23) 野村 渉, 田中智博, 増田朱美, 鳴海哲夫, 玉村啓和.	2 価結合型リガンドの新規デザインによる CXCR4 の細胞表面における機能解析	第4回バイオ関連化学シンポジウム 講演要旨集		9	2010
24) 小森谷真央, 村上 努, 鳴海哲夫, 野村 渉, 山本直樹, 玉村啓和	マトリックスタンパク質を基にした新規抗 HIV ペプチド	第29回メディシナルケミストリーシンポジウム 講演要旨集		28TG -am08	2010
25) 野村 渉	Zinc Finger 融合酵素を用いた革新的ウイルスゲノム改変技術の開発.	第58回日本ウイルス学会学術集会 抄録集		29TF -am03	2010
26) 野村 渉, 増田朱美, 卜部亜里沙, 鳴海哲夫, 玉村啓和	化合物による活性誘導が可能なジンクフィンガーヌクレアーゼの創製	日本薬学会第 131 年会要旨集		29G -am05	2011
27) 森あつみ, 野村 渉, 鳴海哲夫, 大橋南美, 増田朱美, 玉村啓和	細胞内タンパク質の挙動解明を志向したタグ・プローブシステムの開発	日本薬学会第 131 年会要旨集		29G -am06	2011
28) 野村 渉, 卜部亜里沙, 近藤麻美, 増田朱美, 鳴海哲夫, 梁 明秀, 玉村啓和	ジンクフィンガーヌクレアーゼによる EB ウイルス複製阻害効果の検討	日本薬学会第 131 年会要旨集		30Y -am01	2011
29) 山本 純, 前田奈美, 田中智博, 傳田将也, 重永 章, 野村 渉, 玉村啓和, 大高 章	標的タンパク質の効率的濃縮と同定を指向したトレーサブルリンカーの開発	日本薬学会第 131 年会要旨集		31P -0182	2011
30) 鳴海哲夫, 新井啓之, 落合千裕, 吉村和久, 原田恵嘉, 野村 渉, 松下修三, 玉村啓和	HIV-1 外皮タンパク質の構造変化を誘起する低分子 CD4 ミミックの創製研究	日本薬学会第 131 年会要旨集		31P -0419	2011

Bioconjugate Chemistry

ARTICLE

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Fluorescence-Quenching Screening of Protein Kinase C Ligands with an Environmentally Sensitive Fluorophore

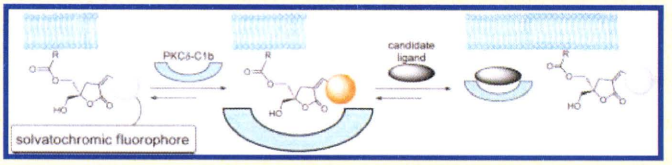
Wataru Nomura,[†] Nami Ohashi,[†] Yoshiaki Okuda,[†,‡] Tetsuo Narumi,[†] Teikichi Ikura,[‡] Nobutoshi Ito,[‡] and Hirokazu Tamamura*[,†,‡]

[†]Department of Medicinal Chemistry, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan

[‡]Graduate School of Biomedical Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

Supporting Information

ABSTRACT: A novel fluorescence-quenching screening method for protein kinase C (PKC) ligands was developed utilizing solvatochromic fluorophores. Solvatochromic dyes, highly sensitive to the presence or the absence of competitive ligands in their binding to the C1b domain of PKCδ (δC1b), were combined with a known pharmacophoric moiety of 1,2-diacylglycerol (DAG) lactones, PKC ligands. Addition of δC1b to the fluorescent compounds caused a gradual increase in the fluorescent intensity in proportion to the increase of δC1b. As a competitive ligand was added to the complex of δC1b domain and fluorescent compounds, a gradual decrease in the fluorescent intensity was observed. The relative binding affinities of known ligands were successfully determined by this fluorescent method and corresponded well to the K_i values measured by a radioisotope method. These results indicate that washing, which is a laborious step in binding evaluations, is not required for this environmentally sensitive fluorophore based system. Screening with the system was performed for 2560 preselected library compounds with possible pharmacophores, and some lead compounds were found. This fluorescence-based method could be applied widely to known ligand−receptor combinations.



INTRODUCTION

Solvatochromic fluorophores are sensitive to immediate changes in their environment such as binding of ligands to proteins,[1] and significant biological issues have been examined by chemical probes with fluorescent dyes.[2,3] The major advantage of the fluorescence-based methods in the observation of physiological phenomena in cells is that detection and evaluation can be performed in real-time directly by microscopy. In these techniques, the signal/background ratio is critical to avoid the observation of artifacts or to obtain clearer images at high resolution. Screening methods of bioactive compounds have been developed as another application of fluorescence.[4] From the aspect of sensitivity, radioisotope (RI) based methods are more efficient but involve laborious experimental procedures. For the development of convenient screening procedures, a simple method for evaluation of binding is needed. By attachment of solvatochromic fluorophores to known ligands, the binding affinity of unknown ligands can be evaluated and related to RI methods.[5] Fluorescent intensity is highly dependent on the binding state to a target protein, and the signal/background ratio would be sufficient without washing steps, which are normally necessary in fluorescent analysis.[6,7]

In this study, we have developed reporter compounds containing solvatochromic dyes for the detection of ligand binding to a target protein and applied them to the screening of chemical libraries. Protein kinase Cδ (PKCδ) was chosen as a target protein because of its importance in physiological phenomena. The 11 isozymes that constitute PKC play pivotal roles in physiological responses to growth factors, oxidative stress, 1,2-diacylglycerol (DAG), and tumor promoters, such as phorbol esters. These responses regulate numerous cellular processes,[8,9] including proliferation,[10] differentiation,[11] migration,[12] and apoptosis.[13,14] Membrane translocation of PKC is caused by binding of ligands to the C1b domain and has been recognized as an important phenomenon in signal transduction because the localization of PKC is key in determining isozyme-specific functions by defining binding partners for downstream signaling.[15] Despite the complex regulatory mechanisms of PKC activation, considerable progress has been made in understanding isozyme-specific functions[16] and several ligands with high specificity for PKC isozymes have been developed as potential drugs.[17−23] Through the development of DAG-lactones, whose design is based on the endogenous ligand DAG, the importance of maintaining intact the pharmacophore triad of two carbonyl groups (*sn*-1 and *sn*-2) and the primary alcohol has been established as a requirement in high affinity ligands for PKC (Figure 1).[16] Various PKC ligands have been synthesized and found to be inhibitors of tumor promotion or of other diseases.

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However, there are limitations to the design of compound templates so far. Thus, in this study, we have attempted to develop the screening methods aimed at discovery of novel template structures for PKCδ ligands as drug leads.

EXPERIMENTAL PROCEDURES

General. ^{1}H NMR spectra were recorded using a Bruker AV500 spectrometer. Chemical shifts were reported in δ (ppm) relative to Me_4Si (in $CDCl_3$) as an internal standard. Low- and high-resolution mass spectra were recorded on a JMS-T1000LC AccuTOF and Bruker Daltonics microTOF-2focus in both positive and negative detection modes. Wakogel C-200 (Wako Pure Chemical Industries, Ltd.) and silica gel 60 N (Kanto Chemical Co., Inc.) were employed for flash chromatography. Fluorescent spectra were recorded on a JASCO FP-6600 spectrofluorometer and JASCO V-650 spectrophotometer using a quartz cell with 1.0 cm path length. Fluorescent intensities of samples in 96-well plates were recorded on Wallac ARVO MX.

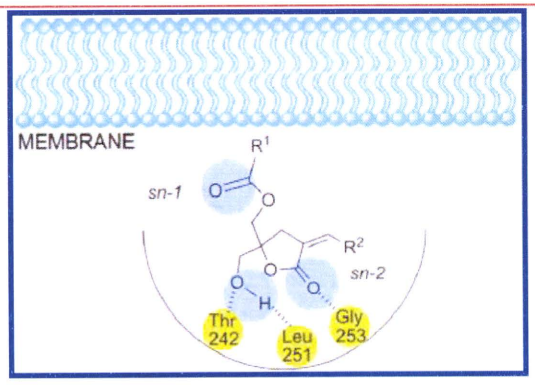


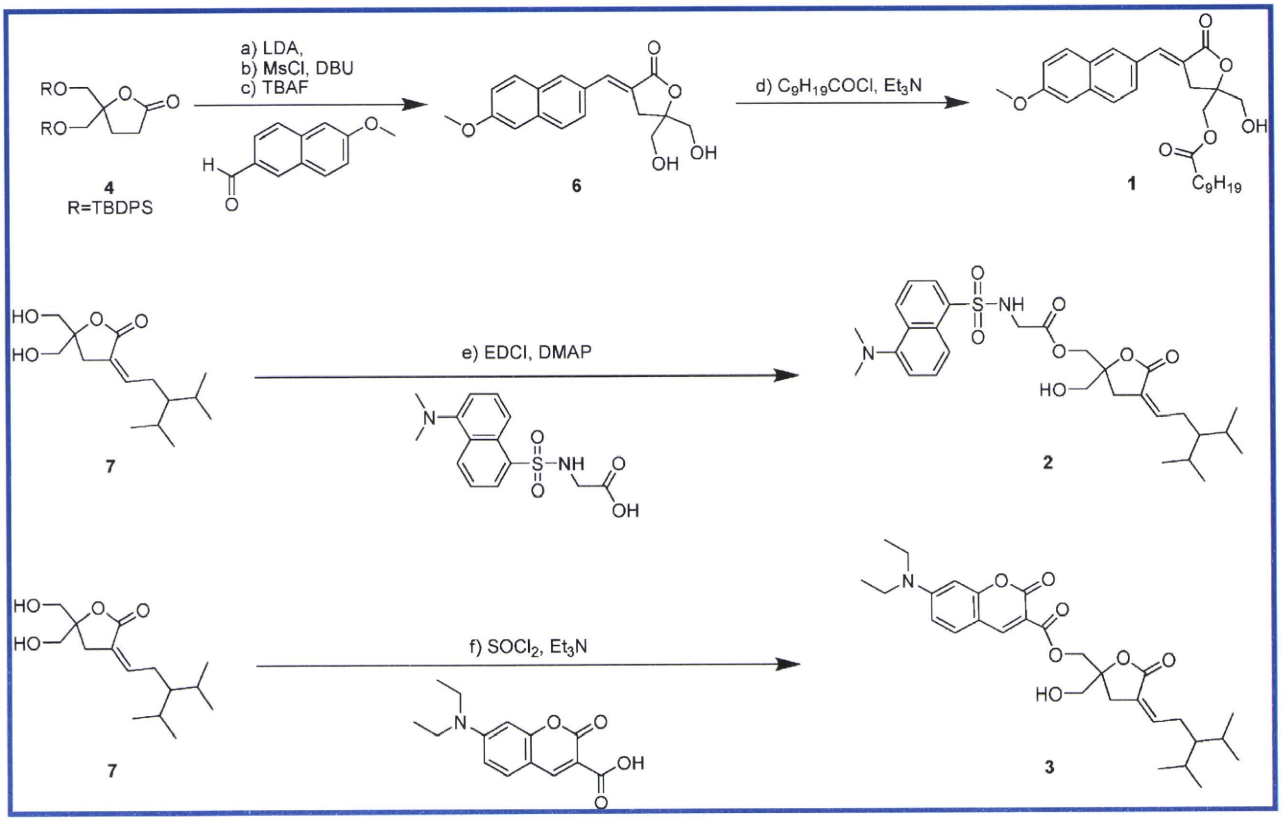
Figure 1. Binding mode of DAG-lactone derivatives to the δC1b domain. The *sn*-1 and *sn*-2 carbonyl groups are indicated. Three important pharmacophores are indicated by light blue spheres. Amino acid residues interacting with these pharmacophores (threonine at 242, leucine at 251, and glycine at 253) are indicated by red spheres.

Standard Monoacylation Procedure. Under argon, Et_3N (3 equiv) was added at 0 °C to a solution containing 5,5-bis(hydroxymethyl)oxalan-2-one in THF, and the mixture was stirred at 0 °C for 30 min. Then 0.2−1.1 equiv of a carboxylic acid derivative (an acyl chloride or carboxylic anhydride) was added. This mixture was stirred at 0 °C for a further 4 h. After evaporation of the solvent, the obtained residue was purified by flash column chromatography.

Preparation of Compounds 7, 10−13, 16, 17, and 19−21. Compounds **7**, **10**−**12**, and **16** were prepared as described in ref 17. Compounds **13** and **17** were prepared as described in refs 24 and 25. The synthesis of **19** is described elsewhere.[26] Compounds **20** and **21** are commercially available.

Expression and Purification of the δC1b Domain. DNA coding C1b domain of mouse PKCδ (231−280)[27] was subcloned into *Bam*H1 and *Eco*RI sites of pGEX-2tk (GE Healthcare) and expressed as gluthatione-*S*-transferase (GST) fusion protein in *Escherichia coli* C41 which contains extension sequences Gly-Ser-Arg-Arg-Ala-Ser-Val-Gly-Ser and Glu-Phe-Ile-Val-Thr-Asp at the N- and C-termini, respectively. The δC1b

Scheme 1. Synthesis of Fluorescent Compounds 1−3[a]



[a] (a) LDA, −78 °C; (b) MsCl, DBU, 0 °C to room temp; (c) TBAF, 0 °C to room temp; (d) $C_9H_{19}COCl$, Et_3N, 0 °C; (e) EDCI, DMAP, 0 °C; (f) $SOCl_2$, Et_3N, 0 °C.

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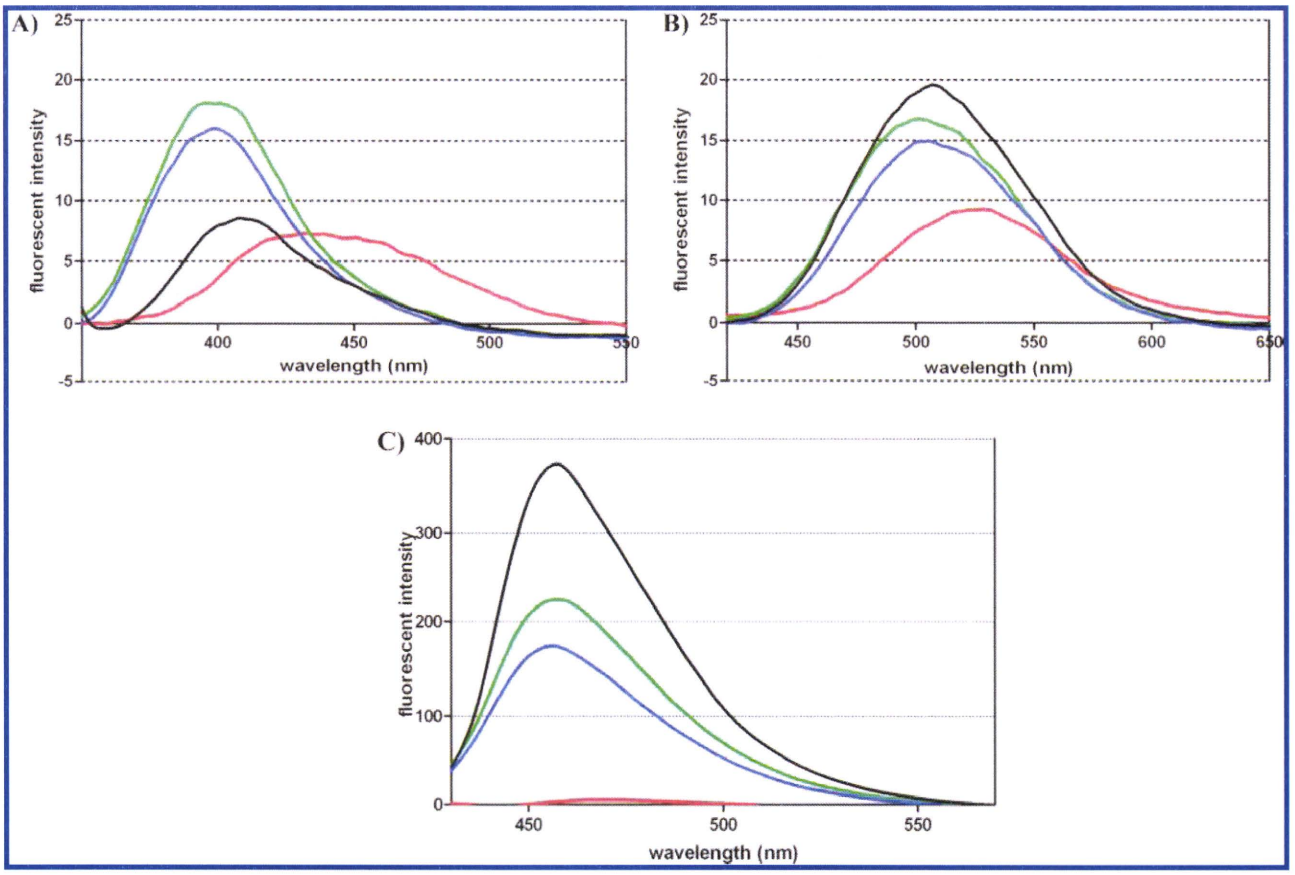

Figure 2. Fluorescent spectra of compounds **1** (A), **2** (B), and **3** (C) in different solvents. The spectra show fluorescence in the solvents color-coded as follows: red, MeOH; blue, THF; black, $CHCl_3$; green, EtOAc. Each ligand was prepared as 1 mM solution in DMSO. In UV measurement, 10 μL of ligand was added to 990 μL of solvents.

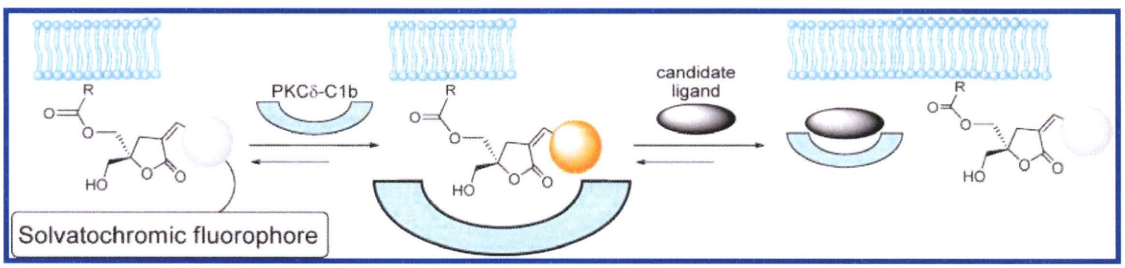

Figure 3. Schematic representation of a fluorescence-quenching screening system. In the presence of the δC1b domain, fluorescence of a solvatochromic fluorophore is enhanced. The replacement by candidate compounds results in fluorescence quenching.

Table 1. Changes of Fluorescent Intensity upon δC1b Binding and Inhibition Constants to δC1b Determined by Competition Assay against [^{3}H]PDBu

compd	ΔF [a]	K_i (nM) [b]
1	3.8	35.9
2	2.3	368
3	0.50	93.3

[a] Fluorescent change by binding of the δC1b domain. The values were determined by titration of the δC1b domain. The ΔF was determined by dividing F_1 by F_0 at the maximum of fluorescent emission in each spectrum. [b] Inhibitory constants determined by competition analysis against [^{3}H]PDBu.[28,36,37]

domain after cleavage contains 65 amino acid residues. Cells were grown at 37 °C in LB medium and induced with 0.3 mM IPTG at growth phase. Cells were cultured overnight at 20 °C after induction. Cells were collected and lysed in 50 mM Tris·HCl buffer (pH 8.0) containing 100 mM NaCl, 1 μg/mL leupeptine, 1 mM PMSF, and 1 mM DTT. Expressed protein was extracted by sonication, then purified by affinity chromatography utilizing glutathione-Sepharose 4B beads resin. GST moiety was cleaved by 300 units of thrombin at 4 °C overnight. The cleaved protein was eluted and further purified on a 2.6 cm × 60 cm Superdex S-75 gel filtration column. Purification was by FPLC system at a flow rate of 1 mL/min utilizing 0.1 M triethanolamine·HCl (pH 7.0) containing 0.5 M NaCl.

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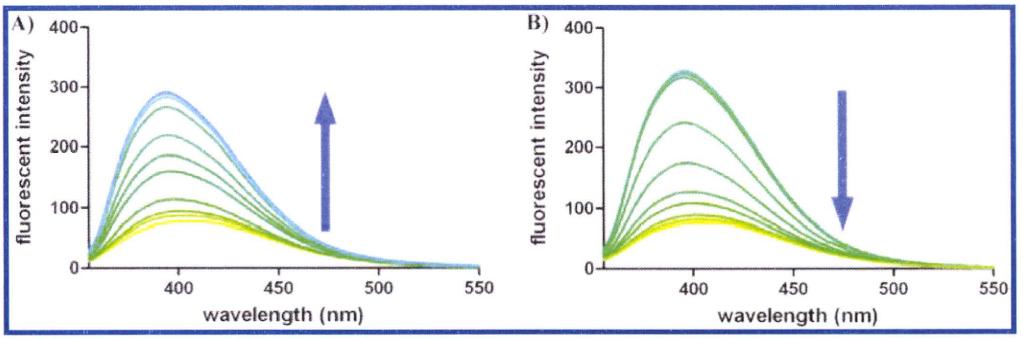

Figure 4. Changes of fluorescent spectra of compound **1** during titration of the δC1b domain (A) and during titration of PDBu (compound **8**) (B) after δC1b binding. The concentration of the δC1b domain was increased to 1.28 μM (6.4 equiv to compound **1**) by titration. The concentration of PDBu was increased to 10.2 μM (51.2 equiv to compound **1**) by titration.

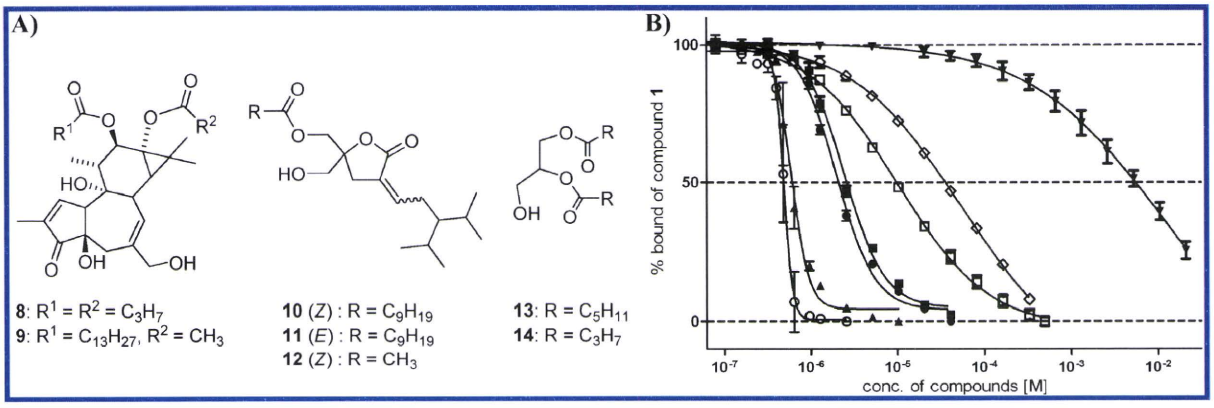

Figure 5. Competitive assay against compound **1** based on fluorescent intensity (spectrofluorometer). (A) Structures of test compounds. (B) Inhibition curves indicate results of compounds **8** (▲), **9** (○), **10** (●), **11** (■), **12** (□), **13** (◇), and **14** (▼).

Determination of K_i of Compounds for Full Length PKCδ. Enzyme–ligand interaction was assessed in vitro as the ability of the ligand to displace bound [20-^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) from recombinant human PKCδ in the presence of phosphatidylserine (PS) in an experimental procedure that was described previously.[28] In constant concentration analysis, the concentrations of compounds were fixed at 100 nM for known compounds and at 10 μM for library compounds. The other procedures were the same as those of the standard evaluation.

Fluorescent Titration of the δC1b Domain. An amount of 25 μL of a solution of PS in chloroform (10 mg/mL) was evaporated to dryness under nitrogen. Then 50 mM Tris·HCl buffer (pH 7.4) was added and the PS was sonicated with a Microtip for a total of 15 s. A stock solution of compound **1** was diluted with 50 mM Tris·HCl buffer (pH 7.4) containing 100 μg/mL PS to prepare a 0.2 μM solution. The recombinant δC1b domain was added to the 0.2 μM solution of compound **1**, and fluorescent spectra (λ_{ex} = 340 nm) were measured at 25 °C. The concentration of the recombinant δC1b domain started at 0.01 μM and increased to 1.28 μM by titration. The final concentrations of compounds **2** and **3** were 0.2 and 0.05 μM, respectively. Change of fluorescent intensity (ΔF) was calculated at the following wavelengths, which showed λ_{max} in each evaluation; compound **1**, 406 (F_0) and 394 (F_1) nm; compound **2**, 520 (F_0) and 480 (F_1) nm; compound **3**, 463 (F_0) and 461 (F_1) nm. Wavelengths recorded before and after the addition of the δC1b domain.

Fluorescent Titration of Candidate Ligands. Compound **1** was diluted with 50 mM Tris·HCl (pH 7.4) containing 100 μg/mL PS to obtain a solution with a 0.2 μM final concentration. The δC1b domain was added to the above solution of compound **1** to be 0.96 μM. A candidate ligand was added to the solution, and fluorescent spectra were recorded with a spectrofluorometer at 25 °C. Fluorescent titration curves (λ_{em} = 407 nm) were analyzed by nonlinear regression, and IC_{50} values were estimated by a nonlinear least-squares curve-fitting method using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, U.S.). In the constant concentration analysis, the concentrations of known compounds were fixed at 100 nM and of library compounds at 10 μM. The other procedures were the same as those in the standard evaluation.

Fluorescence-Quenching Analysis for Library Screening Recorded by Microplate Reader. Fluorescence intensity was recorded on Wallac ARVO MX (PerkinElmer) using a 96-well black plate. Compound **1** was diluted with 50 mM Tris·HCl (pH 7.4) containing 100 μg/mL PS to obtain a solution with a 0.2 μM final concentration. The δC1b domain was added to the above solution of compound **1** to be 0.96 μM. A candidate ligand (10 μM) was added to the compound **1**–δC1b domain complex solution, and fluorescent intensities (λ_{ex} = 355 nm, λ_{em} = 405 nm) were measured at 25 °C.

RESULTS AND DISCUSSION

Synthesis and Fluorescent Properties of Compounds 1–3. Three representative solvatochromic fluorophores, involving 6-methoxynaphthalene,[29] 5-(dimethylamino)naphthalene-1-sulfonyl (dansyl),[6,30,31] and diethylaminocoumarin,[32–35] have

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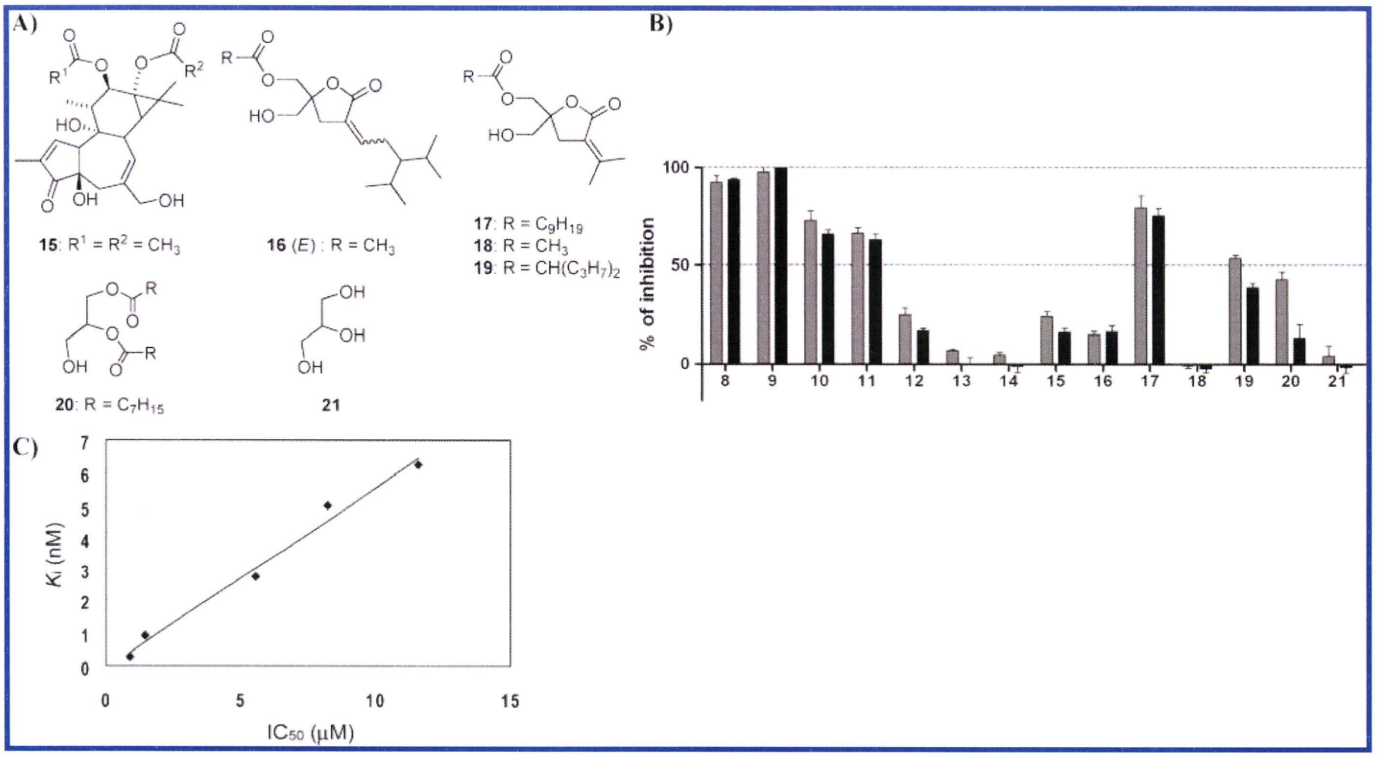

Figure 6. Comparison of inhibition percentages between the fluorescent assay (microplate reader) and the RI assay at a constant concentration of test compounds. (A) Structures of test compounds. (B) Inhibition of binding of compound **1** (fluorescent assay, left gray bars) or [^{3}H]PDBu (RI assay, right black bars) in the presence of test compounds (10 μM in the fluorescent assay and 100 nM in the RI assay). The final DMSO concentration is 4%. (C) Plots of IC_{50} values (μM) by the fluorescent assay versus K_i (nM) by the RI assay for compounds **8**, **9**, **10**, **11**, and **17**.

been utilized. The 6-methoxynaphthalene was incorporated at the α-carbon of the *sn*-1 carbonyl group of the DAG-lactone structure (Scheme 1). The dansyl and diethylaminocoumarin were incorporated into the *sn*-2 carbonyl group. In comparison with the fluorescent spectra of these compounds, obtained in various solvents, compound **1** showed the most variation with solvent polarity (Figure 2). It is known that solvatochromic fluorophores show higher fluorescent intensity in hydrophobic environments and that the fluorophores are sensitive to the environment of a binding pocket in target proteins. The fluorescent intensity of labeled DAG-lactones should reflect its binding to a target protein, PKCδ (Figure 3), and as expected, our reporter compounds **1** and **2** containing solvatochromic fluorophores exibited a remarkable increase of fluorescent intensity in the proximity of the C1b domain of PKCδ (δC1b), which is a specific target of DAG-lactone derivatives (Table 1 and Figure 4A). This indicates that in the binding of compounds **1** and **2** to the δC1b domain, the 6-methoxynaphthalene and dansyl moieties are located in the hydrophobic environment. However, for compound **3**, the fluorescent intensity was decreased as the concentration of δC1b increased. The spectra in various solvents showed dependence of the fluorescent intensity of the compound **3** on solvent polarity, and it was suggested that in the binding of compound **3** to the δC1b domain, the diethylaminocoumarin moiety could be located in the hydrophilic environment. The changes of fluorescent intensity of the synthetic compounds are summarized in Table 1. The binding constants of fluorescent compounds were evaluated by [^{3}H]PDBu competitive assay. Compound **1** showed the highest binding affinity for the δC1b domain. In the fluorescence-based analysis of binding, two factors are key to the sensitivity of the assay system: (i) the fluorescent change as a function of the ligand binding and (ii) the binding affinity of a reporter compound.

Evaluation of Binding to PKCδ by Fluorescent Change of Compound 1. The results obtained supported the selection of compound **1** as a reporter compound for further study. In competitive assays, when candidate ligands of PKCδ are present, they replace the fluorescent compounds, which are formerly bound to δC1b, and the fluorescent intensity should decrease. Practically, addition of PDBu to the complex of compound **1** and δC1b resulted in a remarkable decrease of fluorescent intensity (Figure 4B). By utilization of compound **1** as a reporter, binding analysis of known PKCδ ligands was performed to assess the reliability of the assay system. Compounds **8**−**14** were prepared as test compounds (Figure 5A), and their IC_{50} values, determined by the fluorescence-based competitive assay with a fluorospectrophotometer against compound **1**, were 0.60, 0.49, 1.95, 2.42, 10.0, 54.2, and 1.22 × 10^4 μM, respectively (Figure 5B). The order of the IC_{50} values of compounds **8**−**11** is identical to that of the K_i values (0.91, 0.26, 5.0, and 6.2 nM, respectively) determined by the [^{3}H]PDBu competitive assay.

The Binding Analysis by Competition with Compound 1 Showed Linearity with the Classical RI Assay. To further assess the collinearity of the fluorescent assay on microplates and the [^{3}H]PDBu assay, the inhibition percentages by compounds **8**−**21** in both assays (Figure 6A) at constant concentration were compared. The compound concentrations were set at 10 μM for the fluorescent assay and at 100 nM for the [^{3}H]PDBu assay. As shown in Figure 6B, the inhibition percentages of compounds **8**−**21** are similar in both assays. Comparison of the IC_{50} and K_i of compounds **8**, **9**, **10**, **11**, and **17**, possessing more than 60% inhibition percentages, showed acceptable linearity (Figure 6C).

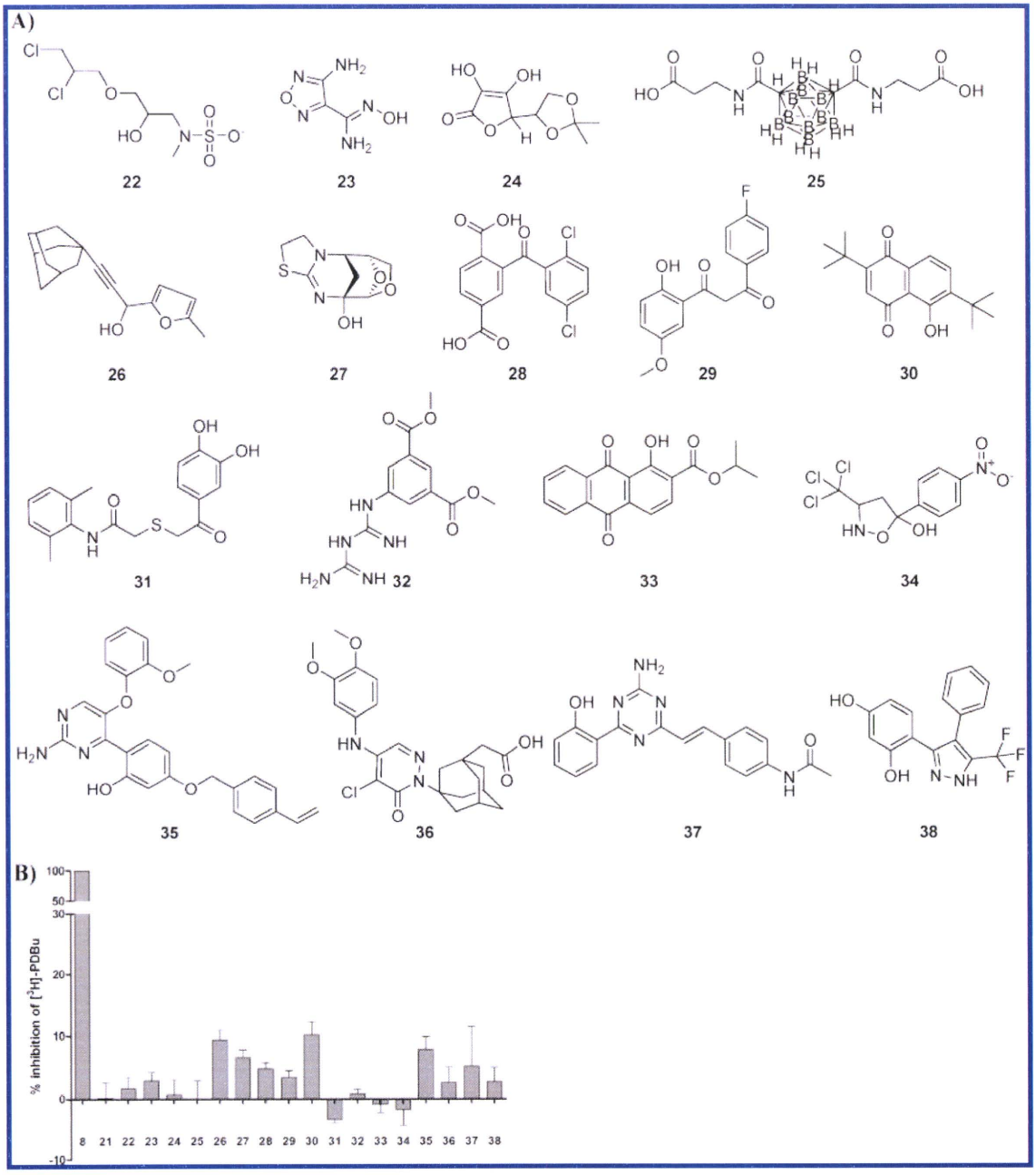

Figure 7. The RI competition assay of positive hit compounds found in fluorescent screening utilizing compound **1**. (A) Structures of test compounds obtained from screening. (B) Results of the inhibition assay based on RI methods. The concentration of compounds was fixed at 10 μM. The *X* axis indicates compound numbers. Columns show average inhibition obtained from triplicated experiments. Bars indicate standard errors.

In view of the above results, the present fluorescence-based evaluation of binding to the δC1b domain on microplates was deemed to be a promising alternative for the classical RI assay.

Expansion of the Fluorescence Evaluation to Screening of Library Compounds. As a proof-of-concept study, compound **1** was utilized in screening of a chemical library, supplied by the Screening Center at the Tokyo Medical and Dental University Screening Center. The structures of the library compounds were analyzed in advance of screening. For binding to the δC1b domain, pharmacophores including the carbonyl and hydroxy groups are necessary and only compounds including these functional groups were chosen. In the analysis of the library on microplates, compounds with more than 20% decrease of fluorescent intensity determined by triplicated assays were selected as candidates for further study. Compounds **22**−**38** were found from 2560 library compounds (Figure 7A) as candidate δC1b ligands. To further assess the binding affinity of these compounds, evaluation of binding by competition with the [^{3}H]PDBu was performed at a fixed concentration (10 μM) (Figure 7B). In this analysis, compounds **30** and **26**, with inhibition percentages at 10 μM of 10.2% and of 9.4%, respectively, showed the most and the second most potent binding affinities for the δC1b domain. The binding affinities of these compounds are still very low compared to that of DAG-lactones, but an SAR study of these compounds could lead to novel structure templates for synthetic PKC ligands. This analysis also revealed that compounds **24**, **25**, **31**, **32**, **33**, and **34** are false positive hits. The results obtained indicate that our screening system with a fluorescent

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DAG-lactone derivative could be utilized for discovery of novel chemical leads for PKCδ.

CONCLUSION

A fluorescence-based method for chemical library screening has been developed. This method, which requires no washing steps, provides a linear response in relative binding affinity between compounds and is compatible with the classical RI assay for PKC ligands. By screening of more than 2500 library compounds, the method was proven to be a reliable means of discovery of compounds that bind to the C1b domain of PKCδ. The method would provide potent lead compounds that bind to the isozyme utilized in the assay. By utilization of the difference of the binding affinity of reporter compounds to different isozymes, it is possible that this screening system can provide an efficient selection of lead compounds highly specific to a target isozyme. This fluorescence-quenching method should be applicable to other known receptor−ligand combinations.

ASSOCIATED CONTENT

Supporting Information. Synthesis procedures and NMR, HRMS, and IR data. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: tamamura.mr@tmd.ac.jp. Phone: +81-3-5280-8036. Fax: +81-3-5280-8039.

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REFERENCES

(1) Loving, G. S., Sainlos, M., and Imperiali, B. (2010) Monitoring protein interactions and dynamics with solvatochromic fluorophores. *Trends Biotechnol. 28*, 73–83.

(2) Rothman, D. M., Shults, M. D., and Imperiali, B. (2005) Chemical approaches for investigating phosphorylation in signal transduction networks. *Trends Cell Biol. 15*, 502–510.

(3) Terai, T., and Nagano, T. (2008) Fluorescent probes for bioimaging applications. *Curr. Opin. Chem. Biol. 12*, 515–521.

(4) Reymond, J. -L. (2008) Substrate arrays for fluorescence-based enzyme fingerprinting and high-throughput screening. *Ann. N.Y. Acad. Sci. 1130*, 12–20.

(5) Nomura, W., Tanabe, Y., Tsutsumi, H., Tanaka, T., Ohba, K., Yamamoto, N., and Tamamura, H. (2008) Fluorophore labeling enables imaging and evaluation of specific CXCR4−ligand interaction at the cell membrane for fluorescence-based screening. *Bioconjugate Chem. 19*, 1917–1920.

(6) Hayashida, O., Ogawa, N., and Uchiyama, M. (2007) Surface recognition and fluorescence sensing of histone by dansyl-appended cyclophane-based resorcinarene trimer. *J. Am. Chem. Soc. 129*, 13698–13705.

(7) Tsutsumi, H., Nomura, W., Abe, S., Mino, T., Masuda, A., Ohashi, N., Tanaka, T., Ohba, K., Yamamoto, N., Akiyoshi, K., and Tamamura, H. (2009) Fluorogenically active leucine zipper peptides as tag-probe pairs for protein imaging in living cells. *Angew. Chem., Int. Ed. 48*, 9164–9166.

(8) Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Sceince 258*, 607–614.

(9) Newton, A. C. (1995) Protein kinase C: structure, function, and regulation. *J. Biol. Chem. 270*, 28495–28498.

(10) Watanabe, T., Ono, Y., Taniyama, Y., Hazama, K., Igarashi, K., Ogita, K., Kikkawa, U., and Nishizuka, Y. (1992) Cell division arrest induced by phorbol ester in CHO cells overexpressing protein kinase C-delta subspecies. *Proc. Natl. Acad. Sci. U.S.A. 89*, 10159–10163.

(11) Mischak, H., Pierce, J. H., Goodnight, J., Kazanietz, M. G., Blumberg, P. M., and Mushinski, J. F. (1993) Phorbol ester-induced myeloid differentiation is mediated by protein kinase C-alpha and -delta and not by protein kinase C-beta II, -epsilon, -zeta, and -eta. *J. Biol. Chem. 268*, 20110–20115.

(12) Li, C., Wernig, F., Leitges, M., Hu, Y., and Xu, Q. (2003) Mechanical stress-activated PKCdelta regulates smooth muscle cell migration. *FASEB J. 17*, 2106–2108.

(13) Ghayur, T., Hugunin, M., Talanian, R. V., Ratnofsky, S., Quinlan, C., Emoto, Y., Pandey, P., Datta, R., Huang, Y., Kharbanda, S., Allen, H., Kamen, R., Wong, W., and Kufe, D. (1996) Proteolytic activation of protein kinase C delta by an ICE/CED 3-like protease induces characteristics of apoptosis. *J. Exp. Med. 184*, 2399–2404.

(14) Alkon, D. L., Sun, M.-K., and Nelson, T. J. (2007) PKC signaling deficits: a mechanistic hypothesis for the origins of Alzheimer's disease. *Trends Pharmacol. Sci. 28*, 51–60.

(15) Wang, Q. J. (2006) PKD at the crossroads of DAG and PKC signaling. *Trends Pharmacol. Sci. 27*, 317–323.

(16) Marquez, V. E., and Blumberg, P. M. (2003) Synthetic diacylglycerols (DAG) and DAG-lactones as activators of protein kinase C (PK-C). *Acc. Chem. Res. 36*, 434–443.

(17) Nacro, K., Bienfait, B., Lee, J., Han, K.-C., Kang, J.-H., Benzaria, S., Lewin, N. E., Bhattacharyya, D. K., Blumberg, P. M., and Marquez, V. E. (2000) Conformationally constrained analogues of diacylglycerol (DAG). 16. How much structural complexity is necessary for recognition and high binding affinity to protein kinase C?. *J. Med. Chem. 43*, 921–944.

(18) Tamamura, H., Bienfait, B., Nacro, K., Lewin, N. E., Blumberg, P. M., and Marquez, V. E. (2000) Conformationally constrained analogues of diacylglycerol (DAG). 17. Contrast between *sn*-1 and *sn*-2 DAG lactones in binding to protein kinase C. *J. Med. Chem. 43*, 3209–3217.

(19) Tamamura, H., Sigano, D. M., Lewin, N. E., Blumberg, P. M., and Marquez, V. E. (2004) Conformationally constrained analogues of diacylglycerol. 20. The search for an elusive binding site on protein kinase C through relocation of the carbonyl pharmacophore along the *sn*-1 side chain of 1,2-diacylglycerol lactones. *J. Med. Chem. 47*, 644–655.

(20) Baba, Y., Ogoshi, Y., Hirai, G., Yanagisawa, T., Nagamatsu, K., Mayumi, S., Hashimoto, Y., and Sodeoka, M. (2004) Design, synthesis, and structure−activity relationship of new isobenzofuranone ligands of protein kinase C. *Bioorg. Med. Chem. Lett. 14*, 2963–2967.

(21) Baba, Y., Mayumi, S., Hirai, G., Kawasaki, H., Ogoshi, Y., Yanagisawa, T., Hashimoto, Y., and Sodeoka, M. (2004) Evaluation of series of isobenzofuranone dimers as PKCalpha ligands: implication for the distance between the two ligand binding sites. *Bioorg. Med. Chem. Lett. 14*, 2969–2972.

(22) Yanagita, R. C., Nakagawa, Y., Yamanaka, N., Kashiwagi, K., Saito, N., and Irie, K. (2008) Synthesis, conformational analysis, and biological evaluation of 1-hexylindolactam-V10 as a selective activator for novel protein kinase C isozymes. *J. Med. Chem. 51*, 46–56.

(23) Nakagawa, Y., Yanagita, R. C., Hamada, N., Murakami, A., Takahashi, H., Saito, N., Nagai, H., and Irie, K. (2009) A simple analogue of tumor-promoting aplysiatoxin is an antineoplastic agent rather than a tumor promoter: development of a synthetically accessible protein kinase C activator with bryostatin-like activity. *J. Am. Chem. Soc. 131*, 7573–7579.

(24) Martin, S. F., Josey, J. A., Wong, Y.-L., and Dean, D. W. (1994) General method for the synthesis of phospholipid derivatives of 1,2-*O*-diacyl-*sn*-glycerols. *J. Org. Chem. 59*, 4805–4820.

(25) Malolanarasimhan, K., Kedei, N., Sigano, D. M., Kelley, J. A., Lai, C. C., Lewin, N. E., Surawski, R. J., Pavlyukovets, V. A., Garfield, S. H., Wincovitch, S., Blumberg, P. M., and Marquez, V. E. (2007) Conformationally constrained analogues of diacylglycerol (DAG). 27. Modulation of membrane translocation of protein kinase C (PKC) isozymes alpha and delta by diacylglycerol lactones (DAG-lactones) containing rigid-rod acyl groups. *J. Med. Chem. 50*, 962–978.

(26) Nomura, W., Narumi, T., Ohashi, N., Serizawa, Y., Lewin, N. E., Blumberg, P. M., Furuta, T., and Tamamura, H. (2011) Synthetic caged DAG-lactones for photochemically-controlled activation of protein kinase C. *ChemBioChem 12*, 535–539.

(27) Mischak, H., Bodenteich, A., Kolch, W., Goodnight, J., Hofer, F., and Mushinski, J. F. (1991) Mouse protein kinase C-delta, the major isoform expressed in mouse hemopoietic cells: sequence of the cDNA, expression patterns, and characterization of the protein. *Biochemistry 30*, 7925–7931.

(28) Kazanietz, M. G., Krausz, K. W., and Blumberg, P. M. (1992) Differential irreversible insertion of protein kinase C into phospholipid vesicles by phorbol esters and related activators. *J. Biol. Chem. 267*, 20878–20886.

(29) List, B., Barbas, C. F., III, and Lerner, R. A. (1998) Aldol sensors for the rapid generation of tunable fluorescence by antibody catalysis. *Proc. Natl. Acad. Sci. U.S.A. 95*, 15351–15355.

(30) Davis, G. A. (1972) Dansylglycine as a fluorescent probe for aqueous solutions of cationic detergents. *J. Am. Chem. Soc. 94*, 5089–5090.

(31) Morii, T., Sugimoto, K., Makino, K., Otsuka, M., Imoto, K., and Mori, Y. (2002) A new fluorescent biosensor for inositol trisphosphate. *J. Am. Chem. Soc. 124*, 1138–1139.

(32) Matlock, D. L., and Heyduk, T. (1999) A real-time fluorescence method to monitor the melting of duplex DNA during transcription initiation by RNA polymerase. *Anal. Biochem. 270*, 140–147.

(33) Kakio, A., Nishimoto, S., Yanagisawa, K., Kozutsumi, Y., and Matsuzaki, K. (2001) Cholesterol-dependent formation of GM1 ganglioside-bound amyloid beta-protein, an endogenous seed for Alzheimer amyloid. *J. Biol. Chem. 276*, 24985–24990.

(34) Timofeevski, S. L., Prusakiewicz, J. J., Rouzer, C. A., and Marnett, L. J. (2002) Isoform-selective interaction of cyclooxygenase-2 with indomethacin amides studied by real-time fluorescence, inhibition kinetics, and site-directed mutagenesis. *Biochemistry 41*, 9654–9662.

(35) Sakamoto, S., and Kudo, K. (2008) Supramolecular control of split-GFP reassembly by conjugation of beta-cyclodextrin and coumarin units. *J. Am. Chem. Soc. 130*, 9574–9582.

(36) Sharkey, N. A., and Blumberg, P. M. (1985) Highly lipophilic phorbol esters as inhibitors of specific [^{3}H]phorbol 12,13-dibutyrate binding. *Cancer Res. 45*, 19–24.

(37) Ohashi, N., Nomura, W., Kato, M., Narumi, T., Lewin, N. E., Blumberg, P. M., and Tamamura, H. (2009) Synthesis of protein kinase Cdelta C1b domain by native chemical ligation methodology and characterization of its folding and ligand binding. *J. Pept. Sci. 15*, 642–646.

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Synthetic Caged DAG-lactones for Photochemically Controlled Activation of Protein Kinase C

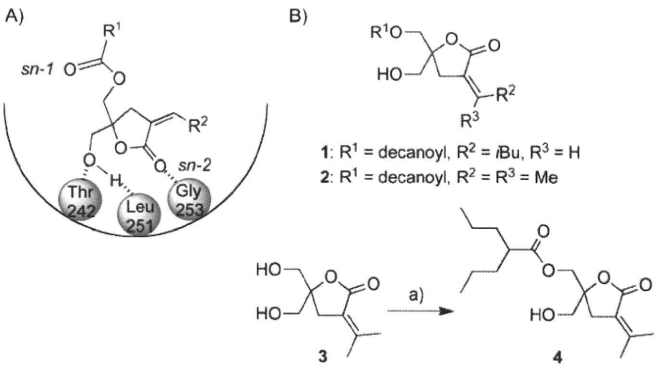
Wataru Nomura,[a] Tetsuo Narumi,[a] Nami Ohashi,[a] Yuki Serizawa,[a] Nancy E. Lewin,[b] Peter M. Blumberg,[b] Toshiaki Furuta,[a, c] and Hirokazu Tamamura*[a]

The signal transduction pathways associated with interactions with small organic molecules attract great interest in the field of chemical biology. To study the action of bioactive compounds in detail, it is necessary to eliminate the signaling complexity caused by multiple combined effects. The development of "caged" compounds, which are not active when the pharmacophore is blocked by a photoactivatable moiety, has been a powerful tool with which to approach this problem. Triggered by photoirradiation to a limited area in the cell, the specific effects of the ligand in that location can then be observed over time. Several strategies for "caging" molecules have been developed and each approach has its own advantages.[1]

The protein kinase C (PKC) isoforms play pivotal roles in physiological responses to growth factors and oxidative stress mediated through the endogenous second messenger 1,2-diacylglycerol (DAG). These responses regulate numerous cellular processes,[2] including proliferation,[3] differentiation,[4] migration,[5] and apoptosis.[6] The tumor-promoting phorbol esters, potent analogues of DAG, have provided a convenient probe of PKC function. Ligand binding to the C1b domain in PKC leads to its membrane translocation. The translocation of PKC is of central importance for its function because the localization of PKC determines the substrates to which it has access.[7] Despite the complex regulatory mechanisms of PKC activation, considerable progress in understanding isozyme-specific functions has been made.[8] Development of ligands with high specificities for PKC isozymes has been a critical issue in the medicinal field.[9a–b] Enhancement of the understanding of targets and signaling pathways would provide important insights contributing to this effort.

As high-affinity ligands for PKC, DAG-lactones have established the importance of the pharmacophore triad of two carbonyl groups (*sn*-1 and *sn*-2) and the hydroxy group being maintained intact.[8] In this study, we have utilized coumarin-based "caging" molecules including 6-bromo-7-hydroxycoumarin (Bhc)[10] and 6-bromo-7-methoxycoumarin (Bmc)[11] to block the binding of DAG-lactones to PKCδ. The caged protecting groups are attached to the primary alcohol, which is an important DAG-lactone pharmacophore. Photolytic uncaging of the blocked DAG-lactone then provides a means of driving PKC activation within the cell at desired specific locations and times. An approach previously used to achieve this goal has been the use of caged diacylglycerols.[1d,12] DAG-lactones have several potential advantages. Extensive medical chemistry investigations have yielded DAG-lactones with substantially enhanced affinities, a range of physiochemical properties, and interesting biological selectivities with regard to diacylglycerol targets.[8]

The DAG-lactones have been developed as ligands for PKC isozymes with low-nanomolar binding affinities by a combination of pharmacophore- and receptor-guided approaches based on the structure of the physiological second messenger DAG (Scheme 1).[8] The DAG-lactones were designed by the



Scheme 1. A) The *sn*-2 binding model of DAG-lactones to the PKCδ C1b domain and membrane. B) Structures of the DAG-lactones **1**, **2**, and **4**. a) 2-Propylpentanoyl chloride, pyridine, CH_2Cl_2, 24%.

pharmacophore-guided approach based on the geometries of bioequivalent pharmacophores present in DAG and in phorbol esters.[13] In the DAG-lactone structure, the glycerol backbone was constrained to a γ-lactone ring to reduce the entropic penalty associated with DAG binding. In binding to PKC, the linear or branched acyl (R^1) or α-alkylidene (R^2) chains contribute to optimized hydrophobic interactions with a group of conserved hydrophobic amino acids located on the top half of the C1 domain. There are two competing binding modes (*sn*-1 and *sn*-2), depending on which carbonyl group is directly involved in binding to the protein (Scheme 1 A). In general, it

[a] *Dr. W. Nomura, Dr. T. Narumi, N. Ohashi, Y. Serizawa, Prof. T. Furuta, Prof. H. Tamamura*
Department of Medicinal Chemistry
Institute of Biomaterials and Bioengineering
Tokyo Medical and Dental University
2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062 (Japan)
Fax:(+81) 3-5280-8039
E-mail: tamamura.mr@tmd.ac.jp

[b] *N. E. Lewin, Dr. P. M. Blumberg*
Laboratory of Cancer Biology and Genetics, Center for Cancer Research
National Cancer Institute, National Institutes of Health
Bethesda, Maryland 20892 (USA)

[c] *Prof. T. Furuta*
Faculty of Science, Toho University
2-2-1 Miyama, Funabashi, Chiba 274-8510 (Japan)

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has been found that DAG favors *sn*-1 binding, whereas the corresponding DAG-lactone analogues favor *sn*-2 binding.[8] In this study, three representative DAG-lactones—**1**, **2**, and **4** (Scheme 1 B)—were investigated. Compound **1** had previously been reported as part of a branched α-alkylidene series that provided the most potent α-alkylidene analogues.[9,14] Comparison of the binding affinities of the stereoisomers showed that the *Z* isomer of lactone **1** had a higher affinity for PKCδ than the *E* isomer. The *Z* isomer of compound **1** was therefore purified by flash chromatography and utilized for experiments. Compound **2** had also previously been synthesized to assess the role of a flexible decanoic acid chain at the acyl chain position (R^1).[9a] Compound **4** was synthesized as a compound with a branched chain as an acyloxy moiety and an isopropyl system as an α-alkylidene group; a similar compound with a more branched acyloxy moiety has been reported.[15] The lactones **2** and **4** each contain an isopropylidene group at the α-alkylidene position, which has been identified as one of the principal determinants for control of biological activity. The effect of the acyl group on the isozyme specificity of the DAG-lactones has been investigated with a series of compounds.

The binding affinities of the DAG-lactones were determined as described previously.[16] The K_i values of the compounds were determined as 8.4 ± 2.9, 6.5 ± 0.8, and 22 ± 1.6 nM (mean ± SEM) for **1**, **2**, and **4**, respectively. The binding affinities of **1** and **2** were compatible with those in the previous reports[9a,15b] (2.3 and 15.9 nM, respectively), whereas that of **4** was weaker than those of the related compounds. It has been reported that branched chains interfere with the interaction with the hydrophobic surface of the C1b domain in the *sn*-2 binding mode of DAG-lactones. As reported previously, the acyl and α-alkylidene moieties affect PKCδ translocation caused by ligand binding. To reveal the translocation caused by the synthesized DAG-lactones, CHO-K1 cells expressing a PKCδ-EGFP fusion construct were prepared and the PKCδ-EGFP was visualized by confocal microscopy as a function of time after ligand addition (Figure 1 A–C). Compound **1** at 10 μM did not cause translocation of PKCδ even 30 min after addition (data not shown). Compound **2** caused rapid translocation, requiring 5 min for complete translocation. Compound **4** caused less complete and slower translocation, perhaps reflecting its weaker potency. As described for other DAG-lactones, translocation was predominantly to internal membrane compartments (nuclear membrane, mitochondria, and other cellular organelles).[17] From these studies, compound **2** was selected as the most suitable for photoactivation analysis with the aid of caged protecting groups.

To study PKCδ activation by photolysis of ligands, caged compounds were synthesized as depicted in Scheme 1 B. As caging moieties, (6-bromo-7-hydroxycoumarin-4-yl)methoxycarbonyl (Bhcmoc) and (6-bromo-7-methoxycoumarin-4-yl)methoxycarbonyl (Bmcmoc) were utilized. Several caged compounds based on these caging moieties have been reported previously.[1c] As one of the most commonly used classes of structures for the protection of phosphate, amine, and carbonyl functional groups, coumarin-based protecting groups including MCM,[18] HCM (and ACM),[19] DMCM,[20] BCMCM,[21] DMACM (and DEACM),[22] and Bhc[10] have been successfully developed. Given its critical role in forming two of the three hydrogen bonds driving binding of the DAG-lactone to the binding pocket of the C1 domain, the hydroxy group of the DAG-lactone was the obvious site for addition of the coumarin-based protecting group in order to disrupt binding (Scheme 1 A).

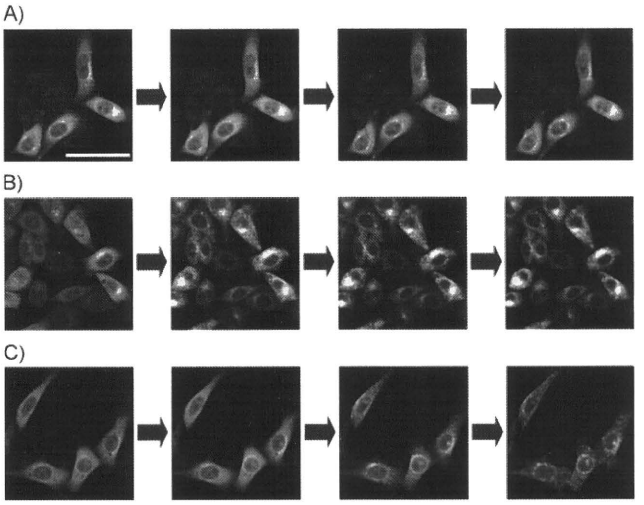

Figure 1. Translocation of PKCδ caused by compounds **1**, **2**, and **4**. A)–C) Time-dependent translocation of compounds 1, **2**, and **4**, respectively (0, 1, 5, and 10 min after addition of compounds). The final concentration of the compounds was 10 μM. The scale bar indicates 50 μm.

The function of the Bhc- and Bmc-protected DAG-lactones **10** and **11** as "phototriggers" (Figure 2 A) was evaluated in terms of several parameters. Firstly, the UV spectra of the compounds were determined (Figure 2 B). The compounds showed clear absorbance originating from the caged moiety. The absorbance maxima for Bhc-**2** and Bmc-**2** were 377 and 329 nm, respectively. Next, photolysis was performed with the aid of a photochemical lamp (RPR3500 Å). The breakdown of the caged compounds and the production of the uncaged compounds were monitored by HPLC and the extent of the reaction was calculated from the peak areas, which were determined as a function of irradiation time (Figure 2 C). Quantitative production of the parent compound **2** was successfully observed. The preferred environment of the caging groups for photolysis is thus hydrophilic, accounting for the superiority of the Bhc group over the Bmc group in photolysis efficiency (Table S1 in the Supporting Information). The photochemical properties of Bmc-**1** and Bmc-**4** were also assessed. The results support the view that compound **2** is suitable for photoactivation analysis (Figures S1, S2, and Table S2).

To evaluate the binding affinities of the caged compounds, competitive binding analysis to PKCδ was performed as described previously.[16] The results indicated that the caged compounds, in which the hydroxy group was protected, showed decreases in binding affinity of more than 100-fold (Table S1). The measured binding affinities of Bhc-**2** (**10**) and Bmc-**2** (**11**) to PKCδ were 431 and 940 nM, respectively. For comparison, compound **2** without the blocking group had an affinity of 6.5 nM. In the proposed binding mode of the DAG-lactones to

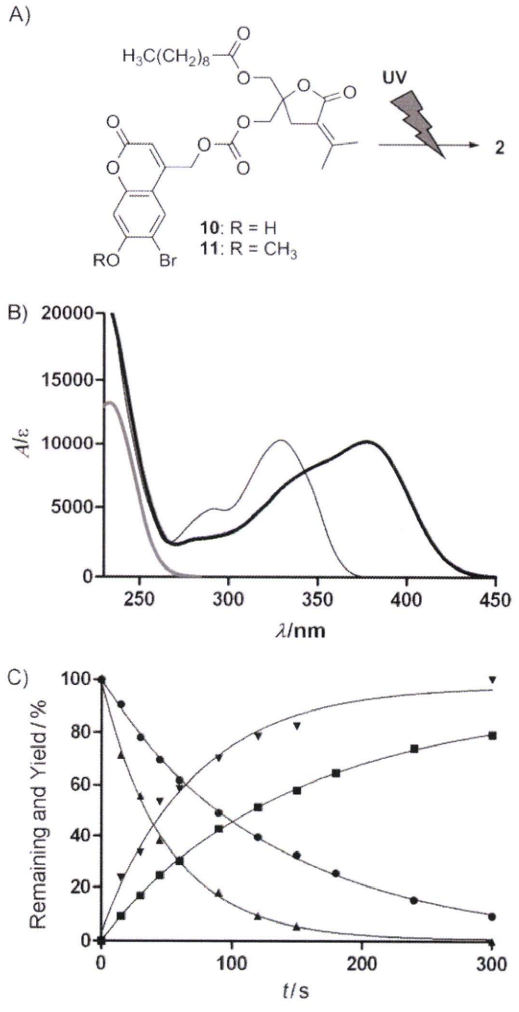

Figure 2. Structures of the caged compounds Bhc-**2** (**10**) and Bmc-**2** (**11**) and their photochemical properties. A) Representation of photochemical cleavage of the caged compounds **2**. B) UV spectra of **2** (gray line), Bhc-**2** (thick black line), and Bmc-**2** (thin black line). C) Plots of one-photon photolysis experiments. The plots show Bhc-**2** remaining (▲), Bhc-**2** product (▼), Bmc-**2** remaining (●), and Bmc-**2** product (■).)

scope. Of the synthetic compounds in this study, Bhc-**2** was most suitable for the purpose in terms of the efficiency of the photolysis reaction and rapid translocation of PKCδ. Translocation and reactivation of kinase activity by uncaged Bhc-**2** were confirmed (Figure S4).

To elucidate PKC-related signal transduction by stimulation of ligand binding, spatially and temporally controlled activation is desirable. To assess this point, photoirradiation at a limited region of interest (ROI) in the cell was performed. Because photolysis will only unblock caged ligand located in the irradiated region, the response following the activation should reflect this time- and location-dependent generation of active ligand. As shown in Figure 3, translocation was observed at

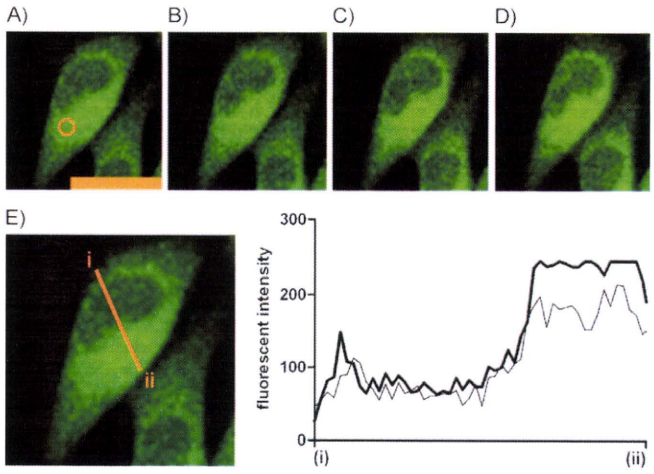

Figure 3. Translocation activated by photolysis of Bhc-**2** (**10**). The region of interest (ROI) is limited to the inside of the circle [orange in (A)]. The panels show the translocation of EGFP-PKCδ fusion protein in a time-course as follows: A) before photoirradiation, B) 2 min, C) 5 min, and D) 10 min after photoirradiation. The image in each panel shows EGFP fluorescence. The scale bar indicates 20 μm. The detailed pictures with pseudocolors are given in Figure S5. E) Fluorescent intensity mapped along the orange bar from (i) to (ii) in the panel. The thin and thick lines show the intensity at 0 and 10 min of photoirradiation, respectively.

PKC, the carbonyl oxygen of the DAG-lactone interacts with Gly253 of PKCδ, and the hydrogen and oxygen of the hydroxy group interact with Leu251 and Thr242, respectively. In the *sn*-2 binding mode, which is known to be preferred by DAG-lactones, the acyl group is proposed to interact with the lipid bilayer of the cellular membranes. In this mode, the caged hydroxy group cannot interact with Leu 251 or Thr242. The caged DAG-lactones thus successfully displayed "loss of function" in binding, a requisite property of caged compounds. To examine the effects on translocation of PKCδ in mammalian cells, the caged compounds Bhc-**2** and Bmc-**2** were added to medium at 10 μM, a concentration at which compound **2** caused rapid translocation. Consistently with their loss of binding affinity for PKCδ, as shown by the in vitro analysis, the caged compounds failed to induce PKCδ translocation, confirming that the activation of PKCδ by compound **2** is successfully retarded by caging (Figure S3). The behavior of Bhc-**2** (**10**) upon photolysis was also evaluated under the confocal microscope. 10 min after photoirradiation of Bhc-**2** (**10**). The pattern of subcellular localization was similar to that of compound **2** as shown in Figure 1. However, the time-course of translocation was dramatically slower, taking 10 min, in contrast with the rapid response seen upon addition of compound **2**. We conclude that the photolysis of compound Bhc-**2** was successful, in that translocation was observed. The slow kinetics are consistent with the concentration of the released DAG-lactone **2** being relatively low, which would be expected because the photolysis reaction occurred in the limited area of the ROI. Additionally, the cells around the ROI target cell also showed translocation of PKCδ. This effect might be caused by the release of uncaged compound and its reuptake by surrounding cells. The DAG-lactones are highly lipophilic, with their computed log*P* values ranging from 3 to 5. This property allows DAG-lactones to complete the hydrophobic surface of the C1b domain of PKCδ and to interact with the lipid bilayer of the membrane. However, the lipophilicity of the DAG-lactones also

allows the compounds to permeate through membranes and to be released back into the medium, making them available for reuptake.

It has been shown that patterns and kinetics of translocation of activated PKCδ depend on the lipophilicity and side-chain structures of DAG-lactones.[8] For compound **2**, it was possible that the translocation caused is to the Golgi apparatus, because the activated PKCδ is accumulated to the perinuclear region. To confirm the translocation to the Golgi apparatus, cellular staining by BODIPY TR ceramide was performed on CHO-K1 cells expressing PKCδ-EGFP. Compound **2** was added to these cells at 10 μM. After 10 min of compound addition, the amount of PKCδ that had colocalized with Golgi apparatus was increased (Figure S6). In the next study to determine the differences in translocation between cell lines, A549 and HeLa were utilized in addition to CHO-K1 cells. As shown in Figure 4, photoirradiation induced translocation of PKCδ in all cell lines. In comparison with the stimulation by compound **2**, translocation was modest because of the low concentration of released compound **2** as discussed above. Of the cell types examined, HeLa cells showed most distinctive translocation to Golgi apparatus and nuclear membrane.

In this study, the Bhc- and Bmc-caged DAG-lactones were prepared and evaluated in terms of photochemical control of PKC activation. DAG-lactone binding to PKCδ in mammalian cells was successfully controlled through photoirradiation. Derivatization at the DAG-lactone hydroxy group was shown to be very effective for diminishing affinity towards PKC. Photoactivation limited to the ROI was successfully regulated. However, there remained effects on other cells near the cells of interest, and this needs to be addressed in future studies. Development of more precise photochemical control methodology will be needed to address the signaling mechanism related to PKCδ in a single cell or at a single location within a single cell. Nonetheless, these results indicate the potential of this approach for studying PKC function.[1b,c]

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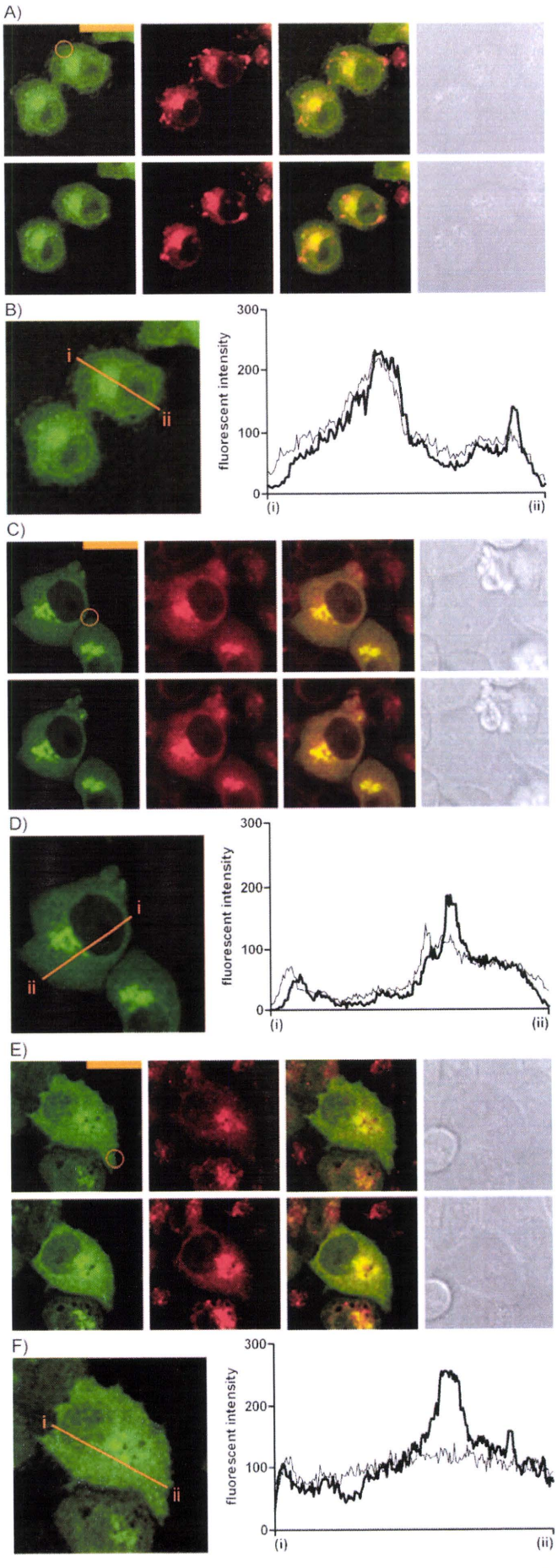

Figure 4. Observation of translocation of activated PKCδ within the Golgi-stained cells by photolysis of Bhc-**2** (**10**) in ROI. The cells are: A) CHO-K1, C) A549, and E) HeLa. The ROI is limited to the inside of the orange circles. Each set of cell images shows before photoirradiation (top) and 10 min after (bottom); EGFP-PKCδ, staining by BODIPY TR ceramide, merged image, and differential interference contrast (DIC) from left to right. The scale bars indicate 20 μm. B), D), and F) Fluorescent intensities mapped along the orange bars from (i) to (ii) in the panels. The thin and thick lines show the intensity at 0 and 10 min of photoirradiation, respectively.

Keywords: cage compounds · diacylglycerol · drug design · enzymes · proteins

[1] a) G. Dormán, G. D. Prestwich, *Trends Biotechnol.* **2000**, *18*, 64–77; b) G. Mayer, A. Heckel, *Angew. Chem.* **2006**, *118*, 5020–5042; *Angew. Chem. Int. Ed.* **2006**, *45*, 4900–4921; c) H.-M. Lee, D. R. Larson, D. S. Lawrence, *ACS Chem. Biol.* **2009**, *4*, 409–427; d) E. J. Quann, E. Merino, T. Furuta, M. Huse, *Nat. Immunol.* **2009**, *10*, 627–637.

[2] a) Y. Nishizuka, *Science* **1992**, *258*, 607–614; b) A. C. Newton, *J. Biol. Chem.* **1995**, *270*, 28495–28498.

[3] T. Watanabe, Y. Ono, Y. Taniyama, K. Hazama, K. Igarashi, K. Ogita, U. Kikkawa, Y. Nishizuka, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 10159–10163.

[4] H. Mischak, J. H. Pierce, J. Goodnight, M. G. Kazanietz, P. M. Blumberg, J. F. Mushinski, *J. Biol. Chem.* **1993**, *268*, 20110–20115.

[5] C. Li, E. Wernig, M. Leitges, Y. Hu, Q. Xu, *FASEB J.* **2003**, *17*, 2106–2108.

[6] a) T. Ghayur, M. Hugunin, R. V. Talanian, S. Ratnofsky, C. Quinlan, Y. Emoto, P. Pandey, R. Datta, Y. Huang, S. Kharbanda, H. Allen, R. Kamen, W. Wong, D. Kufe, *J. Exp. Med.* **1996**, *184*, 2399–2404; b) D. L. Alkon, M.-K. Sun, T. J. Nelson, *Trends Pharmacol. Sci.* **2007**, *28*, 51–60.

[7] Q. J. Wang, *Trends Pharmacol. Sci.* **2006**, *27*, 317–323.

[8] V. E. Marquez, P. M. Blumberg, *Acc. Chem. Res.* **2003**, *36*, 434–443.

[9] a) K. Nacro, B. Bienfait, J. Lee, K.-C. Han, J.-H. Kang, S. Benzaria, N. E. Lewin, D. K. Bhattacharyya, P. M. Blumberg, V. E. Marquez, *J. Med. Chem.* **2000**, *43*, 921–944; b) H. Tamamura, B. Beinfait, K. Nacro, N. E. Lewin, P. M. Blumberg, V. E. Marquez, *J. Med. Chem.* **2000**, *43*, 3210–3217.

[10] a) T. Furuta, S. S.-H. Wang, J. L. Dantzker, T. M. Dore, W. J. Bybee, E. M. Callaway, W. Denk, R. Y. Tsien, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 1193–1200; b) H. Ando, T. Furuta, R. Y. Tsien, H. Okamoto, *Nat. Genet.* **2001**, *28*, 317–325; c) H. J. Montgomery, B. Perdicakis, D. Fishlock, G. A. Lajoie, E. Jervis, J. G. Guillemette, *Bioorg. Med. Chem.* **2002**, *10*, 1919–1927; d) W. Lin, D. S. Lawrence, *J. Org. Chem.* **2002**, *67*, 2723–2726; e) M. Lu, O. D. Fedoryak, B. R. Moister, T. M. Dore, *Org. Lett.* **2003**, *5*, 2119–2122; f) A. Z. Suzuki, T. Watanabe, M. Kawamoto, K. Nishiyama, H. Yamashita, M. Ishii, M. Iwamura, T. Furuta, *Org. Lett.* **2003**, *5*, 4867–4870.

[11] T. Furuta, T. Watanabe, S. Tanabe, J. Sakyo, C. Matsuba, *Org. Lett.* **2007**, *9*, 4717–4720.

[12] a) X. P. Huang, R. Sreekumar, J. R. Patel, J. W. Walker, *Biophys. J.* **1996**, *70*, 2448–2457; b) V. G. Robu, E. S. Pfeiffer, S. L. Robia, R. C. Balijepalli, Y. Pi, T. J. Kamp, J. W. Walker, *J. Biol. Chem.* **2003**, *278*, 48154–48161.

[13] a) Y. Kishi, R. R. Rando, *Acc. Chem. Res.* **1998**, *31*, 163–172; b) P. A. Wender, C. M. Cribbs, K. F. Koehler, N. A. Sharkey, C. L. Herald, Y. Kamano, G. R. Petit, P. M. Blumberg, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 7197–7201; c) S. Wang, G. W. A. Milne, M. C. Nicklaus, V. E. Marquez, J. Lee, P. M. Blumberg, *J. Med. Chem.* **1994**, *37*, 1326–1338.

[14] a) H. Tamamura, D. M. Sugano, N. E. Lewin, P. M. Blumberg, V. E. Marquez, *J. Med. Chem.* **2004**, *47*, 644–655; b) K. Malolanarasimhan, N. Kedei, D. M. Sigano, J. A. Kelley, C. C. Lai, N. E. Lewin, R. J. Surawski, V. A. Pavlyukovets, S. H. Garfield, S. Wincovitch, P. M. Blumberg, V. E. Marquez, *J. Med. Chem.* **2007**, *50*, 962–978.

[15] D. M. Sigano, M. L. Peach, K. Nacro, Y. Choi, N. E. Lewin, M. C. Nicklaus, P. M. Blumberg, V. E. Marquez, *J. Med. Chem.* **2003**, *46*, 1571–1579.

[16] a) N. A. Sharkey, P. M. Blumberg, *Cancer Res.* **1985**, *45*, 19–24; b) M. G. Kazanietz, K. W. Krausz, P. M. Blumberg, *J. Biol. Chem.* **1992**, *267*, 20878–20886.

[17] L. Li, P. S. Lorenzo, K. Bogi, P. M. Blumberg, S. H. Yupsa, *Mol. Cell. Biol.* **1999**, *19*, 8547–8558.

[18] a) T. Furuta, H. Torigai, M. Sugimoto, M. Iwamura, *J. Org. Chem.* **1995**, *60*, 3953–3956; b) B. Schade, V. Hagen, R. Schmidt, R. Herbrich, E. Krause, T. Eckardt, J. Bendig, *J. Org. Chem.* **1999**, *64*, 9109–9117.

[19] a) T. Furuta, A. Momotake, M. Sugimoto, M. Hatayama, M. Iwamura, *Biochem. Biophys. Res. Commun.* **1996**, *228*, 193–198; b) T. Furuta, M. Iwamura, *Methods Enzymol.* **1998**, *291*, 50–63.

[20] T. Eckardt, V. Hagen, B. Schade, R. Schmidt, C. Schweitzer, J. Bendig, *J. Org. Chem.* **2002**, *67*, 703–710.

[21] a) V. Hagen, J. Bendig, S. Frings, T. Eckardt, S. Helm, D. Reuter, U. B. Kaupp, *Angew. Chem.* **2001**, *113*, 1077–1080; *Angew. Chem. Int. Ed.* **2001**, *40*, 1045–1048; b) V. Hagen, S. Frings, J. Bendig, D. Lorenz, B. Wiesner, U. B. Kaupp, *Angew. Chem.* **2002**, *114*, 3775–3777; *Angew. Chem. Int. Ed.* **2002**, *41*, 3625–3628.

[22] a) D. Geißler, W. Kresse, B. Wiesner, J. Bendig, H. Kettenmann, V. Hagen, *ChemBioChem* **2003**, *4*, 162–170; b) V. Hagen, S. Frings, B. Wiesner, S. Helm, U. B. Kaupp, J. Bendig, *ChemBioChem* **2003**, *4*, 434–442.

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Azamacrocyclic Metal Complexes as CXCR4 Antagonists

Tomohiro Tanaka,[a] Tetsuo Narumi,*[a] Taro Ozaki,[a] Akira Sohma,[a] Nami Ohashi,[a] Chie Hashimoto,[a] Kyoko Itotani,[a] Wataru Nomura,[a] Tsutomu Murakami,[b] Naoki Yamamoto,[b, c] and Hirokazu Tamamura*[a]

The chemokine receptor CXCR4 is a member of the seven transmembrane GPCR family, which is implicated in multiple diseases, including HIV infection, cancers, and rheumatoid arthritis. Low-molecular-weight nonpeptidic compounds, including AMD3100 and various pyridyl macrocyclic zinc(II) complexes, have been identified as selective antagonists of CXCR4. In the present study, structure–activity relationship studies were performed by combining the common structural features of alkylamino and pyridiyl macrocyclic antagonists. Several new zinc(II) or copper(II) complexes demonstrated potent anti-HIV activity, strong CXCR4-binding activity, and significant inhibitory activity against Ca^{2+} mobilization induced by CXCL12 stimulation. These results may prove useful in the design of novel CXCR4 antagonists, and the compounds described could potentially be developed as therapeutics against CXCR4-relevant diseases or chemical probes to study the biological activity of CXCR4.

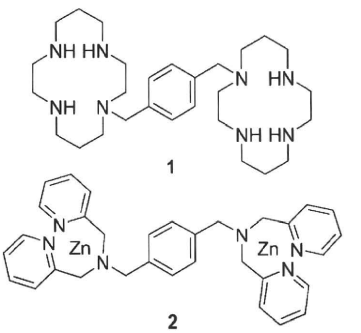
Introduction

The chemokine receptor CXCR4, which transduces signals of its endogenous ligand, CXCL12/stromal cell-derived factor-1 (SDF-1),[1–4] is classified as a member of the seven transmembrane GPCR family, and plays a physiological role via its interaction with CXCL12 in chemotaxis,[5] angiogenesis,[6,7] and neurogenesis[8,9] in embryonic stages. CXCR4 is, however, relevant to multiple diseases including HIV infection/AIDS,[10,11] metastasis of several types of cancer,[12–14] leukemia cell progression,[15,16] and rheumatoid arthritis (RA),[17,18] and is considered an attractive drug target to combat these diseases. Thus, inhibitors targeting CXCR4 are expected to be useful for drug discovery.

Several CXCR4 antagonists have been reported,[19–35] including our discovery of the highly potent CXCR4 antagonist T140, a 14-mer peptide with a disulfide bridge, its smaller derivative, the 5-mer cyclic peptide FC131, and several other potent analogues.[19,24–26,28–30] Clinical development of these peptidic antagonists could be pursued using specific administration strategies involving biodegradable microcapsules.[14,36] However, herein we focus on novel nonpeptidic low-molecular-weight CXCR4 antagonists. To date, AMD3100 (**1**),[20,22] Dpa-Zn complex (**2**),[37] KRH-1636,[27] and other compounds[31–35] have been developed in this and other laboratories as low-molecular-weight nonpeptidic CXCR4 antagonists. The present study reports structure–activity relationship studies based on the combination of common structural motifs, such as xylene scaffolds and cationic moieties that are present in the aforementioned compounds.

Results and Discussion

In order to determine spatially suitable positioning of cationic moieties, *p*- and *m*-xylenes were utilized as spacers. Cationic moieties such as bis(pyridin-2-ylmethyl)amine (dipicolylamine), 1,4,7,10-tetraazacyclododecane (cyclen), and 1,4,8,11-tetraazacyclotetradecane (cyclam) were introduced as R^1 and R^2 (Figure 1). This combination of R^1, R^2, and spacer groups led to the design and synthesis of compounds **12–31**.



The CXCR4 binding activity of synthetic compounds was assessed based on the inhibition of [^{125}I]CXCL12 binding to Jurkat cells, which express CXCR4.[38] The percent inhibition of all compounds at 1 μM is shown in Table 1. Seven compounds (**16**, **17**, **20–22**, **28**, and **29**, Table 1) resulted in greater than 87% inhibition. The high activity of **16** is consistent with re-

[a] *T. Tanaka, Dr. T. Narumi, T. Ozaki, A. Sohma, N. Ohashi, C. Hashimoto, K. Itotani, Dr. W. Nomura, Prof. H. Tamamura*
Institute of Biomaterials and Bioengineering
Tokyo Medical and Dental University
2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062 (Japan)
Fax: (+81) 3-5280-8039
E-mail: tamamura.mr@tmd.ac.jp

[b] *Dr. T. Murakami, Prof. N. Yamamoto*
AIDS Research Center, National Institute of Infectious Diseases
1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640 (Japan)

[c] *Prof. N. Yamamoto*
Department of Microbiology, Yong Loo Lin School of Medicine
National University of Singapore, Singapore 117597 (Singapore)

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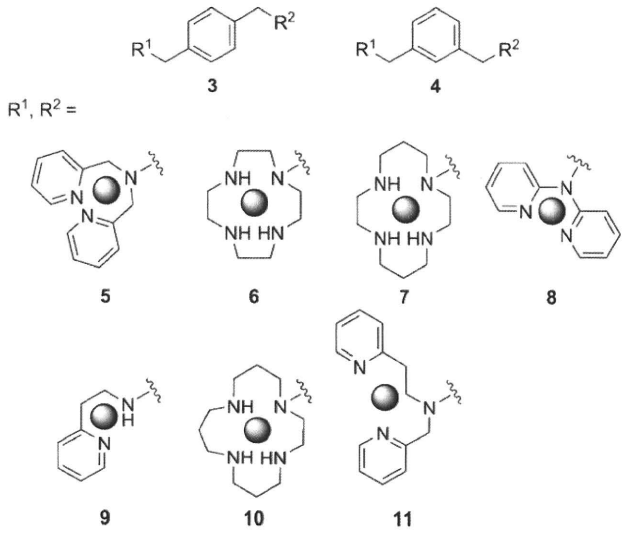


Figure 1. The structures of aromatic spacers (upper) and cationic moieties (R^1 and R^2). The shaded circle represents the position of the metal cation (Zn^{II} or Cu^{II}) in the chelate.

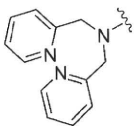
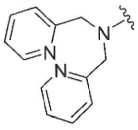
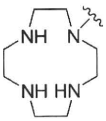
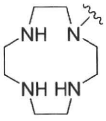
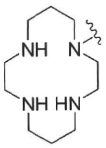
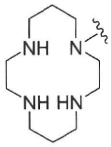
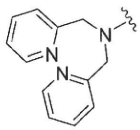
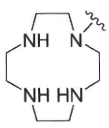
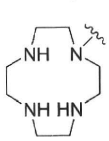
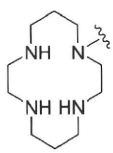
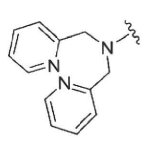
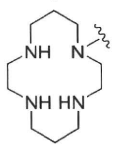
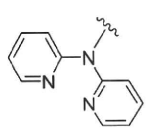
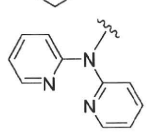
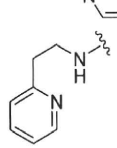
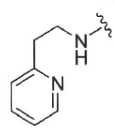
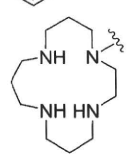
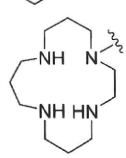
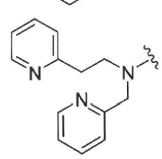
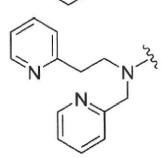
sults reported previously.[20,22] The anti-HIV activities of **17** and **29**, which contain only cyclam or cyclal rings, were reported by De Clercq et al.[39,40] Compounds with only pyridine and/or cyclen rings did not show any high binding activity. The presence of azamacrocyclic rings is presumably indispensable to the interaction of these compounds with CXCR4, and the size of rings appears to be important because not only compounds **16** and **17**, with two cyclam rings in the molecule, but also compounds **28** and **29**, with two cyclal rings, have remarkably more potent CXCR4 binding activity than compounds **14** and **15**, which have two cyclen rings. Compound **22**, with a *p*-xylene moiety, exhibited higher activity than compound **23**, which has an *m*-xylene moiety, indicating that *p*-xylene is more suitable than *m*-xylene as a spacer for approximate positioning of cationic moieties. At 0.1 μM, compound **22** resulted in 86% inhibition of [^{125}I]CXCL12 binding, while the other six compounds exhibited 37–66% inhibition. The IC_{50} value of compound **22** was estimated to be 37 nM.

$ZnCl_2$ was added to phosphate-buffered saline (PBS) solutions of these 20 compounds, **12–31**, to form zinc(II) complexes. The percent inhibition for each compound at 1 μM against [^{125}I]CXCL12 binding was determined and is given in Table 1. Zinc complexation of **12–15**, **18**, **19**, and **23** resulted in a remarkable increase in CXCR4 binding activity compared to the corresponding zinc-free compounds. These molecules contain dipicolylamine and/or cyclen moieties, suggesting that chelation of the nitrogen atoms with the zinc(II) ion significantly affects their interactions with CXCR4. The high activity of the zinc chelates of **12** and **13** is consistent with results provided in our previous paper.[37] Additionally, the anti-HIV activity of zinc complexes of **14** and **15** was reported by Kimura et al.[41] For compounds with only dipicolylamine and/or cyclen macrocycles as cationic moieties (**12–15**, **18**, and **19**), zinc complexation is critical to achieve high binding activity; the corresponding zinc-free compounds exhibit no significant activity. Compounds **16**, **17**, **20–22**, **28**, and **29** demonstrated high binding affinity in metal-free states as well as in zinc complexation states, indicating that zinc complexation of either of the macrocyclic rings in these compounds is not essential for high activity. The CXCR4 binding activity and anti-HIV activity of the zinc complex of **16** were reported previously.[42,43] Measured inhibition percentages for 0.1 μM of the zinc complexes of **12**, **14–23**, **28**, and **29** are given in Table 1. The zinc complexes of **20–22**, **28**, and **29** at 0.1 μM exhibited greater than 79% inhibition of [^{125}I]CXCL12 binding, and the other eight zinc complexes (of **12**, **14–19**, and **23**,) showed less than 55% inhibition. The IC_{50} values of zinc complexes of **20–22**, **28**, and **29** were estimated to be 11, 8.3, 22, 40, and 52 nM, respectively. Zinc complexes of compounds containing a combination of cyclen and cyclam moieties, **20** and **21**, had remarkably potent IC_{50} values.

To form chelates with a copper(II) cation, $CuCl_2$ was added to solutions in PBS of **12-31**. The inhibition percentages of all the compounds at 1 μM against [^{125}I]CXCL12 binding are shown in Table 1. Copper complexes of **14** and **15** exhibited a significant increase in CXCR4 binding activity as compared to the corresponding copper-free compounds, a phenomenon which is also seen in the zinc chelates. These compounds have two cyclen moieties in the molecules, suggesting that zinc or copper complexation is critical for high binding activity. Compounds **16**, **17**, and **20–22** showed high binding affinities in metal-free states and zinc- and copper-complexed states, indicating that metallic complexation of the cyclam rings in these compounds is not necessary for high activity. The CXCR4 binding activity of the copper complex of **16** was previously reported.[42] For compounds **17**, **22**, **23**, **28**, and **29**, copper complexation caused a significant decrease in binding activity compared to the corresponding copper-free compounds, whereas for compounds **14**, **15**, **18**, and **19**, copper complexation caused an increase in binding activity. This phenomenon may be due to the difference in ring sizes and structures of macrocycles, and was not observed upon zinc-complex formation. Inhibition at 0.1 μM of the copper complexes of **16** and **20–22**, which exhibited greater than 85% inhibition of [^{125}I]CXCL12 binding at 1 μM, are given in Table 1. The copper complexes of **16**, **20**, **21**, and **22** at 0.1 μM showed 39, 69, 88, and 39% inhibition, respectively, with the IC_{50} value of the copper complex of **21** estimated to be 16 nM.

Molecular modeling analysis of compound **21** and its zinc(II) and copper(II) complexes predicted that these complexes would form a stable coordinate conformation as shown in Figure 2. In general, zinc(II) complexes are predicted to adopt a tetrahedral conformation, while copper(II) complexes form a planar four coordinate/square conformation. The zinc(II) complex of **21** is predicted to have a tetrahedral conformation and the copper(II) complex a square planar conformation in both the cyclen and cyclam rings. The carboxyl group of either Asp171 or Asp262 in CXCR4 is thought to coordinate strongly with zinc ions but not copper ions in the complexes,[41–43] and as a consequence, the zinc complex of **21** would bind more strongly than **21** or its copper complex. This order of binding

Table 1. CXCR4 binding activity of compounds **12–31** in the metal ion-free form, the zinc complex, and the copper complex.

Compd	Spacer	R^1	R^2	Metal free			Zinc complex			Copper complex		
				Inhibition[a] [%]		IC_{50}[b]	Inhibition[a] [%]		IC_{50}[b]	Inhibition[a] [%]		IC_{50}[b]
				1 μM	0.1 μM	[nM]	1 μM	0.1 μM	[nM]	1 μM	0.1 μM	[nM]
12	*p*-xylene			0	n.d.	n.d.	83 ± 2	24 ± 5	n.d.	10 ± 4	n.d.	n.d.
13	*m*-xylene			0	n.d.	n.d.	31 ± 3	n.d.	n.d.	0	n.d.	n.d.
14	*p*-xylene			30 ± 4	n.d.	n.d.	87 ± 4	0	n.d.	60 ± 2	n.d.	n.d.
15	*m*-xylene			33 ± 2	n.d.	n.d.	94 ± 1	13 ± 6	n.d.	80 ± 3	n.d.	n.d.
16	*p*-xylene			94 ± 4	59 ± 6	n.d.	97 ± 5	28 ± 3	n.d.	98 ± 1	39 ± 3	n.d.
17	*m*-xylene			95 ± 3	49 ± 9	n.d.	98 ± 4	55 ± 7	n.d.	75 ± 1	n.d.	n.d.
18	*p*-xylene			32 ± 0.7	n.d.	n.d.	97 ± 6	0	n.d.	52 ± 3	n.d.	n.d.
19	*m*-xylene			17 ± 5	n.d.	n.d.	91 ± 4	0	n.d.	22 ± 6	n.d.	n.d.
20	*p*-xylene			89 ± 3	62 ± 3	n.d.	> 100	79 ± 1	11	> 100	69 ± 3	n.d.
21	*m*-xylene			89 ± 3	66 ± 3	n.d.	92 ± 3	> 100	8.3	> 100	88 ± 1	16
22	*p*-xylene			94 ± 3	86 ± 3	37	99 ± 8	79 ± 0.6	22	85 ± 3	39 ± 3	n.d.
23	*m*-xylene			58 ± 8	n.d.	n.d.	90 ± 17	37 ± 0.3	n.d.	48 ± 4	n.d.	n.d.
24	*p*-xylene			3 ± 0.9	n.d.	n.d.	0	n.d.	n.d.	0	n.d.	n.d.
25	*m*-xylene			4 ± 3	n.d.	n.d.	0	n.d.	n.d.	0	n.d.	n.d.
26	*p*-xylene			14 ± 2	n.d.	n.d.	10 ± 3	n.d.	n.d.	0	n.d.	n.d.
27	*m*-xylene			10 ± 3	n.d.	n.d.	10 ± 4	n.d.	n.d.	0	n.d.	n.d.
28	*p*-xylene			91 ± 0.4	37 ± 0.9	n.d.	97 ± 4	> 100	40	57 ± 4	n.d.	n.d.
29	*m*-xylene			87 ± 2	50 ± 1	n.d.	> 100	91 ± 4	52	55 ± 1	n.d.	n.d.
30	*p*-xylene			0	n.d.	n.d.	14 ± 3	n.d.	n.d.	14 ± 3	n.d.	n.d.
31	*m*-xylene			24 ± 2	n.d.	n.d.	20 ± 3	n.d.	n.d.	0	n.d.	n.d.
FC-131	*cyclo*-[D-Tyr-Arg-Arg-Nal-Gly-]			100	100	1.8	–	–	–	–	–	–

[a] CXCR4 binding activity was assessed based on inhibition of [^{125}I]CXCL12 binding to Jurkat cells. Percent inhibition for all compounds at 1 and 0.1 μM were calculated relative to the percent inhibition by FC131 (100%). [b] IC_{50} values are the concentrations which correspond to 50% inhibition of [^{125}I]CXCL12 binding to Jurkat cells. All data are mean values ± SEM of at least three independent experiments. n.d. = not determined.

affinities is commonly seen for these compounds and their zinc(II) or copper(II) complexes.

We investigated the CXCR4 antagonistic activity of compound **22** and the zinc complexes of **20**, **21**, **22**, and **28**, all of which possess strong CXCR4 binding activity. The CXCR4 antagonistic activity was assessed based on the inhibitory activity of the compounds against Ca^{2+} mobilization induced by CXCL12 stimulation through CXCR4 (figure S1 in the Support-

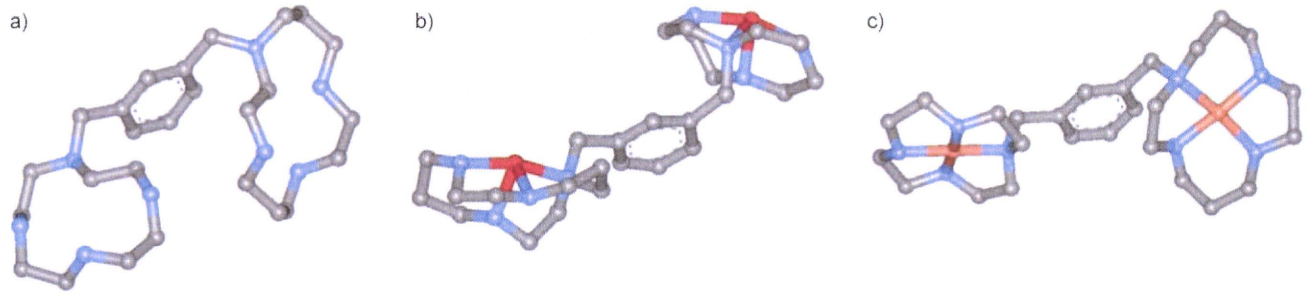


Figure 2. Structures calculated by molecular modeling of a) compound **21**, and its b) zinc and c) copper complexes. Atom color code: nitrogen = blue, carbon = gray, zinc = red, copper = light red.

ing Information). All of the tested compounds showed significant antagonistic activity at 1 μM.

The representative compounds **14**, **16**, **20–23**, **28**, and **29**, as well as their zinc chelates, were evaluated for anti-HIV activity. CXCR4 is the major co-receptor for the entry of T-cell-line-tropic (X4) HIV-1.[10,11] Inhibitory activity against X4-HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells was assessed and is shown in Table 2.[38] A correlation between CXCR4 binding activity and anti-HIV activity was observed. For compound **16** and its zinc complex, anti-HIV activity was significantly stronger than CXCR4 binding activity, and for the zinc complexes of compounds **20–22**, the CXCR4 binding activity is two to four-times stronger than the anti-HIV activity. The anti-HIV activity of the zinc complex of **16** was the most potent (EC_{50} = 8.2 nM). This is comparable to the anti-HIV activities of **16** and its zinc complex that were reported previously.[20,22,42,43] The zinc complex of **21**, which was the most active compound in terms of CXCR4 binding activity, also exhibited potent anti-HIV activity (EC_{50} = 36 nM).

Taken together, these results show that all of the compounds exhibiting CXCR4 binding activity also showed significant anti-HIV activity (EC_{50} values < 300 nM), and none of the tested compounds exhibited significant cytotoxicity (CC_{50} values > 10 μM; Table 2). Conversely, zinc complexes of **20**, **21**, **22**, and **28** did not exhibit significant anti-HIV activity against macrophage-tropic (R5) HIV-1 (NL(AD8) strain)-induced cytopathogenicity in PM-1 cells at concentrations below 10 μM. Since R5-HIV-1 strains use CCR5 instead of CXCR4 as the major co-receptor for entry, this suggests that these compounds do not bind CCR5 but rather are highly selective for CXCR4.

Table 2. Anti-HIV activity and cytotoxicity of representative compounds in the metal ion-free and zinc chelates.

Compd	Metal ion-free		Zinc chelate	
	EC_{50}[a] [nM]	CC_{50}[b] [μM]	EC_{50}[a] [nM]	CC_{50}[b] [μM]
14	200	> 10	200	> 10
16	21	> 10	8.2	> 10
20	38	> 10	39	> 10
21	50	> 10	36	> 10
22	93	> 10	48	> 10
23	290	> 10	220	> 10
28	36	> 10	56	> 10
29	130	> 10	42	> 10
FC131	93	> 10		
AZT	69	> 100		

[a] EC_{50} values are the concentrations corresponding to 50% protection from X4-HIV-1 (NL4–3 strain)-induced cytopathogenicity in MT-4 cells. [b] CC_{50} values are the concentrations at which the viability of MT-4 cells is reduced by 50%. All data are mean values from at least three independent experiments.

Conclusions

The present study introduces a new class of low-molecular-weight CXCR4 antagonists and their zinc(II) or copper(II) complexes, which contain pyridyl or azamacrocycle moieties with *p*-xylene or *m*-xylene spacers. These compounds demonstrated strong CXCR4 binding activity. Zinc complexes of **20** and **21**, which were the two most active compounds, contain cyclen and cyclam rings with *p*- and *m*-xylene spacers and exhibited remarkably potent IC_{50} values (11 and 8.3 nM, respectively). These compounds showed significant CXCR4 antagonistic activity, based on inhibitory activity against Ca^{2+} mobilization induced by CXCL12 stimulation through CXCR4, as well as potent anti-HIV activity, as assessed by protection from X4-HIV-1-induced cytopathogenicity in MT-4 cells. These results provide useful insights into the future design of novel CXCR4 antagonists, complementing information from other CXCR4 antagonists such as T140, FC131, and KRH-1636. Furthermore, these new compounds are useful for the development of therapeutic strategies for CXCR4-relevant diseases and chemical probes to study the biological activity of CXCR4.

Experimental Section

Chemistry

Compounds **12–17**, **20**, **21**, **24**, **25**, **27–29**, and **31** were synthesized as previously reported.[20,22,37,40,41,44–47] Compounds **18**, **19**, **22**, **23**, **26**, and **30** were synthesized in the present study; details are provided in the Supporting Information. A representative compound, **18**, was synthesized by coupling *p*-dibromoxylene (1,4-bis-(bromomethyl)benzene) with tri-Boc-protected 1,4,7,10-tetraazacyclododecane, followed by treatment with trifluoroacetic acid and subsequent coupling with bis(pyridin-2-ylmethyl)amine. All crude compounds were purified by RP-HPLC and identified by FAB/ESI-

HRMS. Zinc(II) or copper(II) complex formation was accomplished by treatment of the above compounds with 10 equiv of $ZnCl_2$ or $CuCl_2$ in PBS. All zinc(II) or copper(II) complexes were characterized by chemical shifts of their methylene protons in ^1H NMR analysis. The pyridyl zinc(II) complex was characterized previously,[37] and zinc(II) or copper(II) complex formation with these macrocyclic compounds has been reported elsewhere.[41,42,48,49] Detailed procedures and data are provided in the Supporting Information.

Biological assays

A CXCR4 binding assay for compounds, based on the inhibition of [^{125}I]CXCL12 binding to Jurkat cells, was performed as reported by Tanaka et al.[38] CXCR4 antagonistic activity was evaluated as described by Ichiyama et al[27], measuring inhibitory activity against Ca^{2+} mobilization induced by CXCL12 stimulation in HOS cells expressing CXCR4. Anti-HIV activity was determined by inhibitory activity against X4-HIV-1(NL4–3)-induced cytopathogenicity in MT-4 cells as reported by Tanaka et al.[38] An X4 HIV-1 infectious molecular clone (pNL4–3) was obtained from the AIDS Research and Reference Reagent Program. The virus NL4–3 was obtained from the culture supernatant of 293T cells transfected with pNL4–3.

Molecular modeling

Molecular modeling calculations were performed using Sybyl (version 7.0, Tripos). Energy minimization was performed using the Tripos force field and Gasteiger–Hückel charge parameters. The lowest energy conformation was obtained by random search methods.

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Keywords: azamacrocycles · Ca^{2+} mobilization · CXCR4 · HIV · structure–activity relationships

[1] T. Nagasawa, H. Kikutani, T. Kishimoto, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 2305–2309.
[2] C. C. Bleul, M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski, T. A. Springer, *Nature* **1996**, *382*, 829–833.
[3] E. Oberlin, A. Amara, F. Bachelerie, C. Bessia, J. L. Virelizier, F. Arenzana-Seisdedos, O. Schwartz, J. M. Heard, I. Clark-Lewis, D. L. Legler, M. Loetscher, M. Baggiolini, B. Moser, *Nature* **1996**, *382*, 833–835.
[4] K. Tashiro, H. Tada, R. Heilker, M. Shirozu, T. Nakano, T. Honjo, *Science* **1993**, *261*, 600–603.
[5] C. C. Bleul, R. C. Fuhlbrigge, J. M. Casanovas, A. Aiuti, T. A. Springer, *J. Exp. Med.* **1996**, *184*, 1101–1109.
[6] K. Tachibana, S. Hirota, H. Iizasa, H. Yoshida, K. Kawabata, Y. Kataoka, Y. Kitamura, K. Matsushima, N. Yoshida, S. Nishikawa, T. Kishimoto, T. Nagasawa, *Nature* **1998**, *393*, 591–594.
[7] T. Nagasawa, S. Hirota, K. Tachibana, N. Takakura, S. Nishikawa, Y. Kitamura, N. Yoshida, H. Kikutani, T. Kishimoto, *Nature* **1996**, *382*, 635–638.
[8] Y. Zhu, Y. Yu, X. C. Zhang, T. Nagasawa, J. Y. Wu, Y. Rao, *Nat. Neurosci.* **2002**, *5*, 719–720.
[9] R. K. Stumm, C. Zhou, T. Ara, F. Lazarini, M. Dubois-Dalcq, T. Nagasawa, V. Hollt, S. Schulz, *J. Neurosci.* **2003**, *23*, 5123–5130.
[10] H. K. Deng, R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. D. Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, N. R. Landau, *Nature* **1996**, *381*, 661–666.
[11] Y. Feng, C. C. Broder, P. E. Kennedy, E. A. Berger, *Science* **1996**, *272*, 872–877.
[12] T. Koshiba, R. Hosotani, Y. Miyamoto, J. Ida, S. Tsuji, S. Nakajima, M. Kawaguchi, H. Kobayashi, R. Doi, T. Hori, N. Fujii, M. Imamura, *Clin. Cancer Res.* **2000**, *6*, 3530–3535.
[13] A. Müller, B. Homey, H. Soto, N. Ge, D. Catron, M. E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S. N. Wagner, J. L. Barrera, A. Mohar, E. Verastegui, A. Zlotnik, *Nature* **2001**, *410*, 50–56.
[14] H. Tamamura, A. Hori, N. Kanzaki, K. Hiramatsu, M. Mizumoto, H. Nakashima, N. Yamamoto, A. Otaka, N. Fujii, *FEBS Lett.* **2003**, *550*, 79–83.
[15] N. Tsukada, J. A. Burger, N. J. Zvaifler, T. J. Kipps, *Blood* **2002**, *99*, 1030–1037.
[16] J. Juarez, K. F. Bradstock, D. J. Gottlieb, L. J. Bendall, *Leukemia* **2003**, *17*, 1294–1300.
[17] T. Nanki, K. Hayashida, H. S. El-Gabalawy, S. Suson, K. Shi, H. J. Girschick, S. Yavuz, P. E. Lipsky, *J. Immunol.* **2000**, *165*, 6590–6598.
[18] H. Tamamura, M. Fujisawa, K. Hiramatsu, M. Mizumoto, H. Nakashima, N. Yamamoto, A. Otaka, N. Fujii, *FEBS Lett.* **2004**, *569*, 99–104.
[19] T. Murakami, T. Nakajima, Y. Koyanagi, K. Tachibana, N. Fujii, H. Tamamura, N. Toshida, M. Waki, A. Matsumoto, O. Yoshie, T. Kishimoto, N. Yamamoto, T. Nagasawa, *J. Exp. Med.* **1997**, *186*, 1389–1393.
[20] D. Schols, S. Struyf, J. Van Damme, J. A. Este, G. Henson, E. DeClarcq, *J. Exp. Med.* **1997**, *186*, 1383–1388.
[21] B. J. Doranz, K. Grovit-Ferbas, M. P. Sharron, S.-H. Mao, M. Bidwell Goetz, E. S. Daar, R. W. Doms, W. A. O'Brien, *J. Exp. Med.* **1997**, *186*, 1395–1400.
[22] G. A. Donzella, D. Schols, S. W. Lin, J. A. Este, K. A. Nagashima, *Nat. Med.* **1998**, *4*, 72–76.
[23] O. M. Z. Howard, J. J. Oppenheim, M. G. Hollingshead, J. M. Covey, J. Bigelow, J. J. McCormack, R. W. Buckheit, Jr., D. J. Clanton, J. A. Turpin, W. G. Rice, *J. Med. Chem.* **1998**, *41*, 2184–2193.
[24] H. Tamamura, Y. Xu, T. Hattori, X. Zhang, R. Arakaki, K. Kanbara, A. Omagari, A. Otaka, T. Ibuka, N. Yamamoto, H. Nakashima, N. Fujii, *Biochem. Biophys. Res. Commun.* **1998**, *253*, 877–882.
[25] H. Tamamura, A. Omagari, S. Oishi, T. Kanamoto, N. Yamamoto, S. C. Peiper, H. Nakashima, A. Otaka, N. Fujii, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2633–2637.
[26] N. Fujii, S. Oishi, K. Hiramatsu, T. Araki, S. Ueda, H. Tamamura, A. Otaka, S. Kusano, S. Terakubo, H. Nakashima, J. A. Broach, J. O. Trent, Z. Wang, S. C. Peiper, *Angew. Chem.* **2003**, *115*, 3373–3375; *Angew. Chem. Int. Ed.* **2003**, *42*, 3251–3253.
[27] K. Ichiyama, S. Yokoyama-Kumakura, Y. Tanaka, R. Tanaka, K. Hirose, K. Bannai, T. Edamatsu, M. Yanaka, Y. Niitani, N. Miyano-Kurosaki, H. Takaku, Y. Koyanagi, N. Yamamoto, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 4185–4190.
[28] H. Tamamura, N. Fujii, *Curr. Drug Targets-Infectious Disorders* **2004**, *4*, 103–110.
[29] H. Tamamura, K. Hiramatsu, S. Ueda, Z. Wang, S. Kusano, S. Terakubo, J. O. Trent, S. C. Peiper, N. Yamamoto, H. Nakashima, A. Otaka, N. Fujii, *J. Med. Chem.* **2005**, *48*, 380–391.
[30] H. Tamamura, T. Araki, S. Ueda, Z. Wang, S. Oishi, A. Esaka, J. O. Trent, H. Nakashima, N. Yamamoto, S. C. Peiper, A. Otaka, N. Fujii, *J. Med. Chem.* **2005**, *48*, 3280–3289.
[31] G. C. Valks, G. McRobbie, E. A. Lewis, T. J. Hubin, T. M. Hunter, P. J. Sadler, C. Pannecouque, E. De Clercq, S. J. Archibald, *J. Med. Chem.* **2006**, *49*, 6162–6165.
[32] W. Zhan, Z. Liang, A. Zhu, S. Kurtkaya, H. Shim, J. P. Snyder, D. C. Liotta, *J. Med. Chem.* **2007**, *50*, 5655–5664.
[33] A. Khan, G. Nicholson, J. Greenman, L. Madden, G. McRobbie, C. Pannecouque, E. De Clercq, R. Ullom, D. L. Maples, R. D. Maples, J. D. Silversides, T. J. Hubin, S. J. Archibald, *J. Am. Chem. Soc.* **2009**, *131*, 3416–3417.
[34] G. J. Bridger, R. T. Skerlj, P. E. Hernandez-Abad, D. E. Bogucki, Z. Wang, Y. Zhou, S. Nan, E. M. Boehringer, T. Wilson, J. Crawford, M. Metz, S. Hatse, K. Princen, E. De Clercq, D. Schols, *J. Med. Chem.* **2010**, *53*, 1250–1260.

[35] R. T. Skerlj, G. J. Bridger, A. Kaller, E. J. McEachern, J. B. Crawford, Y. Zhou, B. Atsma, J. Langille, S. Nan, D. Veale, T. Wilson, C. Harwig, S. Hatse, K. Princen, E. De Clercq, D. Schols, *J. Med. Chem.* **2010**, *53*, 3376–3388.

[36] M. Takenaga, H. Tamamura, K. Hiramatsu, N. Nakamura, Y. Yamaguchi, A. Kitagawa, S. Kawai, H. Nakashima, N. Fujii, R. Igarashi, *Biochem. Biophys. Res. Commun.* **2004**, *320*, 226–232.

[37] H. Tamamura, A. Ojida, T. Ogawa, H. Tsutsumi, H. Masuno, H. Nakashima, N. Yamamoto, I. Hamachi, N. Fujii, *J. Med. Chem.* **2006**, *49*, 3412–3415.

[38] T. Tanaka, H. Tsutsumi, W. Nomura, Y. Tanabe, N. Ohashi, A. Esaka, C. Ochiai, J. Sato, K. Itotani, T. Murakami, K. Ohba, N. Yamamoto, N. Fujii, H. Tamamura, *Org. Biomol. Chem.* **2008**, *6*, 4374–4377.

[39] G. J. Bridger, R. T. Skerlj, D. Thornton, S. Padmanabhan, S. A. Martellucci, G. W. Henson, M. J. Abrams, N. Yamamoto, K. De Vreese, R. Pauwels, E. De Clercq, *J. Med. Chem.* **1995**, *38*, 366–378.

[40] G. J. Bridger, R. T. Skerlj, S. Padmanabhan, S. A. Martellucci, G. W. Henson, M. J. Abrams, H. C. Joao, M. Witvrouw, K. De Vreese, R. Pauwels, E. De Clercq, *J. Med. Chem.* **1996**, *39*, 109–119.

[41] Y. Inouye, T. Kanamori, T. Yoshida, T. Koike, M. Shionoya, H. Fujioka, E. Kimura, *Biol. Pharm. Bull.* **1996**, *19*, 456–458.

[42] L. O. Gerlach, J. S. Jakobsen, K. P. Jensen, M. R. Rosenkilde, R. T. Skerlj, U. Ryde, G. J. Bridger, T. W. Schwartz, *Biochemistry* **2003**, *42*, 710–717.

[43] H. F. Egberink, E. De Clercq, A. L. Van Vliet, J. Balzarini, G. J. Bridger, G. Henson, M. C. Horzinek, D. Schols, *J. Virol.* **1999**, *73*, 6346–6352.

[44] M. Le Baccon, F. Chuburu, L. Toupet, H. Handel, M. Soibinet, I. Dechamps-Olivier, J.-P. Barbier, M. Aplincourt, *New J. Chem.* **2001**, *25*, 1168–1174.

[45] B. Antonioli, D. J. Bray, J. K. Clegg, K. Gloe, K. Gloe, O. Kataeva, L. F. Lindoy, J. C. McMurtrie, P. J. Steel, C. J. Sumby, M. Wenzel, *Dalton Trans.* **2006**, 4783–4794.

[46] S. P. Foxon, D. Utz, J. Astner, S. Schindler, F. Thaler, F. W. Heinemann, G. Liehr, J. Mukherjee, V. Balamurugan, D. Ghosh, R. Mukherjee, *Dalton Trans.* **2004**, 2321–2328.

[47] S. Mandal, F. Lloret, R. Mukherjee, *Inorg. Chim. Acta* **2009**, *362*, 27–37.

[48] M. Soibinet, I. De'champs-Olivier, E. Guillon, J.-P. Barbier, M. Aplincourt, F. Chuburu, M. Le Baccon, H. Handel, *Eur. J. Inorg. Chem.* **2003**, 1984–1994.

[49] R. W. Hay, M. T. Tarafder, *Transition Met. Chem.* **1990**, *15*, 490–492.

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