Chapter V

"Symmetric Covalent Linkage of W-7 Results in Novel Derivatives with Increased Inhibitory Activities against Ca^{2+}/CaM Complex"

V-1 Summary

A useful calmodulin (CaM) antagonist, *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7), was invented by Hidaka *et al.* in 1978 (Hidaka *et al.*, 1978). Here, I have designed new CaM antagonists on the basis of the three-dimensional structure of Ca^{2+}/CaM complexed with W-7. Ten new compounds all share a similar architecture, in which two W-7 molecules are linked between the aminohexyl termini via a linker with different functionalities. A wide range of inhibitory activities against Ca^{2+}/CaM-dependent protein kinase I (CaMKI) has been observed with these self-crosslinked W-7 analogs, $(W\text{-}7)_2$. In vitro competitive CaMKI assays using CaMK-I and NMR studies indicated that one molecule of $(W\text{-}7)_2$ binds to one CaM molecule, with the each chloronaphthalene rings of $(W\text{-}7)_2$ anchored to the N and C-terminal hydrophobic pockets of Ca^{2+}/CaM, respectively. The most potent compound, *N,N'*-bis[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexyl]-*p*-xylene-diamine inhibits CaMKI activity at an IC_{50} value of 0.23 μM; about 75 times more effectively than W-7. The property of the linker sequence in these compounds significantly contributes to inhibitory activity. The present study opens an avenue for developing powerful CaM antagonists that could be used at low doses in vivo.

V-2 Introduction

The significance of CaM in diverse intracellular Ca^{2+}-signaling systems has been recognized in pharmacological studies. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), developed by Hidaka *et al.* (Hidaka *et al.*, 1978), is one of the most widely used CaM antagonists (Hidaka & Yokokura, 1996). However, W-7 binds to Ca^{2+}/CaM much more weakly ($K_d = 1.1 \times 10^{-5}$ M) than target enzymes ($K_d = \sim 1.0 \times 10^{-9}$ M) (Hidaka *et al.*, 1978; Meyer *et al.*, 1992), and this necessitates the use of W-7 in relatively high concentrations for both in vitro and in vivo studies. The IC_{50} values of W-7 for in vitro Ca^{2+}/CaM-dependent activities of PDE type I and smooth muscle myosin light chain kinase (smMLCK) were 7.10 ± 1.92 μM and 36.7 ± 3.8 μM, respectively, while CaM could activate the enzymes at 3 nM or less (Yokokura *et al.*, 1996). In the past, laborious efforts have been made to identify new synthetic compounds with improved binding affinities and inhibitory activities to Ca^{2+}/CaM (MacNeil *et al.*, 1988; Yokokura *et al.*, 1996).

In chapter II and III, I analyzed the local and global structure of Ca^{2+}/CaM complexed with W-7 by NMR (Osawa *et al.*, 1998) and SAXS. The structure consists of one W-7 molecule binding to each of the N and C-terminal domains of Ca^{2+}/CaM. The chloronaphthalene ring in W-7 is deeply inserted into a hydrophobic pocket in each domain of Ca^{2+}/CaM, while its aminohexyl group is not included in the specific interaction with Ca^{2+}/CaM, and appears to be highly flexible. Interestingly, Ca^{2+}/CaM adopts a compact globular conformation upon binding of two W-7 molecules although the relative orientation of both CaM domains may not be firmly fixed. Based on this structural information, I have designed the covalently crosslinked derivatives of W-7, $(W\text{-}7)_2$, in which the aminohexyl termini of two W-7 molecules are covalently linked via various linkers. This is analogous to the 'linked fragment approach' (Christophe *et al.*, 1992) and a more recently introduced application referred to as 'structure-activity relationship (SAR) by NMR'

(Hadjuk *et al.*, 1997; Olejniczak *et al.*, 1997; Shuker *et al.*, 1996).

The concept of the present design is explained as below. The binding constant, K, is an exponential function of the binding energy, ΔG, i.e. $K = \exp(\Delta G / R \cdot T)$, where R and T are the gas constant and the temperature, respectively. This formula shows that the increase in ΔG by linking two W-7 molecules should lead to the compound with an exponentially increased inhibitory activity. When two molecules of free W-7 bind to Ca^{2+}/CaM, the loss of their entropies should be nearly twice that of the binding of one molecule of covalently linked compound to Ca^{2+}/CaM, since the number of binding molecule is twice. Due to this difference in entropy loss, the linked compound could gain more binding energy corresponding to the entropy loss on the binding of second W-7 molecule to Ca^{2+}/CaM, which leads to the increase of its ΔG more than two W-7 molecules.

Although it seems difficult to estimate the changes in ΔG, the discussion of the effective concentration provides estimates of how much the affinity to Ca^{2+}/CaM should be increased (Figure V-1). By tethering two 5-chloro-1-naphthalenesulfonamide moieties within a diameter of r, they should be found within the volume, $V = 4\pi r^3/3$. When the first moiety of $(W\text{-}7)_2$ is bound to Ca^{2+}/CaM, the effective concentration, C_{eff}, of the second moiety is formulated as $C_{eff} = (V \cdot N_A)^{-1}$, where N_A is Avogadro's constant. In the case of a compound with r = 30 Å, which is actually the case of the compound **4** in Table V-1,

$K_2 = [Ca^{2+}/CaM] \cdot [\text{first W-7}] \cdot [\text{second W-7}] / [Ca^{2+}/CaM - 2 \cdot (W\text{-}7)]$,

where [first W-7] and [second W-7] are the concentrations of free W-7 and essentially the same as each other. In the case where $(W\text{-}7)_2$ binds to Ca^{2+}/CaM, [second W-7] corresponds to C_{eff}. Thus, the affinity of the compound should be increased by a factor of C_{eff} / [second W-7], which is estimated to be 10^3, suggesting that it may be possible to obtain a potent CaM antagonist with an affinity of 10^{-8} M (Hidaka *et al.*, 1978).

Thus, I designed ten analogs of $(W\text{-}7)_2$ with a series of linker sequences, which were synthesized and biochemically tested. One of them is found to be 75 times more effective against Ca^{2+}/CaM than W-7. The interactions of these compounds with Ca^{2+}/CaM are investigated by in vitro competitive CaMKI assay and NMR spectroscopy. A possible interaction model is proposed, and the structure-activity relationship of the new compounds is discussed.

V-3 Materials and Methods

V-3-1 CaM Purification

Frozen bovine brain was thawed overnight at 4 °C and homogenized in buffer H (50 mM sodium phosphate (pH 5.7), 5 mM EDTA, and 100 mM phenylmethylsulfonyl fluoride) with a home mixer. The extract was centrifuged at 13,000 x g for 30 min, and 50 %(w/v) trichloroacetic acid (TCA) was added to the supernatant to a final concentration of 3%, followed by stirring for 10 min. Other centrifugations were also run similarly unless indicated otherwise. The suspension was adjusted to a pH value of 5.2 by adding appropriate droplets of 6 M NaOH and stirred for 1 hr. Following centrifugation, 50 % TCA was added to the supernatant again to a final concentration of 3 %. The suspension was stirred for 10 min and centrifuged for another 10 min. The pellet was resuspended in 50 ml of 1 M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), followed by dialysis against buffer A containing 50 mM sodium phosphate (pH 5.7), 0.1 M NaCl, and 1 mM EDTA. Following centrifugation at 13,000 x g for 10 min, the obtained supernatant was mixed with anion-exchange DE-52 celluloses (Pharmacia LKB Biotech., Inc.) previously equilibrated with buffer A. The slurry was stirred for 1 hr, and resin was packed into a column (3.0 x 30 cm). The column was washed with buffer A and subjected to a linear NaCl gradient to 0.4 M. Proteins in the eluate were analyzed by sodium dodecyl sulfate-10 % polyacrylamide gel electrophoresis (Leammli, 1970). CaM was eluted from the column around a point of 0.28 M NaCl (data not shown). To a pool of the CaM-containing fractions, 50 % TCA was once again added to a final concentration of 3 %. The suspension was centrifuged, and the pellet was dissolved in 6 ml of buffer B (20 mM Tris·HCl (pH 7.5), 0.2 M NaCl, and 1 mM EGTA) plus appropriate droplets of 1 M Tris base. Each half of the sample was separately loaded onto a HiLoad 26/60 Sephacryl S-300 HR column (2.6 x 60 cm, Pharmacia LKB Biotech., Inc.) and developed in buffer B at a flow rate of 1 ml/min with a P-

500 pump (Pharmacia LKB Biotech., Inc.). The eluate was monitored for its light absorption at a wavelength of 280 nm. Three protein peaks were observed on the chromatogram, and CaM was eluted in the last peak (data not shown). The CaM-containing fractions were pooled and dialyzed extensively against 10 mM ammonium bicarbonate. The sample was lyophilized and stored at -20 °C. All of the procedures described above were carried out at 4 °C. Proteins in the stored sample were analyzed by reverse-phased high-performance liquid chromatography (HPLC) on a Symmetry C8 column (3.9 x 150 mm, Millipore) using a linear gradient from 90 % solvent A (0.1 %(v/v) trifluoroacetic acid (TFA) and 10 %(v/v) acetonitrile) and 10 % solvent B (0.08 % TFA and 80 % acetonitrile) to 20 % solvent A and 80 % solvent B. Only one peak with an absorbance of 215 nm light was eluted at a point of 49 % acetonitrile (data not shown) and manually collected. This peak was confirmed to correspond to CaM by mass spectrometry (see "Mass spectrometry measurements").

V-3-2 Chemical Synthesis of the $(W\text{-}7)_2$ Derivatives

N,N'-bis[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexanoyl]-*p*-xylenediamine (**10**).

5-Chloro-1-naphthalenesulfonyl chloride (575 mg, 2.2 mmol) and triethyl amine (0.5 ml) were mixed with *N,N'*-bis(6-amino-1-hexanoyl)-*p*-xylenediamine (362 mg, 1 mmol) in pyridine (5 ml) on ice, and the whole was stirred for 30 min at room temperature. The reaction was stopped by pouring the mixture into a saturated NaCl solution, and products were extracted with a sufficient volume of ethyl acetate. The organic extract was washed extensively with the brine and evaporated to dryness under reduced pressure. The residue was subjected to a preparative liquid chromatography on a column of the silica gel, BW-820MH (Fuji Silysia Chemical, Ltd., Tokyo), using an

elution of chloroform-methanol (100:3). The target diamide was finally obtained as 365 mg of white powder. Other diamides were also prepared by almost the same procedures as described here. ^{1}H-NMR δ: 1.14~1.26(4H, m), 1.27~1.40(4H, m), 1.42~1.54(4H, m), 2.08(4H, t, J = 7.0 Hz), 2.84(4H, q, J = 6.0 Hz), 4.38(4H, d, J = 6.0 Hz), 5.29(2H, t, J = 6.0 Hz), 6.17(2H, brt, J = 6.0 Hz), 7.24(2H, s), 7.50(2H, dt, J = 8.5 Hz), 7.63(2H, dt, J = 7.5 Hz), 7.67(2H, d, J = 7.5 Hz), 8.28(2H, dd, J = 7.5 Hz), 8.55(2H, dd, J = 8.5 Hz), 8.59(2H, dd, J = 8.5 Hz).

N,N'-bis[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexanoyl]-1,4-diaminobutane (**7**).

^{1}H-NMR δ: 1.20~1.35(4H, m), 1.35~1.50(4H, m), 1.50~1.65(8H, m), 2.10(4H, t, J = 7.0 Hz), 2.90(4H, q, J = 6.0 Hz), 3.28(4H, br), 5.85(2H, brt, J = 6.0 Hz), 6.13(2H, brt, J = 6.0 Hz), 7.52(2H, t, J = 8.5 Hz), 7.64(2H, t, J = 8.5 Hz), 7.67(2H, d, J = 8.5 Hz), 8.29(2H, dd, J = 8.5 Hz), 8.56(2H, d, J = 8.5 Hz), 8.66(2H, d, J = 8.5 Hz).

N,N'-bis[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexanoyl]-1,6-diaminohexane (**8**).

^{1}H-NMR δ: 1.16~1.34(8H, m), 1.34~1.50(12H, m), 2.06(4H, t, J = 7.0 Hz), 2.88(4H, dt, J = 6.4 Hz), 3.19(4H, q, J = 6.5 Hz), 5.95(2H, t, J = 6.0 Hz), 6.09(2H, brt, J = 6.0 Hz), 7.59(2H, dt, J = 8.5 Hz), 7.62(2H, dt, J = 7.2 Hz), 7.65(2H, dd, J = 7.2 Hz), 8.28(2H, dd, J = 7.2 Hz), 8.54(2H, d, J = 8.5 Hz), 8.65(2H, d, J = 8.5 Hz).

N,N'-bis[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexanoyl]-*p*-phenylenediamine (**9**).

^{1}H-NMR (d_6-DMSO) δ: 1.11~1.22(4H, m), 1.27~1.45(8H, m), 2.13(4H,

t, J = 7.0 Hz), 2.79(4H, q, J = 6.0 Hz), 7.45(4H, s), 7.70(2H, dt, J = 8.5 Hz), 7.82(2H, dt, J = 7.5 Hz), 7.86(2H, d, J = 7.5 Hz), 8.06(2H, t, J = 6.0 Hz), 8.23(2H, dd, J = 7.5 Hz), 8.50(2H, d, J = 8.5 Hz), 8.68(2H, d, J = 8.5 Hz), 9.71(2H, s).

N,N'-bis[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexyl]-*p*-xylenediamine (**4**).

A borane-methyl sulfide complex (543 mg, 7 mmol) was added to an ice-cold solution of *N,N'*-bis[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexanoyl]-*p*-xylene-diamine (290 mg, 0.36 mmol) in tetrahydrofuran (5 ml), and the mixture was stirred for 12 hr at room temperature. To destroy the excess of borane-methyl sulfide complex, a small amount of ice was added. Then, sodium bicarbonate (200 mg) was added to the mixture, followed by a reflux for 4 hr. Products were extracted with a sufficient volume of ethyl acetate, and the organic extract was evaporated to dryness under reduced pressure. The residue was subjected to a preparative thin-layer chromatography on a pre-coated glass plate with silica gel (0.5 mm thick, Merck) using a development of ethyl acetate-methanol (1:1). The diamine of interest was finally obtained as 60.3 mg of white powder. Other diamines were also prepared by almost the same procedures as described here. ^{1}H-NMR δ: 1.05~1.20(4H, m), 1.2~1.4(8H, m), 1.4~1.7(4H, m), 2.51(4H, t, J = 7.0 Hz), 2.89(4H, t, J = 7.0 Hz), 3.73(4H, s), 4.60~5.0(2H, br), 7.24 (4H, s), 7.55(2H, t, J = 8.5 Hz), 7.65(2H, t, J = 7.5 Hz), 7.69(2H, dd, J = 7.5 Hz), 8.30(2H, dd, J = 7.3 Hz, 1.0 Hz), 8.57(2H, d, J = 8.5 Hz), 8.62(2H, d, J = 8.5 Hz).

N,N'-bis[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexyl]-1,4-diaminobutane (**1**).

^{1}H-NMR ($CDCl_3$:CD_3OD = 3:1) δ: 1.13~1.35(8H, m), 1.35~1.50(4H, m), 1.50~1.70(4H, m), 1.70~1.90(4H, m), 2.55~2.75(4H, m), 2.80~2.95(8H,

m), 7.58(2H, t, J = 8.5 Hz), 7.60(2H, t, J = 8.0 Hz), 7.69(2H, d, J = 8.0 Hz), 7.70(2H, t, J = 8.5 Hz), 8.58(2H, d, J = 8.5 Hz), 8.66(2H, d, J = 8.5 Hz).

N,N'-bis[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexyl]-1,6-diaminohexane (**2**).

^{1}H-NMR ($CDCl_3$:CD_3OD = 5:1) δ: 1.15~1.85(24H, m), 2.60~2.75(4H, m), 2.80~2.95(8H, m), 7.58(2H, t, J = 7.2 Hz), 7.68(2H, t, J = 8.5 Hz), 7.70(2H, d, J = 7.2 Hz), 8.29(2H, dd, J = 7.2 Hz, 3.0 Hz), 8.56(2H, d, J = 8.5 Hz), 8.66(2H, d, J = 8.5 Hz).

N,N'-bis[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexyl]-*p*-phenylenediamine (**3**).

^{1}H-NMR δ: 1.10~1.25(8H, m), 1.3~1.45(8H, m), 2.87~3.01(8H, m), 6.53(4H, s), 7.53(2H, t, J = 7.5 Hz), 7.66(2H, d, J = 7.5 Hz), 7.68(2H, t, J = 8.5 Hz), 8.34(2H, d, J = 7.5 Hz), 8.58(2H, d, J = 8.5 Hz), 8.62(2H, d, J = 8.5 Hz).

N-[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexanoyl]-*N'*-[6-(5-chloro-1-naphthalene-sulfonyl)-amino-1-hexyl]-1,4-diaminobutane (**5**).

Borane-methyl sulfide complex (230 mg, 3.0 mmol) was added to an ice-cold solution of *N,N'*-bis[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexanoyl]-1,4-diamino-butane (120 mg, 0.157 mmol) in tetrahydrofuran (5 ml), and the mixture was stirred for 2 hr at room temperature. To destroy the excess of borane-methyl sulfide complex, a small volume of methanol was added. Products were extracted with a sufficient volume of chloroform. The organic extract was washed extensively with the brine and evaporated to dryness under reduced pressure. The residue was subject to a preparative thin-layer chromatography on a pre-coated glass plate with silica gel (0.5 mm thick, Merck) using a development of chloroform-methanol (10:1). The monoamine of interest was obtained as 25.0 mg of white powder. The other

monoamine was also prepared by almost the same procedures as described here. ^{1}H-NMR δ: 1.22~1.85(18H, m), 2.05(2H, brt, J = 6.0 Hz), 2.24(2H, brt, J = 6.0 Hz), 2.80~3.10(6H, m), 3.35(2H, brq, J = 6 Hz), 6.33(1H, brt, J = 6.0 Hz), 6.91(1H, brt, J = 6.0 Hz), 7.14(1H, brt, J = 6.0 Hz), 7.61(1H, t, J = 6.0 Hz), 7.63(1H, t, J = 8.0 Hz), 7.66~7.70(4H, m), 8.13(1H, d, J = 8.0 Hz), 8.14(1H, d, J = 8.0 Hz), 8.37(1H, t, J = 8.0 Hz), 8.38(1H, t, J = 8.0 Hz), 8.80~8.84(2H, m), 8.82(1H, br).

N-[6-(5-chloro-1-naphthalenesulfonyl)-amino-1-hexanoyl]-*N'*-[6-(5-chloro-1-naphthalene-sulfonyl)-amino-1-hexyl]-1,6-diaminohexane (**6**).

^{1}H-NMR δ: 1.20~1.58(10H, m), 1.73~1.85(2H, m), 1.85~1.95(2H, m), 2.15(2H, t, J = 6.5 Hz), 2.80~3.02(8H, m), 3.23(1H, brq, J = 6.5 Hz), 6.41(1H, t, J = 6.5 Hz), 6.49(1H, t, J = 6.5 Hz), 6.51(1H, t, J = 6.5 Hz), 7.51(2H, t, J = 9.0 Hz), 7.61(2H, d, J = 7.3 Hz), 7.63(2H, t, J = 7.3 Hz), 8.27(2H, dd, J = 7.3 Hz), 8.53(2H, d, J = 9.0 Hz), 8.68(2H, d, J = 9.0 Hz).

V-3-3 In Vitro Protein Kinase Assays

CaMKI, which was devoid of part of its C-terminal region, was expressed in *Eschericia coli* as a fusion protein with glutathione S-transferase and purified near homogeneity as described elsewhere (Yokokura *et al.*, 1995). It was kept for both the catalytic and regulatory regions of CaM kinase I and was found to exhibit almost the same Ca^{2+}/CaM-dependent activity as that of a wild-type enzyme (data not shown). The recombinant CaM kinase I was assayed in a final volume of 50 ml, containing 35 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid·NaOH (pH 7.5), 10 mM magnesium acetate, 1 mM dithiothreitol, 0.01 %(v/v) Tween-20, 50 mM syntide-2, plus either 1 mM EGTA or a set of 1 mM EGTA, 1.5 mM $CaCl_2$, and 100 nM CaM. Reactions

were started by adding [γ-^{32}P]ATP (Amersham Corp.) to a final concentration of 100 mM (41.5 cpm/pmol), and the mixtures were incubated for 10 min at 30 °C. ^{32}P incorporation into the peptide substrate, syntide-2, was determined by pipetting a 25-ml aliquot of the mixture onto P-81 phosphocelullose paper (2.3 cm in diameter, Whatman) as described elsewhere (Roskowski, 1983). The paper was given three 10-min washes with 75 mM phosphoric acid, dried, and quantified for its Cerenkov radiations with an LS6500 liquid scintillation counter (Beckman Instruments, Inc.).

V-3-4 Mass Spectrometry Measurements

These were performed on a Sciex API-III triple quadrupole mass spectrometer equipped with an ion-spray ionization interface (Takara Shuzo Ltd., Kyoto). Product ions were detected for the values of molecular masses divided by charge numbers (m/z), scanning a range of mass units from 800 to 2,000. Ion currents as a function of mass units were deconvoluted by the data processing package, BioMultiView (Takara Shuzo Ltd., Kyoto), to give a calculated mass spectrum of singly charged ions. The purified CaM produced a series of 11 significant ion current peaks, which could be assigned to the charged states of CaM by multiple protons from $20H^+$ to $10H^+$, with $(CaM + 16H)^{16+}$ and $(CaM + 17H)^{17+}$ being the most intense (data not shown). From all of the peaks, an average mass of CaM was found to be 16,791 Da, which was identical with the value estimated from its primary structure. Similar results have already been reported for rat and bovine CaM (Hu & Loo, 1995; Quadroni *et al.*, 1994). Quadroni *et al.* succeeded in the purification of non-phosphorylated and phosphorylated forms of CaM separately, and their average masses were determined to be 16,791 Da and 16,870 Da (as well as 16,952 Da and 17,032 Da), respectively, on mass spectra (Quadroni *et al.*, 1994). The sample was found to contain only the non-phosphorylated form of CaM.

V-3-5 NMR Spectroscopy

Uniformly ^{15}N-labeled recombinant *Xenopus laevis* CaM was expressed in E. coli and purified to homogeneity as previously described (Ikura *et al.*, 1990a). CaM was dissolved in 0.4 ml of an unbuffered 95 % H_2O/5 % D_2O solution containing 0.1 M KCl and 10.6 mM $CaCl_2$. The pH of the samples was 6.8 and the CaM concentration 1.5 mM. All the NMR spectra for protein solutions were measured at 35 °C on a Bruker AMX-600 spectrometer. The compound **2** was titrated into a CaM solution by successive addition of aliquots, each with an equivalent to one fourth of the protein in molar ratio. After the addition of each aliquot of **2**, one-dimensional ^1H and two-dimensional ^{15}N-^1H HSQC spectra (Kay *et al.*, 1992; Palmer III *et al.*, 1991) were acquired. Finally, spectra with 0, 0.25, 0.5, 0.75, and 1.0 equivalents of **2** to CaM were collected. Assignment of the backbone resonances of Ca^{2+}/CaM complexed with **2** was achieved by tracing the chemical shift changes in a series of ^{15}N-^1H HSQC spectra and further confirmed by ^{15}N-edited NOESY-HSQC spectra recorded at a 1:1 ratio. The titration of W-7 to CaM and assignment of the CaM - W-7 complex have already been reported (Osawa *et al.*, 1998). ^1H-NMR spectra of W-7 and all the synthesized compounds were measured in $CDCl_3$ solutions with an EX-270 spectrometer (Japan Electron Optic Laboratory, Tokyo). Tetramethyl silane was used as an internal standard in $CDCl_3$, and 3-(trimethylsiryl)propionate-2,2,3,3-d_4 in aqueous solutions.

V-3-6 Miscellaneous

W-7 was synthesized in bulk in the previous study (Hidaka *et al.*, 1978), carefully stored in Hidaka's laboratory, and part of used in this study. Proteins were determined by Bradford assay using bovine serum albumin as a standard (Bradford, 1976). Syntide-2 was synthesized with a 433A peptide synthesizer (Applied Biosystems Inc.), the chemistry of which was based on a

modification of the 9-fluorenylmethoxycarbonyl (Fmoc) methods (Knorr *et al.*,). Products were separated from the resin according to the manufacturer's instructions, and followed by preparative HPLC on the C_{18} column, μBONDASPHERE (1.9 x 15 cm, Millipore). The obtained syntide-2 was lyophilized and stored at -20 °C.

V-4 Results

V-4-1 Structures and Activities of the Novel $(W-7)_2$ Derivatives

'Homo-dimeric' derivatives of W-7, $(W-7)_2$, consist of two W-7 molecules covalently linked together at their terminal amino groups via a series of linker sequences including buthylene (**1**), hexylene (**2**), *p*-phenylene (**3**), and *p*-xylene (**4**) (Table V-1). For the latter four compounds, the structurally-related derivatives were further synthesized by substituting carbonyl group(s) for one of the two methylenes next to the joint amino groups (**5** and **6**) or both of them (**7**, **8**, **9**, and **10**) (Table V-1).

These compounds were examined for their ability to inhibit CaM action by in vitro CaMKI assay (Table V-1). The derivatives **1**, **2**, and **4** inhibited the Ca^{2+}/CaM-dependent activity at IC_{50} values of 0.34 ± 0.04 μM, 0.31 ± 0.06 μM, and 0.23 ± 0.03 μM, respectively, whereas **3** inhibited no more than ~15 % of it in high concentrations up to ~100 μM. Under the same assay conditions, W-7 inhibited the activity at an IC_{50} value of 17 ± 2 μM. Interestingly, **5** and **6** of the monoamine-monoamide type were slightly less potent than the corresponding analogs of the diamine type, **1** and **2**, respectively, and yet remained much more potent than W-7. However, the diamide type derivatives of them, **7** and **8**, were still less potent, but neither as potent as W-7. The compounds **9** and **10**, which are the diamide type ones of **3** and **4**, respectively, inhibited only 9 and 13 % of the activity even at a high concentration of 100 μM.

Next, the study was intended to explain the manner in which the most effective compound, **4**, inhibited the CaM action. In the presence of CaM at a fixed concentration of 0.3 μM, CaMKI was increasingly activated by the addition of free Ca^{2+} (Figure V-2a). The activity reached a plateau after the addition of ~0.1 mM free Ca^{2+}. The compound **4** inhibited the activity in a concentration-dependent manner, and this inhibition was not relieved by

further addition of Ca^{2+} (Figure V-2a). In the presence of Ca^{2+} at a fixed concentration of 0.5 mM, the enzyme was also activated by the addition of CaM, with its activation curve showing a sigmoidal relationship (Figure V-2b). The compound **4** inhibited the activity in a concentration-dependent manner as illustrated by a significant shift of the activation curve. Distinct from the results of the Ca^{2+}-titration, however, the same maximal activity was measured in the presence of 0~0.5 μM of the compound upon the addition of 1.5 mM CaM (Figure V-2b).

V-4-2 Comparison of the Ca^{2+}/CaM Interaction between $(W\text{-}7)_2$ and W-7

The binding properties of the compound **2** to Ca^{2+}/CaM were also examined by NMR spectral changes in Ca^{2+}/CaM. Figure V-3 shows a comparison of selected portions of ^{15}N-^{1}H HSQC spectra obtained with Ca^{2+}/CaM itself (Figure V-3a), and Ca^{2+}/CaM mixed with two equivalent amounts of W-7 (Figure V-3b), one equivalent amount of **2** (Figure V-3c) in molar ratio. Each cross peak means the correlation between ^{15}N and ^{1}H atoms of an amide group in the main-chain of uniformly ^{15}N-labeled CaM. This "fingerprint" NMR spectrum is sensitive to protein conformational changes induced by environmental changes such as pH, temperature, and interaction with other molecules. When W-7 was added to the Ca^{2+}/CaM solution, a number of signals of both domains of Ca^{2+}/CaM were shifted due to the binding of W-7 to both CaM domains (Figures V-3a and b). The spectrum of Ca^{2+}/CaM mixed with **2** at a molar ratio of one to one coincided with that of the Ca^{2+}/CaM - W-7 mixture at a molar ratio of one to two with respect to the chemical shift and line width of each signal (Figures V-3b and c). This result strongly suggests that one molecule of **2** binds to both the N and C-terminal domains of one Ca^{2+}/CaM molecule, where each of the two chloronaphthalene rings in **2** is very similar to that of W-7 interacting with each domain of Ca^{2+}/CaM.

According to these results, the most likely binding model of **2** is depicted in Figure V-3.

V-4 Discussion

Although the high inhibitory activity of ~10^{-8} M has been expected as mentioned above, a wide range of IC_{50} values was observed with $(W\text{-}7)_2$ derivatives (Table V-1), showing that the covalent linkage may cause additional effects on the binding such as novel interactions between linker and protein, as well as an entropy loss due to constraining the conformational flexibility of the CaM domains. Thus, the binding energy and the inhibitory activity of the novel compounds depend strongly on the linker; and optimizing the linkers based on the mode of their interactions with Ca^{2+}/CaM, by, for example, using Strucutre-Activity-Relationship data, is essential for obtaining better CaM antagonists.

First of all, the diamine type derivatives, **1**, **2**, and **4** inhibited the activity ~75 times more effectively than the original compound, W-7 (Table V-1). These compounds all have two W-7 molecules in their structure. Although the compound **3** also has two W-7 molecules in its structure, the basicity of a nitrogen atom attached to an aromatic ring is much less than when to an alkyl group. The compound, **4**, clearly inhibited Ca^{2+}/CaM-binding to CaM kinase I (Figure V-2). In addition, the NMR spectrum of Ca^{2+}/CaM complexed with the compound **2** at a molar ratio of 1:1 was similar to that of Ca^{2+}/CaM bound with two W-7 molecules (Figures V-3b and c). These observations suggest that all three compounds, as expected, bind to Ca^{2+}/CaM with the two chloronaphthalene rings inserting separately into the N and C-terminal hydrophobic pockets (Figure V-4). Thus, the increased inhibitory activity of them is obtained due to escaping the entropy loss by the linkage of two W-7 molecules, in other words, due to the high efficiency of the second chloronaphthalene binding. Although other compounds are ~75 times more potent than W-7, this increase was much lower than expected. This is probably because the binding of the compounds to Ca^{2+}/CaM may also introduce conformational constraints to the protein, which are entropically unfavorable.

Although the compounds **1**, **5**, and **7** all have a similar linker with four methylene, their inhibitory activities differ significantly. Clearly, substituting amine group(s) for amide group(s) at the joint positions of the linker (R') caused unfavorable effects on the potency (Table V-1). This trend was true for other sets of the compounds, i.e., the set of **2**, **6**, and **8**, and the set of **4** and **10**. In general, the nitrogen atom in an amide group has no basicity, and the amide group has low conformational flexibility due to its own planarity. Thus, the tendency of the inhibitory activities among each set can be explained in the light of basicity and/or flexibility at the joint position. As the basicity of the linker is decreased, the partial charge created by protonation of the nitrogen atom is decreased, which might lead to the loss of electrostatic interactions between the nitrogen atom and Ca^{2+}/CaM. Otherwise, as linker flexibility is decreased, the chloronaphthalene rings could not tether both CaM domains, due to changing of the relative orientation of both CaM domains. Although the basicity and flexibility of the amine and amide groups cannot be examined separately, basicity appears to be more important since **4**, which has a less flexible *p*-xylenediamine than butylene or hexylene, is the most potent CaM antagonist among the compounds tested. This is also consistent with the weak inhibitory activities of **3** and **9** with *p*-phenylenediamine and *p*-phenylenediamide, respectively, as linker because the nitrogen atoms attached to the aromatic ring are much less basic than those attached to other methylene groups.

Finally, some of the $(W\text{-}7)_2$ analogs in this work have been found to be at most 5 times more potent than W-7 in established bioassays for observing their pharmacological effects on Ca^{2+}/CaM in cells, probably because they could hardly permeate cell membranes (data not shown). Their use seems limited to cell-free systems, but membrane permeability could be improved by selecting better linkers. The second generation of $(W\text{-}7)_2$ will be synthesized with the purpose of overcoming this problem as well as improving their affinities to

Ca^{2+}/CaM.

V-5 Concluding Remarks

I presented here for the first time the development of new CaM antagonists by making use of the structural information of Ca^{2+}/CaM. This could not have been achieved without knowledge of the three-dimensional structure of Ca^{2+}/CaM complexed with the previous CaM antagonist, W-7 (Osawa *et al.*, 1998). Biochemical and physicochemical studies revealed that the novel compounds can interact with Ca^{2+}/CaM with higher affinities than W-7, where the dynamic nature of Ca^{2+}/CaM is reflected. The representative compound of them showed the ability to inhibit the CaM action 75 times more effectively than W-7, suggesting that there is still a great possibility of obtaining more potent CaM antagonists by further optimization of the linker sequences.

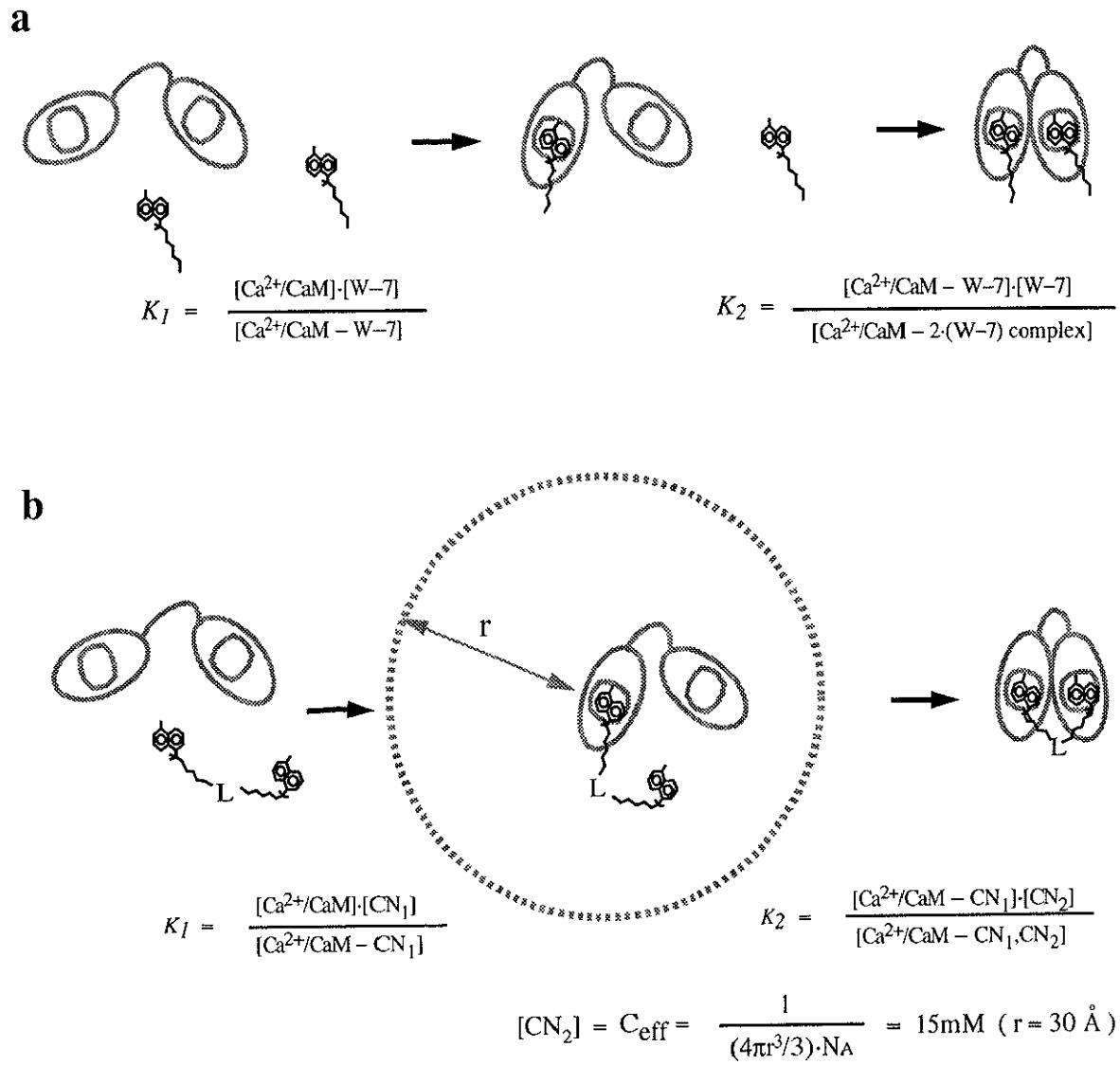
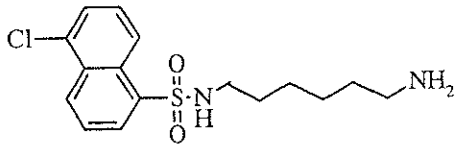
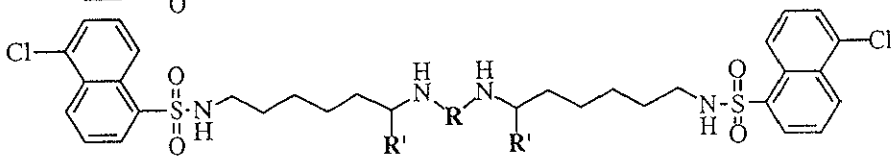
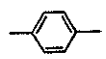
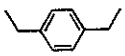
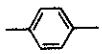
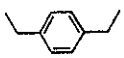


Figure V–1. a, Schematic drawing of the W–7 binding to Ca^{2+}/CaM. The binding constants of first and second W–7 molecules are given as K_1 and K_2, respectively. b, The binding model of $(W-7)_2$. The concentration of each chloronaphthalene group is given as $[CN_1]$ and $[CN_2]$, respectively. When one of the two chloronaphthalene groups of $(W-7)_2$ binds to either hydrophobic pocket of Ca^{2+}/CaM, $[CN_2]$ is estimated to be higher than the effective concentration, C_{eff}, which can be calculated using the longest distance between the two chloronaphthalene groups. In the case of the compound **4** with r = 30 Å, C_{eff} should equal to 15 mM.

Table V-1. IC_{50} values of the covalently linked derivatives of W-7 on CaMK I activation by Ca^{2+}/CaM

	Compound			IC_{50} (μM)[a]
W-7				17 ± 2
				
	R	R'		
1	-$(CH_2)_4$-	2H-	2H-	0.34 ± 0.04
2	-$(CH_2)_6$-	2H-	2H-	0.31 ± 0.06
3		2H-	2H-	(15 ± 6)[b]
4		2H-	2H-	0.23 ± 0.03
5	-$(CH_2)_4$-	2H-	O=	0.99 ± 0.08
6	-$(CH_2)_6$-	2H-	O=	0.75 ± 0.12
7	-$(CH_2)_4$-	O=	O=	(35 ± 5)
8	-$(CH_2)_6$-	O=	O=	31 ± 2
9		O=	O=	(9 ± 6)
10		O=	O=	(13 ± 5)

[a] The IC_{50} values were obtained by three independent experiments, each performed in duplicate (mean ± S.E.).

[b] The values within parentheses stand for inhibitory effects (%) which the compounds showed on the Ca^{2+}/CaM dependent activity of the enzyme at a concentration of 100 μM.

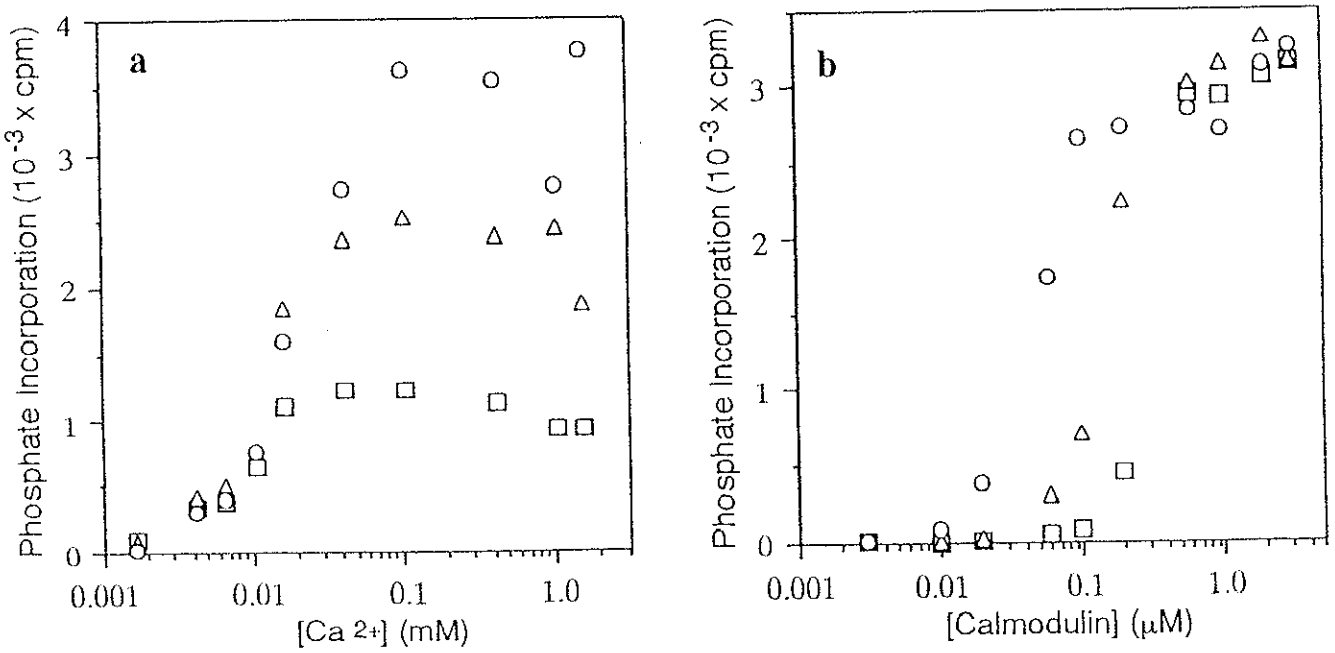


Figure V-2. Ca^{2+}-incompetitive and Ca^{2+}-CaM-competitive inhibition by the compound **4** of Ca^{2+}-CaM-dependent activity. a, Recombinant CaM kinase I (7.5 μg/ml) was incubated with 0~1.5 mM Ca^{2+} and 0.3 μM CaM in the presence of 0 μM (○), 0.5 μM (△), or 1.0 μM (□) **4**. b, The enzyme (7.5 μg/ml) was incubated with 0.5 mM Ca^{2+} and 0~1.5 μM CaM in the presence of 0 μM (○), 0.25 μM (△), or 0.5 μM (□) **4**. Other experimental conditions were identical with the standard ones described in 'Materials and Methods'. The Ca^{2+}-CaM-dependent activity of the enzyme is plotted against a function of added free Ca^{2+} or CaM concentrations.

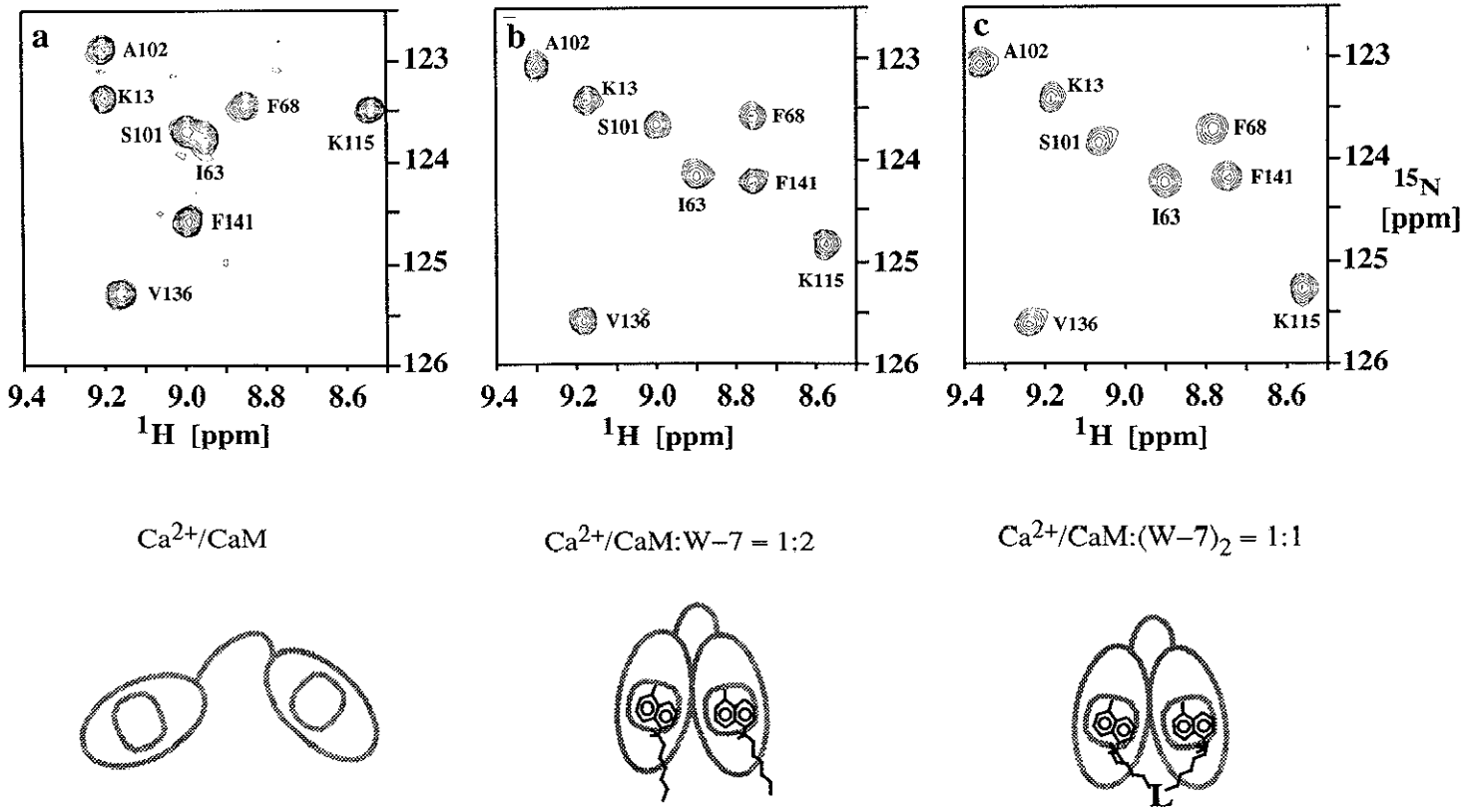


Figure V–3. Comparison of the two–dimensional ^{15}N–^{1}H HSQC spectra. All the peaks are labeled with one–character amino acid code followed by residue number. Each spectrum was acquired for the sample of a, Ca^{2+}/CaM only, b, Ca^{2+}/CaM mixed with two equivalents of W–7, and c, Ca^{2+}/CaM mixed with one equivalent of the compound **2**.

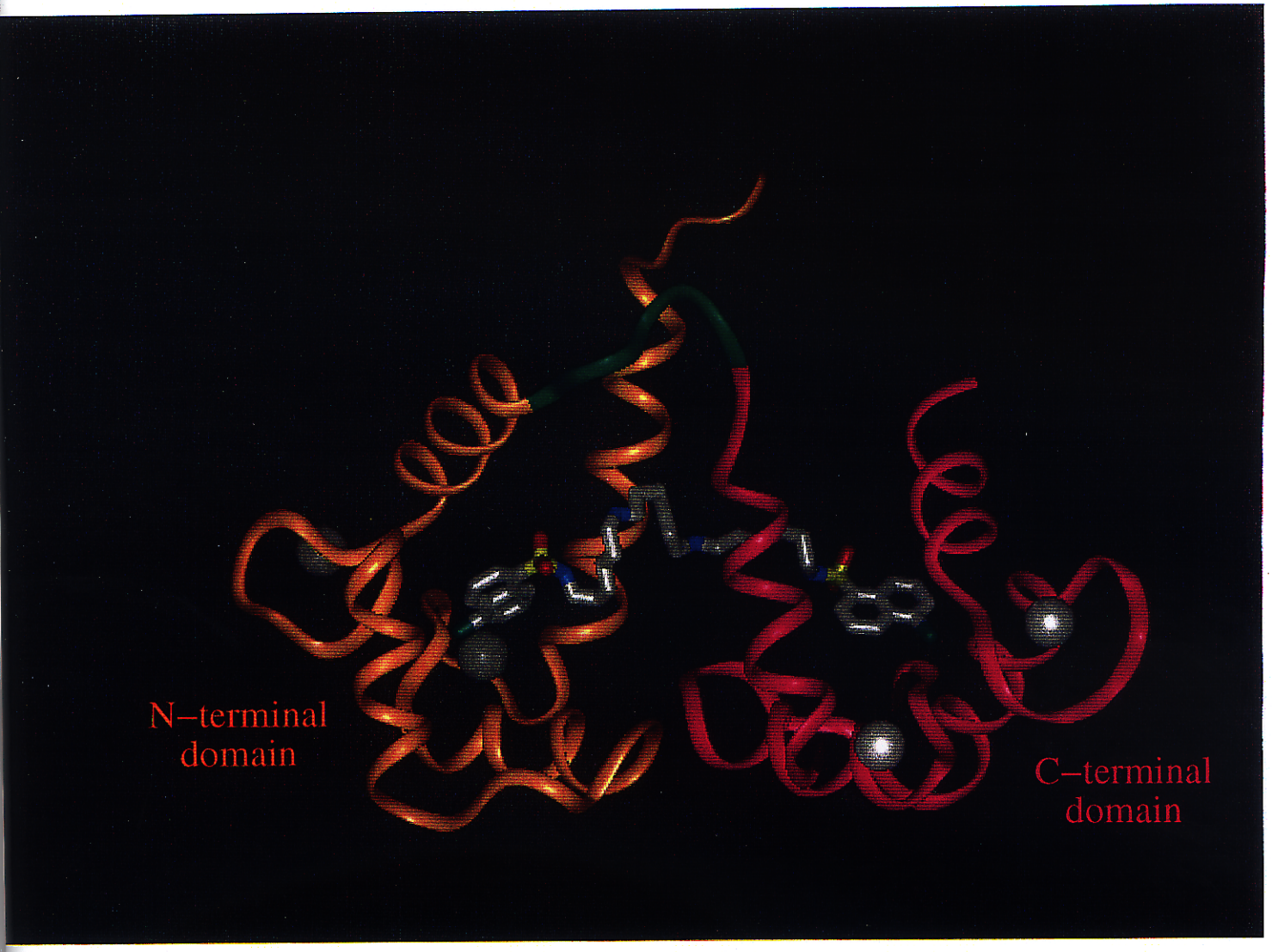


Figure V–4. The proposed model of Ca^{2+}/CaM complexed with the compound **2.** N and C–terminal domain of CaM are shown in orange and magenta, respectively. The compound is drawn as a stick model coloured by atom type: carbon: white, nitrogen: blue, oxygen: red, sulfer: yellow, chlorine: green. The structure was calculated according to the YASAP protocol (Nilges *et al.*, 1988) within X–PLOR (Brunger, 1992) as previously described (Bagby *et al.*, 1994), using the structural restraints obtained by NMR experiments with the Ca^{2+}/CaM–W–7 complex (Osawa *et al.*, 1998).