. The residue was purified by HPLC on YMC SH-342-5 using 60% MeOH to give **2** (3.6 mg, 0.011 mmol, 85%).

Compound **2**: $[\alpha]_D$ −420° (c = 2.3, MeOH, 16.6 °C); UV λ_{max} (MeOH) nm (ε): 340 (20,700), 244 (28,100); ^{1}H NMR δ (400 MHz, 0.037 M, $CDCl_3$, 297 K) ppm: 0.57 (3H, d, J = 6.7 Hz), 0.94 (3H, d, J = 6.4 Hz), 2.58 (1H, m), 2.94 (3H, s), 3.09 (1H, dd, J = 17.6, 3.4 Hz), 3.16 (1H, d, J = 17.6 Hz), 3.60 (1H, dd, J = 11.4, 8.5 Hz). 3.75 (1H, dd, J = 11.4, 3.6 Hz), 4.11 (1H, m), 4.47 (1H, d, J = 10.2 Hz), 6.48 (1H, d, J = 8.4 Hz), 7.01 (1H, s), 7.39 (1H, d, J = 8.4 Hz), 7.47 (1H, s), 8.89 (1H, s); ^{13}C NMR δ (100 MHz, 0.037 M, $CDCl_3$, 297 K) ppm: 14.2, 20.0, 21.3, 26.0, 33.1, 34.0, 55.5, 65.0, 71.2, 106.1, 115.7, 117.1, 118.6, 122.5, 128.6, 139.8, 152.2, 173.1; HR-EI-MS m/z: 326.1751 (M^+, calcd for $C_{18}H_{22}N_4O_2$, 326.1743).

4.2.15. Compound 3. Compound **16** (6.8 mg, 0.015 mmol) was treated similarly as described for the synthesis of **2** to give **3** (4.5 mg, 0.013 mmol, 87%).

Compound **3**: $[\alpha]_D$ −340° (c = 2.0, MeOH, 26.6 °C); UV λ_{max} (MeOH) nm (ε): 361 (20,600), 300 (5500), 249 (14,800); ^{1}H NMR δ (400 MHz, 0.028 M, $CDCl_3$, 297 K) ppm: 0.71 (3H, d, J = 6.7 Hz), 0.99 (3H, d, J = 6.4 Hz), 2.60 (1H, m), 2.66 (3H, s), 2.95 (1H, br s), 3.01 (3H, s), 3.03 (1H, dd, J = 17.6, 3.8 Hz), 3.21 (1H, d, J = 17.6 Hz), 3.61 (1H, m), 3.74 (1H, m), 3.80 (1H, m), 4.60 (1H, d, J = 10.4 Hz), 6.67 (1H, d, J = 8.0 Hz), 7.10 (1H, s), 7.33 (1H, d, J = 1.5 Hz), 7.59 (1H,d, J = 8.0 Hz); ^{13}C NMR δ (100 MHz, 0.028 M, $CDCl_3$, 297 K) ppm: 13.8, 19.0, 20.5, 28.5, 32.1, 33.6, 55.9, 64.9, 73.1, 107.9, 109.6, 113.0, 120.0, 122.4, 122.9, 145.6, 152.0, 153.1, 173.2; HR-EI-MS m/z: 326.1751 (M^+, calcd for $C_{19}H_{24}N_4O_2$, 326.1743).

4.2.16. Compound 4. Compound **17** (12.0 mg, 0.024 mmol) was treated similarly as described for the synthesis of **2** to give **4** (8.4 mg, 0.021 mmol, 88%).

Compound **4**: $[\alpha]_D$ −330° (c = 4.11, MeOH, 25.6 °C); UV λ_{max} (MeOH) nm (ε): 376 (10,800), 302 (2700), 250 (8000); ^{1}H NMR δ (400 MHz, 0.053 M, CD_3OD, 298 K) ppm: 0.76 (3H, d, J = 6.7 Hz), 1.01 (3H, d, J = 6.4 Hz), 2.61 (1H, m), 3.12 (3H, s), 3.25 (1H, m), 3.31 (1H, m), 3.55 (1H, m). 3.64 (1H, m), 3.64 (1H, m), 4.64 (1H, d, J = 10.4 Hz), 7.04 (1H, d, J = 8.4 Hz), 7.64 (1H, s), 7.75 (1H, d, J = 8.4 Hz); ^{13}C NMR δ (100 MHz, 0.053 M, CD_3OD, 298 K) ppm: 19.3, 20.5, 29.8, 32.7, 34.1, 57.3, 65.3, 73.7, 106.9, 108.6, 115.1, 121.3, 123.0 (q, J = 269 Hz), 124.7, 128.0, 142.8 (q, J = 38.6 Hz), 145.3, 154.4, 174.1; HR-EI-MS m/z: 394.1616 (M^+, calcd for $C_{19}H_{21}F_3N_2O_2$, 394.1617).

4.3. Inhibition of specific binding of [^{3}H]PDBu to the PKC C1 peptides

The binding of [^{3}H]PDBu to the PKC C1 peptides was evaluated using the procedure of Sharkey and Blumberg[17] with slight modifications, as reported previously,[16] under the following conditions: 50 mM Tris–

maleate (pH 7.4 at 4 °C), 50 μg/ml 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine, 3 mg/ml bovine γ-globulin, 20 nM [^{3}H]PDBu (15.6 Ci/mmol), 5–40 nM of each PKC C1 peptide, and various concentrations of **2**, **3**, or **4**. The binding affinity was evaluated as the concentration required to cause 50% inhibition of the specific binding of [^{3}H]PDBu, IC_{50}, which was calculated by a computer program (SAS) with a probit procedure.[34] The binding constant, K_i, was calculated using the method of Sharkey and Blumberg.[17]

4.4. Fluorescence spectra of 4 when bound to PKCδ-C1B

The basic protocol was similar to the method that was used to evaluate the binding of **4** to the PKC C1 peptide. The standard assay mixture (250 μl), in 1.5 ml Eppendorf tubes, contained 50 mM Tris–maleate (pH 7.4 at 4 °C), 200 nM of PKCδ-C1B peptide, and $10^{-6.5}$ M of **4** in the absence of phospholipid. In the presence of phospholipid, the assay mixture contained 50 mM Tris–maleate (pH 7.4 at 4 °C), 200 nM of PKCδ-C1B peptide, 10^{-7} M of **4**, and 50 μg/ml 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine. In the competition assay, 10 μM of PDBu was added to assay mixture. After 10 μl of the peptide solution was diluted with 990 μl of helium-purged distilled water, the resultant solution (58 μl) was added to the standard assay mixture described above (192 μl), and the solution was incubated at 4 °C for 20 min. After the incubation, 50 μl of solution was removed and fluorescent spectra were monitored. The excitation wavelength was 377 nm, the excitation maximum of **4**.

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