[Chem. Pharm. Bull. 35(1) 256—261 (1987)]

Photoaffinity Labeling of a δ-Receptor Component of NG 108-15 Cells with a New Enkephalin Analog[1)]

KUNIO OKUMURA, YASUMARU HATANAKA, HITOSHI NAKAYAMA and YUICHI KANAOKA*

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

(Received May 19, 1986)

A new leucine-enkephalin analog containing *p*-azidophenylalanine was synthesized. It binds to the δ-opioid receptors of NG 108-15 cells with high affinity. Iodination of the compound decreased the affinity by 5 times, but the iodinated derivative still retains significant binding activity to the cells. Photochemical reactions revealed that the derivative predominantly labeled a 58 kilodaltons (kDa) polypeptide in the cells (possibly a δ-receptor component).

Keywords——photoaffinity labeling; δ-opioid receptor; NG 108-15 cell; enkephalin analog; polypeptide

It has been well established, by pharmacological and by receptor-radioligand binding approaches, that the endogenous opioid peptides interact with at least three types of putative receptors (μ, δ, and κ).[2)] Relatively little is known, however, about the molecular constituents of such receptors, although several pioneering studies were made by solubilization and affinity purification[3-6)] and by affinity labeling.[7)] The photoaffinity labeling technique has been widely used in studies of a variety of biomolecules[8)] and has been quite useful in the case of

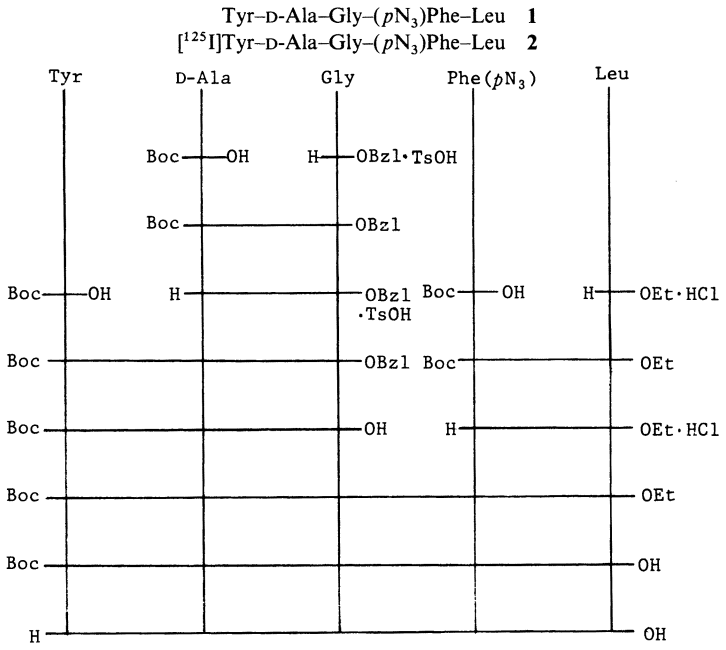

Chart 1. A Synthetic Route for Tyr-D-Ala-Gly-(*p*N$_3$)Phe-Leu

transmembrane receptor systems.[9] As an example of its application to the opioid receptor, we previously reported[10] an enkephalin analog photoaffinity reagent, [D-Ala^2, Leu^5]enkephalin *N*-[(2-nitro-4-phenyl)-amino]ethylamide, which labels opioid receptors of μ-type as well as δ-type, in accord with the findings[11,12] that carboxyl-terminal modification of leucine-enkephalin destroys the δ-selectivity to elicit rather a μ- and δ-preference. As a logical extension of that work, we describe here the synthesis of new photoactivable leucine-enkephalin analogs of carboxyl terminal-free ([D-Ala^2, (pN$_3$)Phe^4, Leu^5]enkephalin **1** and its iodinated derivative **2**) as well as their binding properties, and a successful covalent labeling of δ-receptor protein in a neuronal hybrid cell line (NG 108-15) with **2**.

Experimental

[D-Ala^2, (pN$_3$)Phe^4, Leu^5] **1** was synthesized by conventional solution methods employing (3+2) fragment condensation (Chart 1).

Boc-(pN$_3$)Phe-Leu-OEt (3)——Boc-(pN$_3$)Phe-OH[13,14] (3.06 g, 10 mmol) was dissolved in THF (10 ml) containing *N*-methylmorpholine (1.01 g, 10 mmol), and then BCC (1.37 g, 10 mmol) was added to the solution under cooling at -15 °C. After 10 min, the mixture was added to a chilled THF solution of Leu-OEt·HCl (1.96 g, 10 mmol) and *N*-methylmorpholine (1.01 g, 10 mmol) under stirring. The reaction mixture was stirred at -15 °C for 1 h and then at room temperature for 2 h. The mixture was concentrated *in vacuo* and the residue was dissolved in AcOEt; the AcOEt layer was washed successively with 10% citric acid, 4% $NaHCO_3$, and H_2O–NaCl, dried over Na_2SO_4 and then concentrated *in vacuo*. The residue was crystallized from Et_2O–pet. ether; yield 4.14 g (93%), yellowish prisms, mp 106—108 °C, $[\alpha]_D^{20}$ -6.2 ($c=1$, MeOH). *Anal.* Calcd for $C_{22}H_{35}N_5O_5$: C, 59.04; H, 7.43; N, 15.65. Found: C, 59.18; H, 7,43; N, 15.57. IR (Nujol): 3300, 2100, 1720 cm^{-1}.

Boc-Tyr-D-Ala-Gly-(pN$_3$)Phe-Leu-OEt (4)——Boc-(pN$_3$)Phe-Leu-OEt (**3**) (1.79 g, 4 mmol) was treated with 5 N HCl–AcOEt (4 ml, 20 mmol) for 4 h at room temperature. After the mixture was concentrated *in vacuo*, the residue was dried *in vacuo* over KOH pellets and dissolved in THF (15 ml) containing *N*-methylmorpholine (404 mg, 4 mmol) at -15 °C. By addition of Boc-Tyr-D-Ala-Gly-OH[15] (1.64 g, 4 mmol), peptide coupling was performed as in the case of the preparation of **3**, to give **4**, which was recrystallized from AcOEt–Et_2O; yield 2.66 g (90%), pale yellow powder, mp 112—114 °C (dec.), $[\alpha]_D^{20}$ +15.3 ($c=1$, MeOH). *Anal.* Calcd for $C_{36}H_{50}N_8O_9$: C, 58.52; H, 6.82; N, 15.17. Found: C, 58.38; H, 6.76; N, 15.09. IR (Nujol): 3300, 2130, 1730 cm^{-1}.

Tyr-D-Ala-Gly-(pN$_3$)Phe-Leu ([D-Ala^2, (pN$_3$)Phe^4, Leu^5]enkephalin (1)——The protected peptide **4** (738 mg, 1 mmol) was hydrolyzed with 1 N NaOH (2.1 ml) in MeOH for 1.5 h at room temperature. After the solution was concentrated *in vacuo*, 5 ml of water was added, followed by acidification with 10% citric acid. The product was extracted with AcOEt; the AcOEt layer was washed with H_2O–NaCl, dried over Na_2SO_4 and then concentrated *in vacuo*. The residue was treated with 2 N HCl–AcOEt (5 ml, 10 mmol) for 1 h at room temperature. After concentration *in vacuo*, the residual material was washed thoroughly with Et_2O, followed by column chromatography on silica using $CHCl_3$–MeOH (2:1) as an eluent; yield 520 mg (75%, recrystallized from 50% aqueous MeOH), pale-yellow fine needles, mp 172—175 °C (dec.), $[\alpha]_D^{20}$ +81.5 ($c=1$, H_2O). *Anal.* Calcd for $C_{29}H_{38}N_8O_7 \cdot 1/2H_2O$: C, 53.12; H, 6.61; N, 17.09. Found: C, 53.33; H, 6.31; N, 17.25. IR (Nujol): 3240, 3000, 2130 cm^{-1}.

Iodination of 1——Lactoperoxidase (1 μg, Boehringer #107174), Na[^{125}I] (1.6 mCi, 0.8 nmol; Amersham), and NaI (1.2 μg, 8 nmol) were added to a 100 μl solution of the enkephalin (**1**) (10 μg, 15 nmol), to make 134 μl of 0.1 M sodium acetate buffer (pH 5.6) in a polypropylene tube. Then 3 μl of H_2O_2 (0.02% solution) was added twice at 10 min intervals, and the mixture was allowed to stand for 10 min at room temperature. Monoiodinated derivative (**2**) was purified on a Biogel P-2 column (0.8 × 14 cm); it was eluted just after the non-iodinated **1** with 1 mM AcOH. The specific radioactivity was 115 μCi/nmol. Two other derivatives of **2** with lower radioactivity (14 μCi/nmol) and no radioactivity, were similarly prepared for use in binding experiments.

Cells——NG 108-15 neuroblastoma-glioma hybrid cells, a generous gift from Dr. T. Amano of Mitsubishi-Kasei Institute for Life Science, were grown as described.[16,17] The confluent cells were harvested in Dulbecco's phosphate-buffered saline [137 mM NaCl, 5.4 mM KCl, 7.76 mM Na_2HPO_4, 1.47 mM KH_2PO_4], followed by centrifugation at 100 × g for 5 min. The pellet was resuspended in a 25 mM Tris-HCl (pH 7.5) buffer containing 0.3 M sucrose (1—3 × 10^6 cells/ml), and used for experiments.

Binding Experiment in the Dark——(i) Competition Assay: One milliliter of cell suspension (3 × 10^6 cells) was incubated with 10 μl of [^{3}H]DADLE (40 Ci/mmol; NEN) at the fixed concentration of 2 nM in the presence of various concentrations of **1**. Bacitracin (0.1 mg/ml) was added to the incubation mixture and the whole was incubated at 25 °C for 30 min in the dark. Then 0.4 ml aliquots were removed (in duplicate) and filtered through Whatman GF/C filter paper under vacuum. The filter paper was quickly washed three times with 2 ml of ice-cold buffer (25 mM Tris–HCl, pH 7.5 containing 0.3 M sucrose), then transferred to a scintillation vial where the radioactivity was measured after mixing with 10 ml of scintillation cocktail (ACS-II; Amersham) and 1 ml of 10% Triton X-100. Nonspecific binding

was determined in the presence of a large excess (1 μM) of DALE.

ii) Direct Binding Assay of **2**: Various concentrations of **2** (14 μCi/nmol) were added to 1 ml of cell suspension (1.5×10^6 cells) with or without 1 μM of DALE. Bacitracin was included (0.1 mg/ml) and the sample was incubated at 25 °C for 30 min in the dark. Filtration (0.4 ml aliquots in duplicate) was performed as in (i) and the filter paper was counted in a Packard 5230 γ-counter. The specific binding of **2** to the cells was calculated from the difference between total binding and nonspecific binding in the absence and presence of DALE, respectively.

Photolabeling of Cells——After preincubation of cell suspension (9×10^5 cells/ml) with 28 nM **2** (115 μCi/nmol) in the presence of bacitracin (0.1 mg/ml) at 25 °C for 15 min, the mixture was placed on ice and irradiated with a 100 W low-pressure Hg lamp at a 15 cm distance for 2 min. The irradiated sample was then transferred into a 1.5 ml polypropylene tube and centrifuged at $150 \times g$ for 5 min. After three cycles of washing and centrifuging, the pellet was solubilized with 250 μl of solubilizing solution [3% SDS, 62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 0.75%DTT] at 100 °C for 5 min and subjected to gel electrophoresis. As a control, nonspecific photolabeling was carried out similarly except for the addition of DALE (10 μM) to the preincubation mixture.

Gel Electrophoresis——SDS-polyacrylamide gel electrophoresis was performed by a modification of the reported method.[18] Disc gels (0.7×13 cm) were prepared as 10% polyacrylamide and the solubilized samples (250 μl) described above were applied to upper gels of 3% polyacrylamide. After electrophoresis at 1.5 mA/tube, the gels were stained in 0.25% Coomassie blue R-250, 50%MeOH and 10% AcOH for 1 h, then destained in 25% 2-propanol and 10% AcOH. The gels were sliced at 1 mm thickness and each slice was counted in a Packard γ-counter. Protein molecular weight on the gel was estimated by the use of standard proteins (BioRad) simultaneously electrophoresed.

Protein Concentration——Protein concentration was determined by Peterson's modification[19] of the Lowry method, with bovine serum albumin as standard.

Results and Discussion

Photolysis of 1

The photoactivable enkephalin analog **1** has an absorption maximum at 252 nm ($\varepsilon = 15500$). When **1** (23 μM) was photolyzed with a 100 W low-pressure Hg lamp (which has its main output near 254 nm), the absorption peak at 252 nm disappeared after 1 min at 0 °C (Fig. 1). Under the irradiation conditions, more than 95% of the [^{3}H]DADLE binding activity of NG 108-15 cells was retained in the absence of **1**, and we concluded that this irradiation essentially did not affect the enkephalin binding activity of the cells.

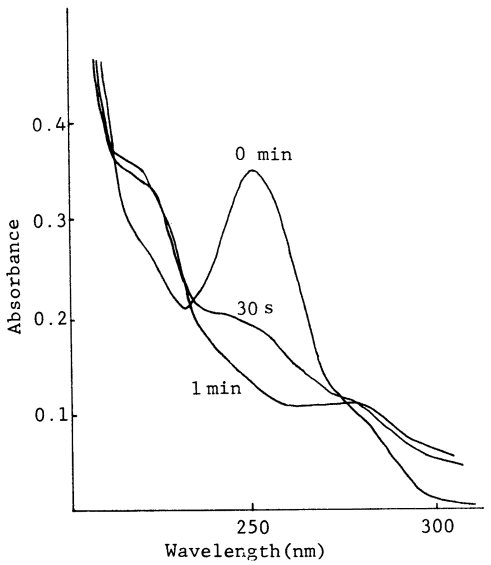

Fig. 1. Absorption Spectra of **1** before and after Photolysis

Compound **1** (23 μM) in water was photolyzed with a 100 W low-pressure Hg lamp.

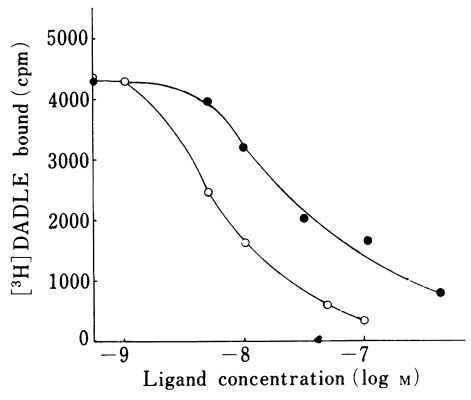

Fig. 2. Displacement Curves of **1** (○) and Its Non-radioactively Iodinated Derivative (●), Using 2 nM [^{3}H]DADLE as a Radioligand

Binding of 1 and Its Iodinated Derivatives to Cultured NG 108-15 Cells in the Dark

Binding of **1** to the opioid receptors of NG 108-15 cells is of high affinity ($IC_{50}=5.4$ nM) and is competitive with respect to [^{3}H]-DADLE (Fig. 2). The K_d value was calculated as 3.0 nM from the following equation[20]:

$$IC_{50}=K_d\,(1+[L]/K_L)$$

where $IC_{50}=5.4$ nM, [L] represents the concentration of [^{3}H]DADLE used (2 nM) and K_L is the dissociation constant of [^{3}H]DADLE (2.5 nM, obtained from a separate experiment; this agrees well with the value reported by Gerber *et al.*[16]). The K_d obtained was very close to that of DALE,[21] which indicates that the introduction of an azido group into the Phe^4 of DALE does not much change its binding affinity to the cells. Although iodination of **1** decreases the affinity by 5 times ($IC_{50}=29$ nM, therefore $K_d=16$ nM by calculation), the iodinated **1** still retains binding activity (Fig. 2). This was confirmed by using **2**, a radioiodinated derivative of **1**, in a direct binding assay. Figure 3 shows that **2** binds to the cells in a saturable manner. A Scatchard plot of the specific binding (Fig. 4) is linear, suggesting homogeneous binding of compound **2** to the cells, and values of $K_d=12$ nM and $B_{max}=316$ fmol/mg of protein were obtained. The former value is close to the affinity estimated above in the competitive binding assay, and the latter agrees well with the reported value for the cell line.[16,17] Taken together, these properties suggest that **1** and **2** are promising reagents for photolabeling the opioid receptors in NG 108-15 cells.

δ-Receptor Component(s) Photolabeled with 2

After irradiation of the cultures NG 108-15 cells with 28 nM **2**, a sample was analyzed by disc SDS-polyacrylamide gel electrophoresis. As shown in Fig. 5a, a 58 kilodaltons (kDa) polypeptide was predominantly labeled in a covalent manner. The labeling was markedly suppressed in the presence of DALE (10 μM) during the irradiation (Fig. 5b). These results suggest that the labeled polypeptide is a receptor component in NG 108-15 cells. Broad bands between 35—20 kDa which are labeled in the absence of DALE might be candidates for the receptors claimed to be present in rat brain, based on the results of affinity chromatography.[4,5] Occasionally, however, labeling of these bands was not observed. We cannot yet conclude that these smaller polypeptides are also receptor components. NG 108-15 cells are

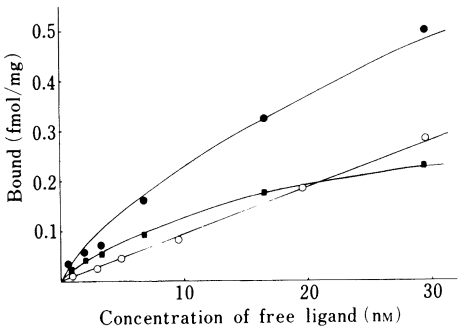

Fig. 3. Saturation Assay for the Binding of **2** to NG 108-15 Cells

Specific binding (■) was obtained by subtracting nonspecific (○) from total (●) binding.

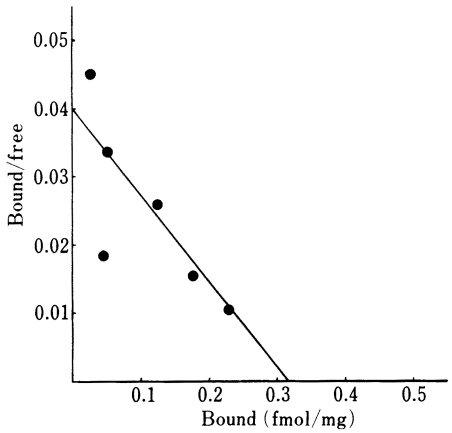

Fig. 4. Scatchard Analysis of the Specific Binding

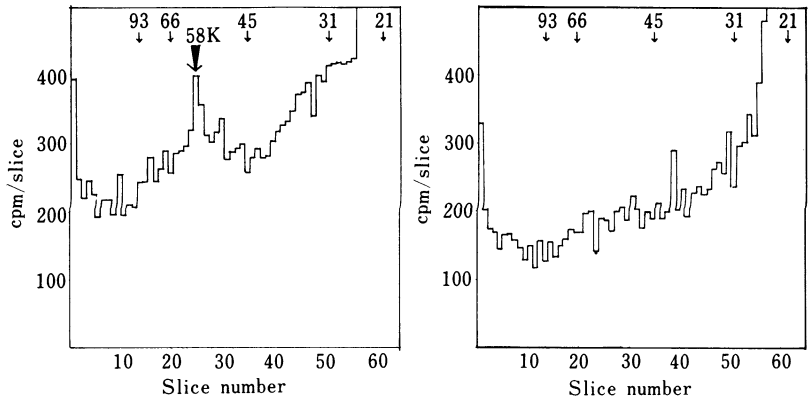

Fig. 5. Distribution of Radioactivity Following SDS Gel Electrophoresis of NG 108-15 Cells Photolyzed with **2** in the Absence (a) and Presence (b) of 10 μM DALE

known to carry only a single type of opioid receptor, with δ-subtype characteristics.[22] The nonradioactive counterpart of **2** binds to the cell line in a competitive manner with respect to the typical δ-ligand, DADLE, as shown in Fig. 3, and the photolabeling with **2** was effectively prevented by DALE. Therefore, the labeled polypeptide (58 kDa) here is a strong candidate for the ligand-binding component of δ-receptor in the cells. Although Klee and his colleagues reported that an opiate (fentanyl) analog labeled a similar 58 kDa protein in the same cell line,[23] our observation is the first identification of the opioid receptor component(s) of the cells by using an enkephalin analog which is structurally similar to the endogenous ligand, leucine-enkephalin.

It was reported that a 58 kDa protein from rat brain was also affinity-labeled by an enkephalin analog with μ- and δ-preference.[12] The authors claimed the labeled 58 kDa protein is a component of μ-receptor. It is an intriguing question whether the 58 kDa protein carries functional sites of both μ- and δ-receptors in rat brain. It should be possible to assess what size of protein(s) is labeled as a δ-receptor component(s) of rat brain by using the δ-directed reagent described here.

Acknowledgement This work was supported in part by Grants-in-Aid for Scientific Research (58105003, 58470116) from the Ministry of Education, Science and Culture, Japan, and grants from Sankyo Foundation of Life Science and from Suzuken Memorial Foundation.

References and Notes

1) Photoaffinity Labeling. V. Part IV: H. Nakayama, E. Yoshida and Y. Kanaoka, *Chem. Pharm. Bull.*, **34**, 2486 (1986).
2) *a*) R. S. Zukin and S. R. Zukin, *Trends NeuroSci.*, **7**, 160 (1984); *b*) M. Nozaki, M. Niwa, J. Hasegawa, E. Imai, M. Hori and H. Fujimura, *Life Sci.*, **31**, 1399 (1982).
3) W. F. Simons, G. Koski, R. A. Streaty, L. M. Hjelmedland and W. A. Klee, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 4623 (1980).
4) J. M. Bidlack, L. G. Abood, P. Osei-Gylmah and S. Archer, *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 636 (1981).
5) R. Maneckjee, R. S. Zukin, S. Archer, J. Michael and P. Osei-Gylmah, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 594 (1985).
6) T. Fujioka, F. Inoue and M. Kuriyama, *Biochem. Biophys. Res. Commun.*, **131**, 640 (1985).
7) For a review: A. E. Takemori and P. S. Portoghese, *Ann. Rev. Pharmacol. Toxicol.*, **25**, 193 (1985).
8) *a*) H. Nakayama and Y. Kanaoka, *J. Syn. Org. Chem.* (in Japanese), **40**, 912 (1982); *b*) H. Bayley, "Photogenerated Reagents in Biochemistry and Molecular Biology," Elsevier, Amsterdam, 1983.
9) *a*) H. Nakayama, E. Yoshida and Y. Kanaoka, *Chem. Pharm. Bull.*, **34**, 2486 (1986); *b*) A. E. Ruoho, A.

Rashidbaigi and P. E. Roehder, "Receptors: Biochemistry and Methodology," Vol. 1, ed. by J. C. Venter and L. C. Harrison, Alan R. Liss, New York, 1984, p. 119.
10) T. Fujioka, T. Matsunaga, H. Nakayama, Y. Kanaoka, Y. Hayashi, K. Kangawa and H. Matsuo, *J. Med. Chem.*, **27**, 836 (1984).
11) H. W. Kosterlitz and S. J. Peterson, *Proc. R. Soc. Lond. B*, **210**, 113 (1980).
12) E. L. Newman and E. Barnard, *Biochemistry*, **23**, 5385 (1984).
13) The following abbreviations are used: Boc = *tert*-butyloxycarbonyl, pN$_3$Phe = *p*-azidophenyl alanyl, THF = tetrahydrofuran, BCC = isobutylchloroformate, DALE = [D-Ala^2,Leu^5]enkephalin, DADLE = [D-Ala^2,D-Leu^5]enkephalin, SDS = sodium dodecylsulfate. All the amino acids used in this paper are of L-configuration except where otherwise noted.
14) R. Schwyer and M. Caviezel, *Helv. Chim. Acta*, **54**, 395 (1971).
15) J. Pless, W. Bauer, F. Cardinax, A. Closse, D. Hauser and R. Huguenin, *Helv. Chim. Acta*, **62**, 398 (1979).
16) L. P. Gerber, S. Stein, M. Rubinstein, J. Wideman and S. Udenfriend, *Brain Res.*, **151**, 117 (1978).
17) W. A. Klee and M. Nirenberg, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 3474 (1974).
18) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
19) G. L. Peterson, *Anal. Biochem.*, **83**, 346 (1977).
20) Y.-C. Cheng and W. H. Prusoff, *Biochem. Pharmacol.*, **22**, 3099 (1973).
21) K.-J. Chang, R. J. Miller and P. Cuatrecasas, *Mol. Pharmacol.*, **14**, 961 (1978).
22) K.-J. Chang and P. Cuatrecasas, *J. Biol. Chem.*, **254**, 2610 (1979).
23) *a*) W. A. Klee, W. F. Simonds, F. W. Sweat, T. R. Burke, Jr., A. E. Jacobson and K. C. Rice, *FEBS Lett.*, **150**, 125 (1982); *b*) W. F. Simonds, T. R. Burke, Jr., K. C. Rice, A. E. Jacobson and W. A. Klee, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 4974 (1985).