

Properties of Astaxanthin/ Ca^{2+} Complex Formation in the Deceleration of Cis/Trans Isomerization

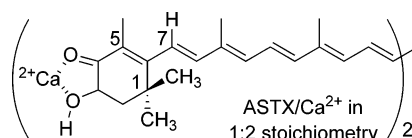
Chien-Sheng Chen,^{†‡} Shih-Hsiung Wu,^{*‡} Ying-Ying Wu,[§] Jim-Min Fang,[†] and Tzu-Hua Wu^{*§}

Department of Chemistry, National Taiwan University, Taipei 106, Taiwan, Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan, and College of Pharmacy, Taipei Medical University, Taipei 110, Taiwan

shwu@gate.sinica.edu.tw; thwu@tmu.edu.tw

Received April 24, 2007

ABSTRACT

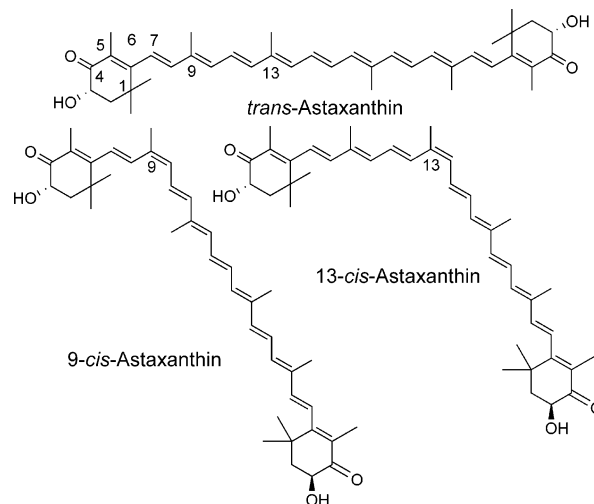


Deceleration of the regioselective cis/trans isomerization of *all-trans*-astaxanthin (ASTX) in the presence of Ca^{2+} was shown by HPLC analysis. The NMR and ITC analyses provided evidence for complexation of ASTX with Ca^{2+} in 1:2 stoichiometry via chelation at both carbonyl and hydroxyl groups. The rotation across torsion ω_6 (C5–C6–C7–C8) upon complexation is consistent with the NOE between 7-H and 5- CH_3 . This study supports the inhibitory effect of ASTX on calcium-induced turbidity of lens crystallins.

Astaxanthin (3,3'-dihydroxy- β,β' -carotene-4,4'-dione, ASTX, Scheme 1) is one of the most common xanthophylls found in the red pigment of crustacean shells (e.g., of crab and shrimp), salmon, and asteroideans.¹ ASTX has several biological activities, including immuno-modulating activity,² hepatoprotection,³ and protection of islet β -cells,⁴ and may be used in disease prevention, e.g., antihypertension, and in protection against ischemia-induced memory impairment.⁵ ASTX appears to be a more powerful antioxidant than other carotenoids and vitamin E in conferring numerous health benefits.⁶

In nature, carotenoids exist predominately in the all-trans configuration. The long chain of conjugated carbon–carbon double bonds within carotenoids is susceptible to light,

Scheme 1. Structure of *trans*-Astaxanthin, 9-*cis*-Astaxanthin, and 13-*cis*-Astaxanthin



[†] National Taiwan University.

[‡] Academia Sinica.

[§] Taipei Medical University.

(1) (a) Miki, W.; Yamaguchi, K.; Konosu, S. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **1982**, *71*, 7–11. (b) Ohgami, K.; Shiratori, K.; Kotake, S.; Nishida, T.; Mizuki, N.; Yazawa, K.; Ohno, S. *Vestn. Ophthalmol. Vis. Sci.* **2003**, *44*, 2694–2701.

(2) (a) Chen, C. J.; Tsai, C. C.; Hsieh, J. F.; Chien, C. M.; Wu, T. H.; Chen, S. T. *Comb. Chem. High Throughput Screening* **2006**, *9*, 777–784. (b) Chew, B. P.; Park, J. S. *J. Nutr.* **2004**, *134*, 257S–261S.

(3) (a) Kang, J. O.; Kim, S. J.; Kim, H. *Methods Find Exp. Clin. Pharmacol.* **2001**, *23*, 79–84. (b) Kozuki, Y.; Miura, Y.; Yagasaki, K. *Cancer Lett.* **2000**, *151*, 111–115.

(4) Uchiyama, K.; Naito, Y.; Hasegawa, G.; Nakamura, N.; Takahashi, J.; Yoshikawa, T. *Redox Rep.* **2002**, *7*, 290–293.

(5) Hussein, G.; Sankawa, U.; Goto, H.; Matsumoto, K.; Watanabe, H. *J. Nat. Prod.* **2006**, *69*, 443–449.

oxygen, heat, and acid degradation. The cis/trans configuration of the geometrical isomers greatly influences the overall molecular shape. The all-trans isomer of ASTX predominates in nature, but the 9-cis, 13-cis, and 15-cis isomers are also encountered. The occurrence of the isomerization of the all-trans isomer to the cis-isomers during the extraction of pigments, saponification of the *trans*-ASTX ester, purification of *trans*-ASTX, and preparation of *trans*-ASTX standard solutions has been studied by using various organic solvents.⁷ The facilitation of the isomerization of *trans*-ASTX to the cis isomers induced by copper ions in ethanol has also been reported; however, the UV spectroscopy and HPLC analysis data are limited, and no detailed information on the structure of ASTX complexes has been provided.⁸

We previously showed that ASTX inhibits calcium-induced lens turbidity and that the ASTX/Ca²⁺ interaction may be responsible for part of this protective effect.⁹ In fact, calcium and calcium-dependent enzymes play specific roles in the development of human diseases and symptoms, including cataracts, impaired cognition, and insulin resistance.¹⁰ Therefore, we aimed to investigate the chelation of calcium by ASTX and the chemical interactions between calcium and ASTX in order to provide meaningful interpretation for the *in vivo* and *in vitro* activities of ASTX.

Effect of Ca²⁺ on Isomerization. The stoichiometry of ASTX/Ca²⁺ in acetonitrile (ACN)/chloroform (1:1, v/v) and ACN/CH₂Cl₂ (1:1, v/v) was analyzed by NMR and isothermal titration calorimetry (ITC), respectively. ACN was used since it increased the solubility of Ca(ClO₄)₂. The formation of the ASTX/Ca²⁺ complex within 3 min of adding calcium ions was examined by using UV/vis spectroscopy. The absorbance increased slightly as the molar ratio of calcium ions increased, but the increase was not linear. The observed slight red-shift of the λ_{\max} from 480 to 484 nm (see the Supporting Information) might result from a slightly better conjugation system in the polyene-backbone of this keto-carotene owing to the ASTX/Ca²⁺ complex formation. The results are consistent with (i) the lack of an apparent charge transfer because of the binding of two calcium ions at both ends of ASTX and (ii) the retention of the all-trans configuration of ASTX during the titration. In comparison, incubation of *trans*-ASTX with Cu²⁺ shows a large blue-shift from 480 to 373 nm with decreased intensity in ethanol,⁸ presumably due to the accelerated isomerization to 9-*cis*- and 13-*cis*-ASTX having the polyene chromophore twisted. The cis/trans isomerization of *trans*-ASTX to 9-*cis* or 13-*cis* forms has been reported with first-order and reversible reaction kinetics in both CH₂Cl₂ and ACN solutions,¹¹ and such an isomerization can be accelerated by thermal incubation.⁷ Since we incubated samples under 37 °C in earlier

lens studies, we also incubated the samples at 37 °C in the current study. The *trans*-ASTX isomerization upon chelation with calcium ions was then analyzed by HPLC. Isomerization decelerated when *trans*-ASTX was incubated with calcium ions at an ASTX/Ca²⁺ molar ratio of 1:2 (Figure 1); similar

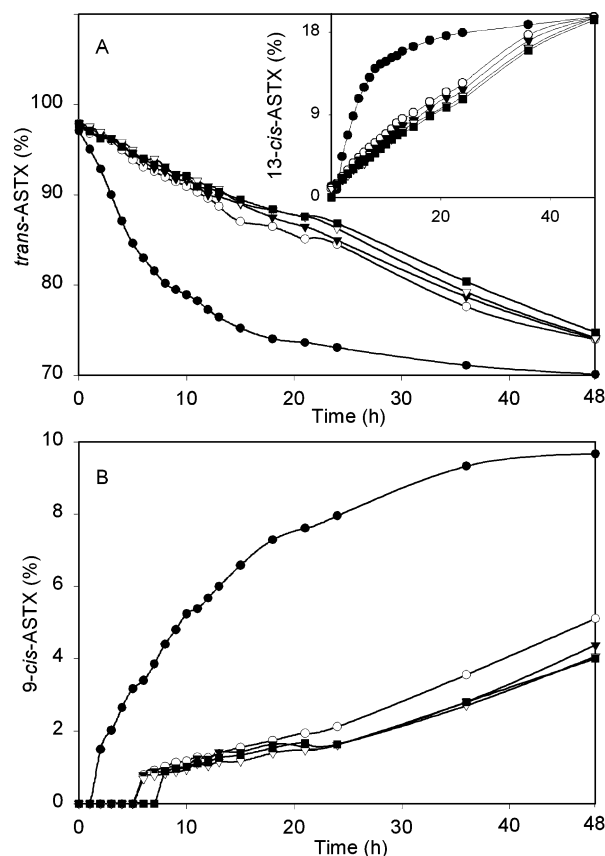


Figure 1. The cis/trans isomerization of ASTX in the presence of calcium at 37 °C. The relative amount of (A) *trans*-astaxanthin, 13-*cis*-astaxanthin (insert), and (B) 9-*cis*-astaxanthin in CH₂Cl₂/ACN (1/1, v/v) in the presence or absence of Ca(ClO₄)₂ are shown: (●) ASTX; (○) ASTX/Ca²⁺ (1:2, mol/mol); (▼) ASTX/Ca²⁺ (1:4, mol/mol); (▽) ASTX/Ca²⁺ (1:8, mol/mol); and (■) ASTX/Ca²⁺ (1:16, mol/mol).

results were obtained with a molar ratio of 1:4. These results differed from the previously reported acceleration of cis/trans isomerization of ASTX induced by NaCl⁷ and by copper ions,⁸ both of which were for different reaction times and at different temperatures. However, the observed regioselectivity, e.g., production of predominantly 13-*cis*-ASTX (~18%) after thermal incubation and a decrease (~6%) in 9-*cis*-ASTX formation in the presence of calcium after 48 h, was similar to the results of other studies.^{7,8}

Interaction of ASTX with Ca²⁺. The binding properties of ASTX (2 mM) with calcium ions in ACN/chloroform (1:1, v/v) were evaluated by NMR. Addition of Ca²⁺ to ASTX at various molar ratios (ASTX/Ca²⁺ 1:1 to 1:128) caused downfield shifts in all proton signals of cyclohexenone moiety (Table 1). The most significant proton chemical shifts

(6) Guerin, M.; Huntley, M. E.; Olaiyola, M. *Trends Biotechnol.* **2003**, *21*, 210–216.

(7) Yuan, J. P.; Chen, F. *J. Agric. Food Chem.* **1999**, *47*, 3656–3660.

(8) Zhao, L.; Chen, F.; Zhao, G.; Wang, Z.; Liao, X.; Hu, X. *J. Agric. Food Chem.* **2005**, *53*, 9620–9623.

(9) Wu, T. H.; Liao, J. H.; Hou, W. C.; Huang, F. Y.; Maher, T. J.; Hu, C. C. *J. Agric. Food Chem.* **2006**, *54*, 2418–2423.

(10) Carragher, N. O. *Curr. Pharm. Des.* **2006**, *12*, 615–638.

(11) Yuan, J. P.; Chen, F. *Food Chem.* **2001**, *73*, 131–137.

Table 1. ^1H and ^{13}C NMR Chemical Shifts (ppm) of ASTX and ASTX/ Ca^{2+} Complex in ACN/Chloroform^a

proton	$\delta_{\text{ASTX}}^{\text{H}}$	$\delta_{\text{complex}}^{\text{H}}$	$\Delta\delta_{\text{shift}}^{\text{H}}$ ^b	carbon	$\delta_{\text{ASTX}}^{\text{C}}$	$\delta_{\text{complex}}^{\text{C}}$	$\Delta\delta_{\text{shift}}^{\text{C}}$ ^b
$\text{CH}_3^{\text{pro-S}}$	1.32	1.35	0.02	$\text{CH}_3^{\text{pro-S}}$	24.75	24.46	-0.29
$\text{CH}_3^{\text{pro-R}}$	1.20	1.25	0.05	$\text{CH}_3^{\text{pro-R}}$	29.39	29.27	-0.12
H2	2.14	2.16	0.02	C1	35.69	36.41	0.72
H2'	1.76	1.91	0.15	C2	44.40	44.16	-0.24
H3	4.29	4.73	0.44	C3	68.16	70.42	2.26
3-OH	3.61	5.29	1.68	C4	199.32	201.92	2.60
H7	6.46	6.56	0.10	C5	125.85	125.79	-0.06
H8	6.27	6.29	0.02	C6	160.96	164.74	3.78
8- CH_3	1.89	1.91	0.02	C7	140.95	142.42	1.47
H10	6.45	6.49	0.04	C8	122.78	122.17	-0.61
H12	6.33	6.38	0.05	C9	133.97	133.92	-0.05

^a ASTX, astaxanthin (1 mM) in ACN-*d*₃/chloroform-*d*₁ (1:1). The complexation of ASTX/ Ca^{2+} , ASTX (1 mM) and $\text{Ca}(\text{ClO}_4)_2$, (1:32, mol/mol). ^b $\Delta\delta_{\text{shift}}^{\text{H}} = \delta_{\text{ASTX}/\text{Ca}}^{\text{H}} - \delta_{\text{ASTX}}^{\text{H}}$; $\Delta\delta_{\text{shift}}^{\text{C}} = \delta_{\text{ASTX}/\text{Ca}}^{\text{C}} - \delta_{\text{ASTX}}^{\text{C}}$.

were the hydroxyl (3-OH, $\Delta\delta_{\text{shift}} = 1.68$ ppm) and α proton of the ketone group (H3, $\Delta\delta_{\text{shift}} = 0.44$ ppm). The 2-D heteronuclear ^1H - ^{13}C HMQC (1J) and HMBC (2J and 3J) also showed downfield shifts in the carbon nuclei, including C3 ($\Delta\delta_{\text{shift}} = 2.26$ ppm) and C4 ($\Delta\delta_{\text{shift}} = 2.60$ ppm). These results strongly indicate that the α -hydroxyketone utilizes the lone pairs at the 3-oxygen and 4-carbonyl groups as the functional site involved in calcium chelation.

A clockwise rotation of the polyene chain across bond C6-C7 was suggested by the ROESY spectra of ASTX with and without calcium. The observed additional NOE of the ASTX/ Ca^{2+} (1:32) complex, 7-H/5- CH_3 (Figure 2B), was used in the molecular simulation with a distance restraint set at medium range (3.0–4.0 Å). The results led to a model with a torsion ω_6 (C5-C6-C7-C8) of -117.0° . This significant twist also reflects the nuclear downfield shift of C6 ($\Delta\delta_{\text{shift}} = 3.78$ ppm), C7 ($\Delta\delta_{\text{shift}} = 1.47$ ppm), and H7 ($\Delta\delta_{\text{shift}} = 0.10$ ppm). This structure contrasts the previously reported X-ray analysis of uncomplexed ASTX with ω_6 torsion of 59.3° (Figure 2A).¹² Our chelation model of ASTX(Ca^{2+})₂ complex indicated a better π -electron conjugation system of the cyclohexenone moiety to polyene,¹³ and the metal ion induced conformational rigidification¹⁴ thus provided a reasonable explanation for the increase in absorbance. Because the 9-alkenyl group is closer to the calcium-chelation site, it would be more easily stabilized in the presence of calcium ions. This model of calcium-chelated ASTX could also explain why the isomerization of *trans*-ASTX gave more 13-cis isomer than 9-cis isomer in the presence of calcium ions.

Thermodynamics of the ASTX/ Ca^{2+} Complex. The measured absorbance of a chromophore can usually be used to characterize the binding properties of a ligand/metal

(12) (a) Moss, G. P. *Pure Appl. Chem.* **1979**, *51*, 507–514. (b) <http://xray.bmc.uu.se/hicup/>.

(13) (a) Weesie, R. J.; Jansen, F. J.; Merlin, J. C.; Lugtenburg, J.; Britton, G.; de Groot, H. J. *Biochemistry* **1997**, *36*, 7288–7296. (b) Fiedor, J.; Fiedor, L.; Kammhuber, N.; Scherz, A.; Scheer, H. *Photochem. Photobiol.* **2002**, *76*, 145–152. (c) Gao, Y.; Focsan, A. L.; Li, Y. Y.; Kispert, L. D. *J. Phys. Chem. A* **2006**, *110*, 10091–10097. (d) Samokyszyn, V. M.; Freyaldenhoven, M. A.; Chang, H. C.; Freeman, J. P.; Compadre, R. L. *Chem. Res. Toxicol.* **1997**, *10*, 795–801.

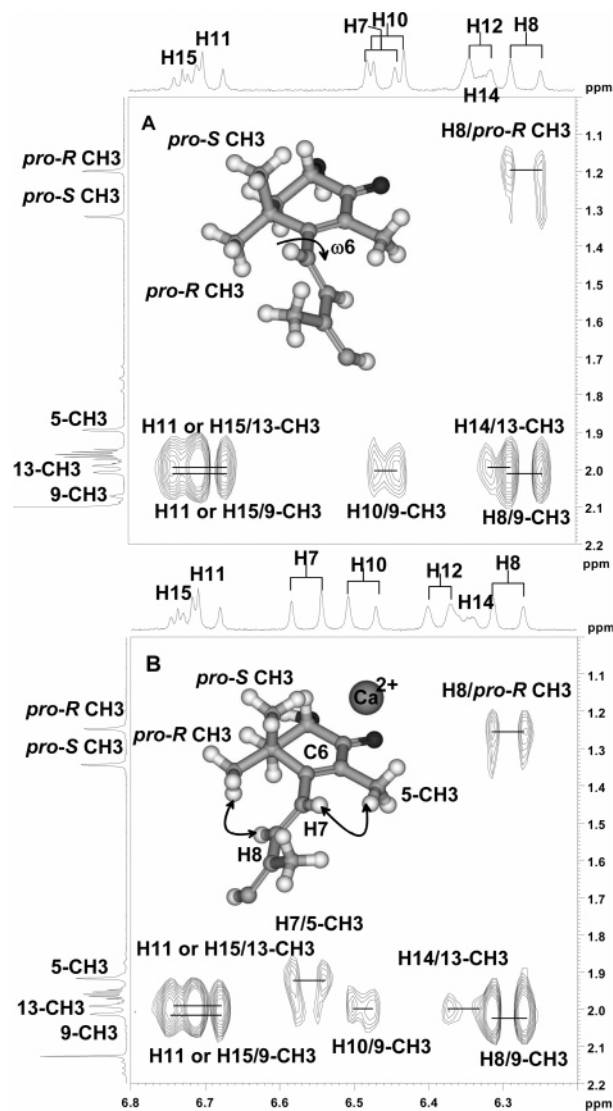


Figure 2. Partial ROESY spectra of ASTX (2 mM) alone (A) and with 32 molar equiv of calcium ions (B) with a mixing time of 300 ms. The structural model in part A shows the partial 3-D structure of ASTX from the X-ray databank that matches the data obtained by ROESY. The structural model of ASTX/ Ca^{2+} in part B is based on molecular simulation and indicates a clockwise rotation of the polyene chain across bond C6-C7 when compared to the native ASTX.

complex,¹⁴ but it is difficult to obtain this characterization without an isobestic point in the UV/vis spectra. We used a Job plot to study the various concentrations of ligand/metal pairs following a design similar to that used in NMR analyses.¹⁵ The results obtained by monitoring the downfield shifts of H3 of ASTX suggested that ASTX complexes two calcium ions (Table 1). By comparison with the literature,¹⁶

(14) (a) Liao, J. H.; Chen, C. T.; Chou, H. C.; Cheng, C. C.; Chou, P. T.; Fang, J. M.; Slanina, Z.; Chow, T. J. *Org. Lett.* **2002**, *4*, 3107–3110. (b) Kuo, L. J.; Liao, J. H.; Chen, C. T.; Huang, C. H.; Chen, C. S.; Fang, J. M. *Org. Lett.* **2003**, *5*, 1821–1824.

(15) Braun, S.; Kalinowski, H.-O.; Berger, S. *100 and More Basic NMR Experiments*; VCH Publishers: New York, 1996.

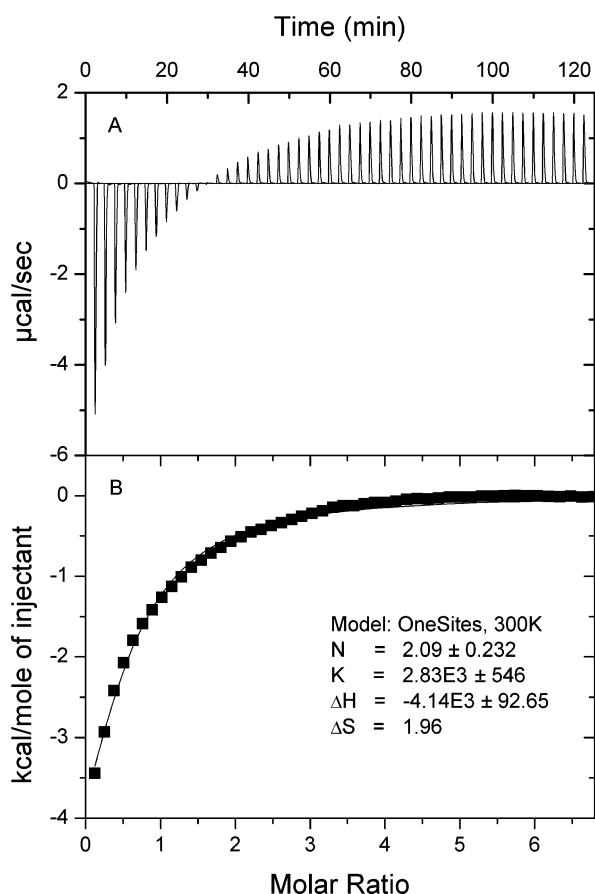


Figure 3. Isothermal titration calorimetry of the complex formation of ASTX (0.25 mM) and calcium ions (7.5 mM) at 300 K. (A) Raw data obtained from 48 automatic injections (6 μL each) of Ca^{2+} and (B) the integrated fitting curve showing the experimental points with the value of the parameter, $n = 2$, by using a one-site binding fitting function.

our ITC results (Figure 3) indicated that the association constant (K_{ass}) of the ASTX/ Ca^{2+} complex prepared in $\text{CH}_2\text{-Cl}_2/\text{ACN}$ (1:1) was 2830 ± 546 (M^{-2}) at 300 K and the molar ratio was 1:2. In addition, the results of ITC showed not only a favorable enthalpy change ($\Delta H = -4140 \pm 95.65$ cal/mol) but also an increase in entropy ($\Delta S = 1.96$ cal/

(16) (a) Haj-Zaroubi, M.; Mitzel, N. W.; Schmidtchen, F. P. *Angew. Chem., Int. Ed. Engl.* **2002**, *41*, 104–107. (b) Linton, B. R.; Goodman, M. S.; Fan, E.; van Arman, S. A.; Hamilton, A. D. *J. Org. Chem.* **2001**, *66*, 7313–7319. (c) Davis, A. P.; Wareham, R. S. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 2978–2996.

(mol K)). We therefore speculated that the calcium ion might coordinate with several solvents or counter molecules before complexation with ASTX. Once the ASTX/ Ca^{2+} complex formed, the boundary molecules on Ca^{2+} were liberated, and this would account for the positive entropy change.

In summary, the present study demonstrates that *trans*-ASTX provides binding capacities for two calcium ions and therefore provides a reasonable explanation for the calcium-induced slight increase in absorbance in the UV/vis spectra. The main chelating site is the α -hydroxycyclohexenone. Such a calcium-chelating activity of ASTX may provide a rationale for the protective effects of ASTX against calcium-induced lens turbidity⁹ because the presence of ASTX would result in less residual free calcium for calpain activation and would indirectly protect against proteolysis. Moreover, at body temperature (37 $^\circ\text{C}$), a regioselective isomerization of ASTX in the presence of calcium did not change the proportion of 13-*cis*-ASTX at 48 h. This result may partially explain the reported relative accumulation of the 13-*cis* isomer over 9-*cis* and all-*trans* ASTX in human plasma after supplementation with dietary ASTX.¹⁷ Nonetheless, the information on how temperature or endogenous metals other than Ca^{2+} affect the isomerization of ASTX and influence its biological activity in mammals is still limited. We are currently studying the effects of various divalent metals on *cis/trans* ASTX at various temperatures.

Acknowledgment. The authors thank Dr. Shu-Chuan Jao, Institute of Biological Chemistry, Academia Sinica in Taiwan for helpful discussions on ITC and NMR analyses. This work was supported by the National Science Council, Taiwan (NSC93-2320-B038-053), and the Shin Kong Wu Ho-Su Memorial Hospital, Taiwan (SKH-TMU-96-14).

Note Added after ASAP Publication: In Table 1, sixth column head, the H should have been a C, this was incorrect in the version published July 13, 2007; it was corrected in the version published July 23, 2007.

Supporting Information Available: UV/vis spectra, Job plot, and HMQC and HMBC spectra of complex formation studies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

OL0709533

(17) (a) Osterlie, M.; Bjerkeng, B.; Liaaen-Jensen, S. *J. Nutr. Biochem.* **2000**, *11*, 482–490. (b) Coral-Hinojosa, G. N.; Ytrestoyl, T.; Ruyter, B.; Bjerkeng, B. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2004**, *139*, 99–110.