A Study of the Calcium Complex of a Glucosylceramide, Soya-cerebroside II

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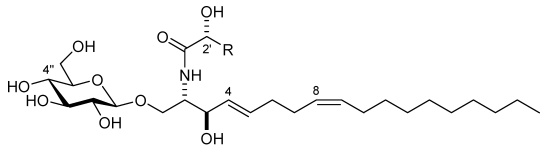
In order to study calcium ion complex of soya-cerebroside II (1), an ionophoretic glucosylceramide isolated from soybean, C8-cerebroside (3) and 3,3″,6″-trideoxy-C8-cerebroside (4) are designed and synthesized. On the basis of extensive ^{1}H-NMR studies in the presence of Ca^{2+} and a continuous variation method *via* ^{1}H-NMR, soya-cerebroside II is suggested to form a calcium complex with 1/Ca^{2+} ratio of 1 : 1. Soya-cerebroside II serves as a tridentate chelating ligand for Ca^{2+}; the amide carbonyl, C2′-hydroxy, and C2″-hydroxy oxygens are responsible for the Ca^{2+} binding. Soya-cerebroside II is structurally analogous to a neural glucosylceramide. Thus, the accumulated neural glucosylceramide inside of endoplasmic reticulum (ER) membrane may serve as an endogenous Ca^{2+}-binding and -transport molecule (ionophore) that result in mobilization of Ca^{2+} from intracellular calcium stores.

Key words sphingoglycolipid; calcium ion-chelator; calcium ion binding-affinity; endogenous ionophore; continuous variation method

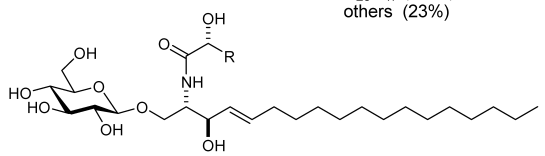
Sphingolipids (SLs) are structural components of eukaryotic cell membranes and a large number of recent reports have indicated that SLs are involved in a number of important regulatory processes in cell development.[1)] As represented by Gausher disease (metabolitic disorder caused by defective activity of glucosylceramide β-glucosidase), accumulation of the glucosylceramide (GlcCer) in brain tissue results in a significant increase in the rate of calcium ion release from endoplasmic reticulum (ER) that is responsible for neuronal cell death through an apoptotic cell death mechanism.[2—8)] Thus, the control of calcium homeostasis in nerve cells to prevent a number of neurological disorders (*i.e.* cerebral ischaemia, trauma, epilepsy and chronic neurodegenerative diseases) has been received a good deal of attention over the past several decades.[9—12)] Interestingly, exogenously added complex glycosphingolipids (*i.e.* ganglioside GM1 and GM3) were also shown to mobilize Ca^{2+} from intracellular stores.[13—17)] It has been discussed that the ryanodine receptor (RyaR), the Ca^{2+}-release channel of ER, is responsible for the mobilization of Ca^{2+} from ER. However, the activation of RyaR due to the elevation of intracellular GlcCer levels has never been proven experimentally.[18—20)] An alternative plausible mechanism is that the GlcCer serves as an endogenous Ca^{2+}-transport molecule (ionophore), translocating Ca^{2+} across the membrane.[21,22)]

We reported that soya-cerebroside II (SC-II, **1**), a GlcCer isolated from soybean, exhibited a Ca^{2+}-binding activity in a glass-cell apparatus (W-08) and a Ca^{2+}-permeation ability across the human erythrocyte membrane. The basic structure of soya-cerebroside II including the absolute stereochemistries of (2*R*)-hydroxy fatty acids are identical to one of the neural GlcCer, **2**. However, the main long-chain base (sphingosine moiety) in **1** is C18-4,8-diunsaturated (*E*/*Z*), and the carbon-length and compositions of 2-hydoxy fatty acids are different from **2** (Fig. 1).[23)] We established a flexible synthetic route for SC-II and its analogs, and conducted structure calcium-ionophoretic activity relationship of SC-II analogs. It was realized that the altering the stereochemistry or removing the C2′-hydroxy group of the (2*R*)-hydroxy fatty acid significantly decreased Ca^{2+}-binding and -permeation activities.[23)] In this note, we report that the model compounds **3** and **4**, which designed to study the Ca^{2+} complex of SC-II as well as GlcCer, form a 1 : 1 complex with Ca^{2+} and exhibited Ca^{2+}-binding and -transport activities.

In the ^{1}H-NMR analysis of SC-II in DMSO-d_6, the C2′- and C2″-hydroxy (OH) protons, and the amide proton were downshifted by 0.06, 0.05, and 0.04 ppm, respectively, in the presence of $CaCl_2$ (**1** : $CaCl_2$ = 1 : 1) compared to those in the absence of $CaCl_2$. In the ^{1}H-NMR of a 1 : 1 mixture of **1** and $Ca(ClO_4)_2$ in pyridine-d_5, the amide and C2′-protons were significantly downshifted by 0.55, and 0.14 ppm, respectively. On the basis of the low field changes in chemical shift observed for the hydroxy, amide, and C2′-protons of **1** in the presence of Ca^{2+} and the coordination geometry of divalent calcium ion (in general, a hexacoordination metal), the amide carbonyl, C2′-OH, and C2″-OH oxygens of SC-II were considered to involve in the complexation of Ca^{2+}.[24)] However, detailed studies of the calcium complex of SC-II were signif-



Soya-cerebroside II (**1**): R = $C_{14}H_{29}$ (62%)
$C_{21}H_{43}$ (10%)
$C_{23}H_{47}$ (7.0%)
others (23%)



Glucosylceramide (**2**): R = $C_{22}H_{45}$ (35%)
$C_{20}H_{41}$ (25%)
$C_{21}H_{43}$ (12%)
others (28%)

Fig. 1. Structures of Soya-cerebroside II (**1**) and a Neural Glucosylceramide (**2**)

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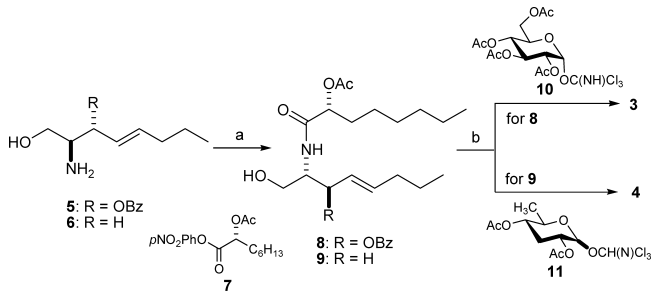

Reagents and conditions: (a) **7**, pyridine, 82% (for **5**), 84% (for **6**); (b) 1. **10** (for **8**) or **11**(for **9**), BF_3•OEt_2, CH_2Cl_2, 2. KOH, MeOH 78% (for **8**), 82% (for **9**).

Chart 1. Syntheses of C8-Cerebroside (**3**) and 3,3″,6″-Trideoxy-C8-cerebroside (**4**)

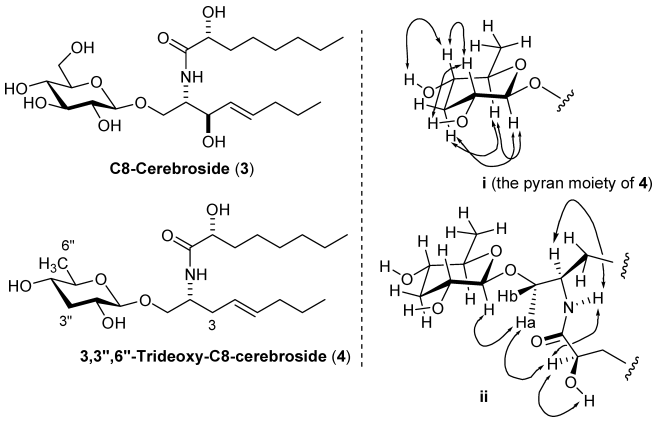

Fig. 2. Structures of C8-Cerebroside (**3**) and 3,3″,6″-Trideoxy-C8-cerebroside (**4**), and NOESY Data for **4** in the Presence and Absence of Ca^{2+}

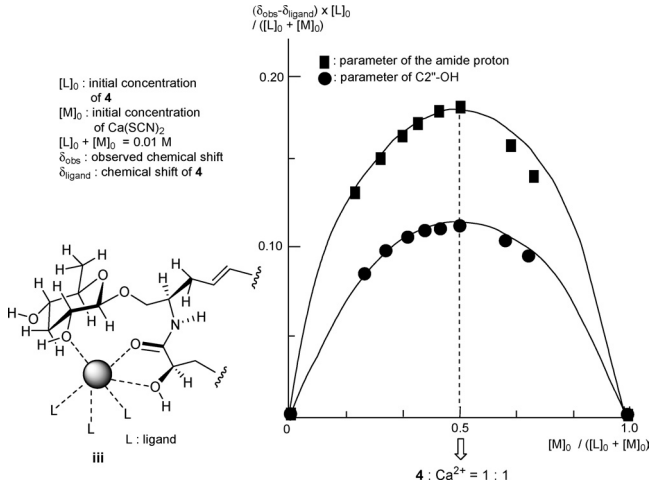

Fig. 3. A Continuous Variation Method for the Identification of Complex Stoichiometry of the **4**–Ca^{2+} Complex

icantly hampered by amphiphilic physicochemical properties of SC-II. Thus, we designed and synthesized two model compounds, C8-cerebroside (**3**) and 3,3″,6″-trideoxy-C8-cerebroside (**4**), for the elucidation of the SC-II (**1**)–Ca^{2+} (and **2**–Ca^{2+}) complex, in which 1) hydrophobicity of SC-II was decreased by shortening the carbon length of ceramide moiety (for **3**), and 2) the non-participating hydroxy groups of SC-II for the Ca^{2+}-complex formation were removed (for **4**).[25]

As illustrated in Chart 1, the synthesis of the C8-ceramide analogs, **8** and **9**, began with the C3-benzoylated D-*erythro*-C8-sphingoshine (**5**)[26] and its C3-deoxy derivative **6**.[27] The amide formations of **5** and **6** with the *p*-nitrophenyl ester **7** in the presence of pyridine provided **8** and **9** in 82% and 84%, respectively. The coupling reaction between **8** and the glucosyl imidate **10** smoothly underwent at 0 °C under Schmidt's conditions to provide C8-cebroside (**3**) exclusively in 78% overall yield after global saponifications. Similarly, 3,3″,6″-trideoxy-C8-cerebroside (**4**) were synthesized by using the imidate **11** in 82% overall yield from **9**.

Indeed, C8-ceramide and its 3,3″,6″-trideoxy analog, **3** and **4**, exhibited excellent solubility in various solvents such as acetone-d_6, DMSO-d_6, and $CDCl_3$ and provided sharp and well-resolved ^{1}H-NMR signals. It has been ascertained that the glucose moiety of **3** exists exclusively in the $^4C_1$ chair conformation in the presence or absence of Ca^{2+}. In NOESY experiments[28] of 3,3″,6″-trideoxy-C8-cerebroside in acetone-d_6, the strong NOEs of 1,3-diaxial protons and the NOE correlations between the hydroxyl protons on C2′, C2″, C4″ and their adjacent protons (C2′-H, C2″-H and C4″-H) were observed in the presence or absence of $Ca(SCN)_2$. Thus, the pyran moiety of **4** exists in the same $^4C_1$ chair conformation as observed in **3** (i in Fig. 2). In addition, the NOE correlations between 1) C1″-H (anomeric proton) and C1-H_a, 2) C1-H_a and C2′-H, 3) C2′-H and –NHCO–, and 3) –NHCO– and C2-H were observed. The dihedral angle of –O–C1–C2– was revealed to be around 42 degrees from the coupling constant of C1-H_a and C1-H_b (C1-H_a/C2-H: J=4.3 Hz, C1-H_b/C2-H: J=6.6 Hz).[29] As observed in the ^{1}H-NMR spectrum of **3** in the presence of Ca^{2+}, the amide, C2′-OH, and C2″-OH protons of **4** were shifted downfield. Therefore, overall conformation of **4** in the presence of calcium ions was established to be ii (Fig. 2).

The identification of complex stoichiometry of the calcium complex of **4** could be established *via* a continuous variation method (Job's method).[30] We carried out by ^{1}H-NMR analysis of the samples containing different amounts of equimolar Ca^{2+} and **4** solutions (in acetone-d_6) with a constant final concentration (0.01 M). The results were plotted in a graph of the difference in chemical shifts ($[(\delta_{obs}-\delta_{ligand})\times[L]_0/([L]_0+[M]_0)]$) of the amide or C2″-OH protons *versus* Ca^{2+} molar fraction. As shown in Fig. 3, the complex stoichiometry was determined as Ca^{2+} : **4** = 1 : 1; the graphic inflection point was observed in 0.5 and no other stoichiometries were identified. It was concluded that 3,3″,6″-trideoxy-C8-cerebroside serves as a tridentate ligand in the complexation with Ca^{2+} and forms a 1 : 1 Ca^{2+}–**4** complex (iii in Fig. 3) on the basis of NOESY experiments of **4** in the presence of Ca^{2+} and the result obtained from a continuous variation method.

We confirmed that C8-cerebroside (**3**) and 3,3″,6″-trideoxy-C8-cerebroside (**4**) are good to excellent model compounds for Ca^{2+}-binding and -transport studies *in vitro*. The calcium ion-binding and transport activity studies of **3** and **4** using the glass-cell apparatus (W-08) revealed that **3** exhibited the Ca^{2+}-binding activity; the Ca^{2+}-binding affinity of **3** in 1-octanol is lower than that of SC-II-(2*R*)-hydroxypalmitoyl analog[23] (the major component of SC-II). However, a detectable level of Ca^{2+}-transport activity of **3** was not observed. On the other hand, 3,3″,6″-trideoxy-C8-cere-

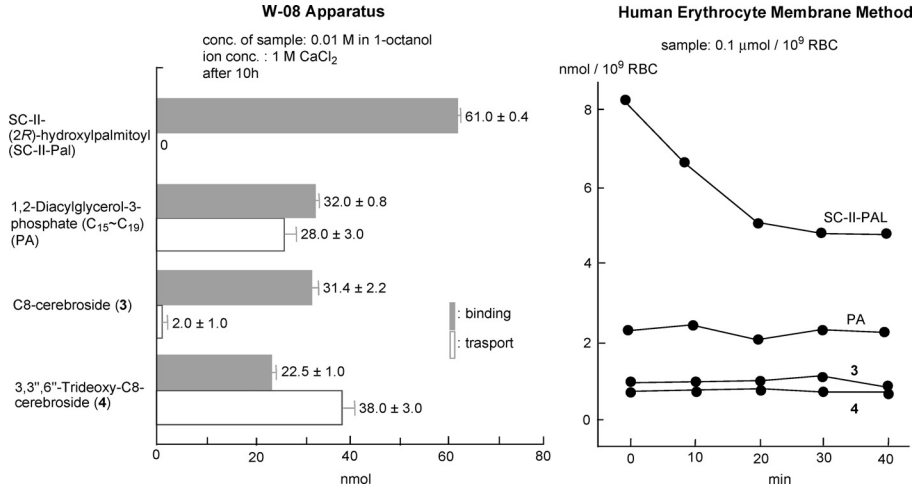

Fig. 4. Calcium Ionophoretic Activities of SC-II-(2*R*)-Hydroxypalmitoyl (SC-II-Pal), PA, **3**, and **4** by Using the W-08 Apparatus[34,35] and the Human Erythrocyte Membrane Method[36]

broside exhibited both the Ca^{2+}-binding and transport activities in the W-08 system, and the Ca^{2+}-binding and transport activities of **4** is nearly equal to an endogenous Ca^{2+} ionophore, 1,2-diacylglycerol-3-phosphate (PA)[31—33] (Fig. 4). However, the model molecules, **3** and **4**, exhibited lower Ca^{2+}-permeation activity than SC-II-(2*R*)-hydroxypalmitoyl analog[23] in the human erythrocyte membrane method.

In conclusion, from extensive ^{1}H-NMR studies with C8-cerebroside (**3**) and 3,3″,6″-trideoxy-C8-cerebroside (**4**) in the presence of Ca^{2+}, SC-II forms a Ca^{2+} complex with a ratio of 1 : 1 in which the amide carbonyl, C2′-OH, and C2″-OH oxygens are responsible for the coordination of Ca^{2+}. The short chain-length molecules **3** and **4** showed lower Ca^{2+}-permiation activity than SC-II in the human erythrocyte membrane method. Thus, C18 carbon chain length or increased hydrophobicity of the model molecules is essential for the Ca^{2+}-permiation activity across the biological membrane. As mentioned above, SC-II is structurally analogous to a neural GlcCer except for the degree of unsaturation in the sphingosine moiety and the compositions of fatty acids. Thus, a neural GlcCer 1) should have similar physicochemical properties, and 2) may possess equal Ca^{2+}-binding and -transport activities *in vivo* to those of SC-II. *In vivo* or *in vitro* studies with neural cells are indispensable to prove unequivocally that the accumulated GlcCer serves as an endogenous Ca^{2+}-transport molecule and increases Ca^{2+} transfer from ER. Nevertheless, we unambiguously demonstrated, for the first time, that the Ca^{2+}-binding and -transport activities of a GlcCer not through the formation of reverse micelles but through a 1 : 1 complexation. Thus, soya-cerebroside II and other related molecules exhibit selectivity for binding divalent metal cations such as Ca^{2+}.[23,34,35]

Experimental

General Experimental Procedures Reactions with air sensitive materials were carried out by standard syringe techniques. Commercially available reagents were used as received without further purification except for acetone-d_6. Acetone-d_6 was dried over $CaSO_4$ and distilled. Thin layer chromatography was performed using 0.25 mm silica gel 60 (F254, Merck) plates visualizing at 254 nm, or developed with potassium permanganate solutions by heating with a hot-air gun. Specified products were purified by flash column chromatography using silica gel 60 (230—400 mesh, Merck). IR absorptions on KBr plates were run on a Nippon Bunko FT-IR 5300. ^{1}H-NMR spectral data were obtained using JEOL 270 or 500 MHz instruments. The residual solvent signal was utilized as an internal reference. ^{13}C-NMR spectral data were obtained using a JOEL 67.5 or 125 MHz spectrometer. For all NMR spectra, δ values are given in ppm and J values in Hz. Mass spectra were obtained using Nippon Denshi JMS-D300 or JMS-SX102. Optical rotations were taken using Nippon Bunko DIP-370.

Synthesis of (2*R*,2′*R*,4*E*)-2-(2′-Acetoxyactanamido)-oct-4-ene-1-ol (9) To a stirred solution of **6** (170 mg, 1.28 mmol) in pyridine (2.0 ml) was added **7** (499 mg, 1.54 mmol). After 6 h at 50 °C, all volatiles were evaporated *in vaccuo*. Purification by silica gel chromatography (hexane : EtOAc = 1 : 2) gave **9** (351 mg, 84%) as a white solid: $[\alpha]_D$ +16.1° (c=0.1, $CHCl_3$, 24 °C); ^{1}H-NMR (270 MHz, C_5D_5N) 8.31 ppm (1H, d), 5.7—5.3 (3H, m), 4.5—4.3 (1H, m), 4.0—3.8 (2H, m), 2.6—2.4 (2H, m), 1.96 (3H, s), 1.9—1.8 (2H, m), 1.5—1.0 (12H, s), 0.76 (3H each s); ^{13}C-NMR (77.5 MHz, $CDCl_3$) 17.3 ppm, 170.2, 132.9, 127.1, 74.7, 63.6, 51.9, 34.9, 32.5, 31.7, 39.1 (2C), 25.3, 22.7, 20.6, 14.0, 13.7; IR (KBr) 3322 cm^{-1}, 2959, 2863, 1746, 1659; HR-EI-MS Calcd for $C_{18}H_{33}NO_4$: 327.2406. Found: 327.2394 (M^+).

Synthesis of *O*-(3,6-Dideoxy-β-D-glucopyranosyl)-(1→1)-(2*R*,2′*R*,4*E*)-2-(2′-hydroxyactanamido)-oct-4-ene-1-ol (4) To a stirred suspension of **9** (160 mg, 0.49 mmol), **11**[37] (340 mg, 0.91 mmol), and MS 4 Å (300 mg) in $ClCH_2CH_2Cl$ (15 ml) was stirred for 2 h at r.t. and cooled to −20—−25 °C. Into the reaction mixture $BF_3 \cdot OEt_2$ (278 mg, 1.96 mmol) was added. The reaction mixture was stirred for 1 h and diluted with CH_2Cl_2 and filtered through celite. The organic phase was washed with sat. aq. $NaHCO_3$, dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was dissolved in MeOH (3 ml) and 5% KOH (0.8 ml) was added. After 30 min, the reaction mixture was neutralized with Dowex 50W×8(H^+), filtered, and evaporated *in vaccuo*. The crude product was purified by silica gel chromatography ($CHCl_3$: MeOH = 10 : 1) to give **4** (165 mg, 82%) as a colorless needle: $[\alpha]_D$ −27.3° (c=0.1, $CHCl_3$, 23 °C); ^{1}H-NMR (270 MHz, C_5D_5N) 6.78 ppm (1H, d), 5.51 (1H, dt, J=15.2, 6.6 Hz), 5.32 (1H, 1H, dt, J=15.2, 6.9 Hz), 4.19 (1H, d, J=7.6 Hz), 4.2—4.1 (1H, m), 4.1—4.0 (1H, m), 3.80 (1H, dd, J=10.6, 7.3 Hz), 3.62 (1H, dd, 1H, J=10.6, 3.3 Hz), 3.5—3.3 (3H, m), 2.4—2.1 (3H, m), 2.0—1.9 (2H, m), 1.8—1.2 (16H, br s), 0.88 (3H each s); ^{13}C-NMR (77.5 MHz, $CDCl_3$) 175.0 ppm, 134.5, 124.7, 105.5, 77.2, 76.0, 72.6, 72.5, 70.6, 68.4, 48.4, 39.1, 34.7, 34.6, 31.7, 29.1, 25.1, 22.6, 22.5, 17.6, 14.1, 13.7; IR (KBr) 3333 cm^{-1}, 2957, 2872, 1647.; HR-FAB-MS Calcd for $C_{22}H_{41}NO_6$+H: 416.3012. Found: 416.3010 ($M+H^+$).

***O*-(β-D-Glucopyranosyl)-(1→1)-(2*S*,2′*R*,3*R*,4*E*)-2-(2′-hydroxyactanamido)-oct-4-ene-1-ol (3)** Data for **3**: $[\alpha]_D$ −4.4° (c=0.2 at 25 °C); ^{1}H-NMR (C_5D_5N, 270 MHz): δ 8.31 (1H, d), 5.5—5.0 (2H, m), 4.85 (1H, d, J=7.3 Hz), 3.8—4.8 (11H, m), 1.5—2.2 (4H, m), 1.1—1.4 (10H, br s), 0.76 (6H, t); ^{13}C-NMR (C_5D_5N, 77.5 MHz): δ 175.6, 132.3 (2C), 131.7, 105.4, 78.3, 78.2, 74.9, 72.1, 71.3, 69.9, 62.4, 54.4, 35.4, 34.5, 31.9, 29.4, 25.6, 22.7, 22.5, 14.1 (2C), 13.7; IR (KBr): 3335 cm^{-1}, 2957, 1643.; HR-MS (FAB) Calcd for $C_{22}H_{41}NO_9$+Na: 486.2679. Found: 486.2683.

Determination of Complex Stoichiometry of the Calcium Complex of

Table 1. Determination of Complex Stoichiometry of the Calcium Complex of **4** *via* a Continuous Variation Method

Amide proton

$[M]_0/([L]_0+[M]_0)$	0	0.2	0.25	0.3	0.33	0.4	0.5	0.66	0.75	1
$(\delta_{obs}-\delta_L)=\Delta\delta$ (ppm)	0	0.097	0.123	0.148	0.165	0.198	0.238	0.309	0.335	—
$[L]_0/([L]_0+[M]_0)$	1	0.8	0.75	0.7	0.66	0.6	0.5	0.33	0.25	0
$\Delta\delta\cdot[L]_0/([L]_0+[M]_0)$	0	0.078	0.098	0.104	0.109	0.118	0.119	0.102	0.084	0

2′-OH

$[M]_0/([L]_0+[M]_0)$	0	0.2	0.25	0.3	0.33	0.4	0.5	0.66	0.75	1
$(\delta_{obs}-\delta_L)=\Delta\delta$ (ppm)	0	0.138	0.178	0.209	0.233	0.280	0.333	0.436	0.471	—
$[L]_0/([L]_0+[M]_0)$	1	0.8	0.75	0.7	0.66	0.6	0.5	0.33	0.25	0
$\Delta\delta\cdot[L]_0/([L]_0+[M]_0)$	0	0.110	0.134	0.146	0.154	0.168	0.167	0.144	0.188	0

$[L]_0$: initial concentration of **4**; $[M]_0$: initial concentration of $Ca(SCN)_2$ ($[L]_0+[M]_0=0.01$ M). δ_{obs}: observed chemical shift; δ_L: chemical shift for **4**.

4 *via* a Continuous Variation Method (Job's Method) The 0.01 M concentrations of **4** and $Ca(SCN)_2$ in acetone-d_6 were prepared. The NMR samples containing different amounts of equimolar $Ca(SCN)_2$ and **4** solutions (500 μl in NMR tube) with a constant final concentration of 0.01 M were prepared. ^{1}H-NMR analyses of the 2′-OH and amide protons for ach sample were conducted. The results were summarized in Table 1.

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27) The detailed syntheses of **6** will be reported elsewhere.
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37) Data for **11**: ^{1}H-NMR ($CDCl_3$, 270 MHz): δ 6.27 (1H, d, J=3.3 Hz), 4.94 (1H, dt, J=12.5, 3.3 Hz), 4.56 (1H, dt, J=14.5, 4.6 Hz), 4.0—3.9 (1H, m), 2.4—1.0 (2H, m), 1.98 (3H, s), 1.91 (3H, s), 1.10 (3H, d, J=6.3 Hz).; IR (KBr): 3187 cm^{-1}, 2986, 2940, 1745, 1670.; HR-MS (FAB) Calcd for $C_{12}H_{16}NO_6Cl_3$+H: 376.0121. Found: 376.0098 ($M+H^+$).