Cyclic Peptides from Higher Plants. XXVIII.[1] Antitumor Activity and Hepatic Microsomal Biotransformation of Cyclic Pentapeptides, Astins, from *Aster tataricus*

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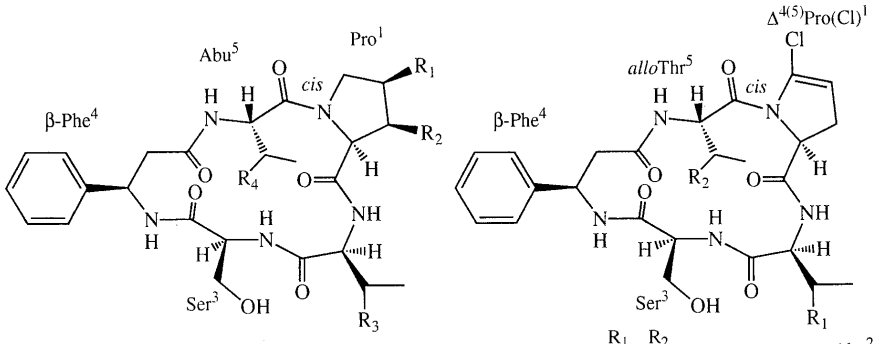
Antitumor activities on Sarcoma 180A of a series of cyclic pentapeptides, astins, isolated from the roots of *Aster tataricus*, were examined. The activities on various congeners of the dichlorinated proline residues prepared by chemical conversion and a hepatic microsomal biotransformation in rats suggested that 1, 2-*cis* dichlorinated proline residues of astins A, B and C play an important role in the antitumor activity of astins.

Key words antitumor activity; astin; *cis*-dichlorinated proline; *Aster tataricus*; hepatic microsomal biotransformation

Based on the existence of many naturally occurring cyclic peptides with unique structures and biological activities, we have focused our attention on new cyclic peptides with various biological activities from higher plants.[1,2] As part of this program, we reported in 1993 on the structures of antitumor cyclic pentapeptides, called astins A (**1**), B (**2**) and C (**3**), containing a 16-membered ring system with a unique 3,4-dichlorinated proline [Pro(Cl_2)], an *allo* threonine [*allo*Thr], a serine [Ser], a β-phenylalanine [β-Phe] and an α-aminobutyric acid [Abu], from the roots of *Aster tataricus* (Compositae).[3] Astins D—I with derivatives of Pro(Cl_2) were thereafter isolated as minor constituents.[4]

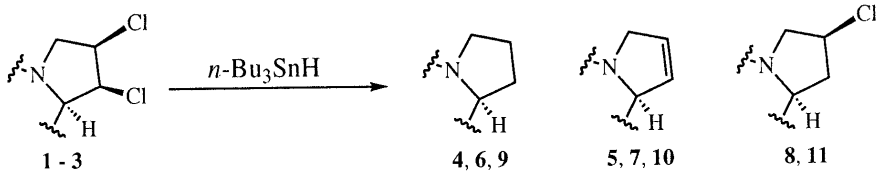
The conformation of astin B in solid state[5–7] was earlier shown to be different from that of cyclochlorotine,[8] a toxic principle, isolated from *Penicillium islandicum* SOPP., with a *trans* proline amide bond and a typical type I β-turn between Pro^1 and Abu^2, by a comparison of X-ray diffraction studies. In solution, the conformations of astin B, and astins A and C were shown to be different from each other by the nuclear Overhauser effect (NOE) correlation studies around the residues of 2 and 3 in Fig. 1.[7,9] The replacement of amide bonds in **1**, **2** and **3** with thioamide bonds was recently reported and the produced thioastins showed more promising antitumor activities than their parent compounds.[10]

The present paper describes antitumor activities of a series of astins and various congeners at the Pro(Cl_2) residues of **1**—**3**. The importance of the presence of the Pro(Cl_2) residues for antitumor activity is also mentioned on the basis of their activities and that of a metabolite of **3** by hepatic microsomal biotransformation in rats.



	R_1	R_2	R_3	R_4
Astin A (**1**):	Cl	Cl	H	OH
B (**2**):	Cl	Cl	OH	H
C (**3**):	Cl	Cl	H	H
F:	H	Cl	H	H
G:	H	H	H	H
I:	Cl	OH	H	H

Fig. 1. Structures of Astins A—I, Pro Was Provisionally Numbered as the First Amino Acid



1 - 3 **4, 6, 9** **5, 7, 10** **8, 11**

Chart 1. Compounds **4** and **5** Were Derived from Astin A (**1**) and **6**, **7** and **8** from Astin B (**2**), and **9**, **10** and **11** from Astin C (**3**)

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Table 1. ^{1}H-NMR Chemical Shifts (ppm) for Derivatives (**4**, **6** and **9**)

Proton	**4**	**6**	**9**
Pro[1]			
H_α	5.62 (d, 8.2)	4.93 (m)	4.86 (d, 7.7)
$H_{\beta1}$	2.21 (m)	2.07 (m)	2.10 (m)
$H_{\beta2}$	2.55 (m)	2.85 (m)	2.66 (m)
$H_{\gamma1}$	1.74 (m)	1.79 (2H, m)	1.79 (2H, m)
$H_{\gamma2}$	1.87 (m)		
$H_{\delta1}$	3.81 (2H, m)	3.77 (2H, t, 7.2)	3.73 (m)
$H_{\delta2}$			3.78 (m)
Abu[2](*allo* Thr[2])			
H_α	5.14 (m)	5.21 (t, 9.8)	5.07 (m)
H_β	2.21 (2H, m)	4.93 (m)	2.12 (2H, m)
H_γ	1.07 (t, 7.3)	1.63 (d, 6.3)	1.03 (t, 7.3)
NH	8.57 (d, 8.1)	9.40 (d, 9.8)	8.74 (d, 8.8)
Ser[3]			
H_α	4.63 (m)	4.73 (m)	4.68 (m)
$H_{\beta1}$	4.50 (dd, 4.5, 10.4)	4.21 (dd, 3.8, 10.9)	4.39 (dd, 4.5, 11.0)
$H_{\beta2}$	4.59 (dd, 6.3, 10.4)	4.42 (dd, 4.8, 10.9)	4.49 (dd, 5.9, 11.0)
NH	9.35 (d, 6.0)	10.00 (d, 4.9)	8.84 (d, 6.0)
β-Phe[4]			
$H_{\alpha1}$	2.82 (dd, 9.1, 13.6)	2.57 (t, 12.7)	2.69 (dd, 10.3, 13.2)
$H_{\alpha2}$	3.10 (dd, 4.9, 13.6)	3.17 (dd, 4.8, 12.7)	3.11 (dd, 4.9, 13.2)
H_β	5.53 (ddd, 4.9, 6.1, 9.1)	5.52 (ddd, 4.8, 5.6, 12.7)	5.53 (ddd, 4.9, 6.2, 10.3)
H_δ	7.47 (2H, d, 7.5)	7.35 (2H, d, 7.1)	7.41 (2H, d, 7.3)
H_ε	7.25 (2H, t, 7.5)	7.14 (2H, t, 7.1)	7.18 (2H, t, 7.3)
H_ζ	7.15 (t, 7.5)	7.11 (t, 7.1)	7.13 (t, 7.3)
NH	9.14 (d, 6.1)	8.29 (d, 5.6)	8.83 (d, 6.2)
allo Thr[5](Abu[5])			
H_α	5.16 (m)	4.93 (m)	4.82 (m)
H_β	4.41 (m)	1.85 (2H, m)	1.79 (2H, m)
H_γ	1.52 (d, 6.1)	1.00 (t, 7.4)	0.94 (t, 7.4)
NH	9.73 (d, 6.4)	9.90 (d, 5.6)	9.78 (d, 4.7)

Measurements were performed in pyridine-d_5 at 400 MHz. Multiplicity and coupling constants (J/Hz) are shown in parentheses.

Table 2. ^{1}H-NMR Chemical Shifts (ppm) for Derivatives (**5**, **7** and **10**)

Proton	**5**	**7**	**10**
Pro[1]			
H_α	6.37 (m)	5.69 (m)	5.58 (m)
H_β	5.84 (m)	5.92 (m)	5.93 (m)
H_γ	6.15 (m)	6.33 (m)	6.22 (m)
$H_{\delta1}$	4.44 (m)	4.23 (m)	4.44 (m)
$H_{\delta2}$	4.76 (m)	4.42 (m)	4.69 (m)
Abu[2](*allo* Thr[2])			
H_α	5.20 (m)	5.10 (m)	5.10 (m)
$H_{\beta1}$	2.04 (m)	4.87 (m)	2.04 (m)
$H_{\beta2}$	2.12 (m)		2.12 (m)
H_γ	1.05 (t, 7.4)	1.56 (d, 6.1)	1.01 (t, 7.3)
NH	8.35 (d, 7.9)	9.22 (d, 9.8)	8.48 (d, 8.4)
Ser[3]			
H_α	4.57 (m)	4.70 (m)	4.62 (m)
$H_{\beta1}$	4.59 (2H, m)	4.23 (dd, 4.0, 10.9)	4.47 (m)
$H_{\beta2}$		4.42 (dd, 5.0, 10.9)	4.53 (m)
NH	9.63 (d, 6.8)	10.02 (d, 4.8)	9.20 (d, 5.8)
β-Phe[4]			
$H_{\alpha1}$	2.88 (dd, 7.8, 13.7)	2.62 (dd, 11.4, 13.0)	2.80 (dd, 9.0, 13.4)
$H_{\alpha2}$	3.07 (dd, 5.2, 13.7)	3.20 (dd, 5.0, 13.0)	3.07 (dd, 5.2, 13.4)
H_β	5.49 (ddd, 5.2, 6.1, 7.8)	5.57 (ddd, 5.0, 6.0, 11.4)	5.53 (ddd, 5.2, 6.3, 9.0)
H_δ	7.50 (2H, d, 7.4)	7.36 (2H, d, 7.1)	7.45 (2H, d, 7.3)
H_ε	7.28 (2H, t, 7.4)	7.15 (2H, t, 7.1)	7.23 (2H, t, 7.3)
H_ζ	7.18 (t, 7.4)	7.12 (t, 7.1)	7.16 (t, 7.3)
NH	9.30 (d, 6.1)	8.30 (d, 6.0)	9.08 (d, 6.3)
allo Thr[5](Abu[5])			
H_α	5.27 (m)	5.17 (m)	4.99 (m)
$H_{\beta1}$	4.39 (m)	1.83 (2H, m)	1.71 (m)
$H_{\beta2}$			1.81 (m)
H_γ	1.53 (d, 6.1)	1.03 (t, 7.4)	0.95 (t, 7.4)
NH	9.63 (d, 6.8)	9.90 (d, 3.5)	9.68 (d, 5.1)

Measurements were performed in pyridine-d_5 at 400 MHz. Multiplicity and coupling constants (J/Hz) are shown in parentheses.

Results and Discussion

Derivatives of Dichlorinated Proline Residues of Astins A, B and C To clarify the relationship between the antitumor activity and the presence of dichlorinated proline residues of **1**—**3**, derivatives with dechlorinated prolines were prepared by dechlorination of **1**—**3** with tributyltinhydride in tetrahydrofuran (THF) containing α,α′-azobisisobutylonitrile. Three types of proline derivatives (**4**—**11**) were produced after purification using reversed phase HPLC (Chart 1). On reaction of **1**, only the corresponding proline and dehydroproline derivatives (**4** and **5**) were produced and monochlorinated derivatives were not observed. The spectroscopic data of the proline derivative **9** prepared from **3** was completely identical with those of astin G,[4] previously isolated from the roots of *A. tataricus* as one of the minor constituents. The positions of each double bond and monochlorine atom were determined by ^{1}H-NMR spectra as follows. The ^{1}H resonance assignments of **4**—**11** were achieved by use of ^{1}H–^{1}H correlation spectroscopy (COSY) spectra and are summarized in Tables 1, 2 and 3. Compounds **4**, **6** and **9** were elucidated to be the corresponding proline derivatives by FAB-MS spectra and ^{1}H coupling sequence from H_α in each proline residue. In compounds **5**, **7** and **10**, each H_α was coupled with an olefinic proton and also long-range coupled with another olefinic proton, and the two olefinic protons were coupled with each other. Therefore, the position of double bonds was found to be $\Delta^{3(4)}$. In compounds **8** and **11**, which were deduced to be monochlorinated derivatives by FAB-MS spectra, each H_α proton was coupled with methylenic protons, which were also coupled with a methine proton attached to monochlorinated bearing carbon.

Furthermore, dihydroxylation at the olefinic bonds of the above dehydroproline derivatives (**5**, **7** and **10**) by osmium tetraoxide produced the corresponding *cis* dihydroxy derivatives (**12**, **13** and **14**) (Chart 2). The configurations of hydroxyl groups at β and γ positions of **12**—**14** were determined to be α by small coupling constants between H_α and H_β (1.7 Hz), by comparison with those of **1**—**3** (5.9 Hz) and the presence of NOE between H_β and H_γ in a phase sensitive NOESY spectra in Fig. 2.[11] The ^{1}H chemical shifts and coupling constants of **12**—**14** are summarized in Table 4.

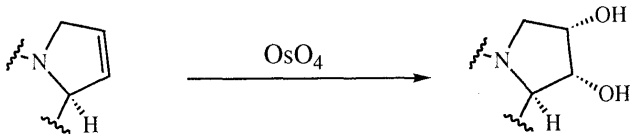
To clarify the influence of the other halogen on antitumor activity, the substituent effect of bromine was examined (Chart 3). Bromination at the olefinic bonds of **5**, **7** and **10** also produced the corresponding *trans* β, γ-brominated derivatives (**15**—**17**). Each configuration at β and γ positions of **15**—**17** was elucidated by the coupling constants around the proline residue as shown in Tables 5 and 6. The H_α and H_β protons in **15**—**17** were observed as singlets. While, in **1**—**3**, the coupling constants between H_α and H_β, and between H_β and H_γ were observed as 5.9 and 4.4 Hz, respectively. Therefore, the configuration of β bromo position was α and that of γ was β. Upon bromination of **3**, a minor bromopeptide (**18**) was also obtained in 13% yield. In the compound **18**, the coupling constant between H_α and H_β was observed to be 5.9 Hz, as in **1**—**3**, and that between H_β and H_γ to be 0.0 Hz. Therefore, the configurations of β, γ-bromo positions of **18** were determined to be 3β, 4α in Fig. 3.

Antitumor Activity of Astins and Their Derivatives Antitumor activity was examined by the total packed cell volume method using Sarcoma 180 ascites in mice.[12]

Table 3. ^{1}H-NMR Chemical Shifts (ppm) for Derivatives (**8** and **11**)

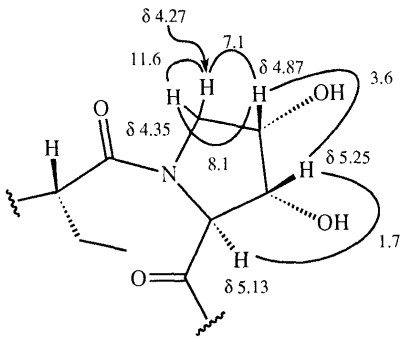
Proton	8	11
Pro(Cl)[1]		
H_α	5.11 (d, 9.1)	5.05 (d, 8.8)
$H_{\beta1}$	3.34 (d, 14.3)	3.26 (d, 14.3)
$H_{\beta2}$	2.75 (ddd, 5.2, 9.1, 14.3)	2.78 (ddd, 5.0, 8.8, 14.3)
H_γ	4.74 (m)	4.72 (m)
$H_{\delta1}$	4.13 (d, 14.2)	4.12 (d, 14.0)
$H_{\delta2}$	4.34 (dd, 5.3, 14.2)	4.34 (dd, 5.0, 14.0)
allo Thr[2](Abu[2])		
H_α	5.27 (m)	5.15 (m)
$H_{\beta1}$	5.02 (m)	2.23 (2H, m)
H_γ	1.75 (d, 6.1)	1.13 (t, 7.3)
NH	9.61 (d, 9.9)	9.18 (d, 9.3)
Ser[3]		
H_α	4.74 (m)	4.72 (m)
$H_{\beta1}$	4.19 (dd, 3.7, 10.9)	4.32 (dd, 4.8, 11.0)
$H_{\beta2}$	4.41 (dd, 4.5, 10.9)	4.46 (dd, 5.3, 11.0)
NH	10.13 (d, 4.8)	8.45 (d, 5.4)
β-Phe[4]		
$H_{\alpha1}$	2.57 (t, 12.6)	2.67 (dd, 11.9, 12.9)
$H_{\alpha2}$	3.32 (dd, 4.9, 12.6)	3.17 (dd, 4.9, 12.9)
H_β	5.60 (ddd, 4.9, 5.9, 12.6)	5.62 (ddd, 4.9, 6.3, 11.9)
H_δ	7.34 (2H, d, 6.9)	7.38 (2H, d, 7.1)
H_ε	7.14 (2H, t, 6.9)	7.16 (2H, t, 7.1)
H_ζ	7.11 (t, 6.9)	7.12 (t, 7.1)
NH	8.11 (d, 5.9)	8.40 (d, 6.3)
Abu[5]		
H_α	5.23 (m)	5.08 (m)
H_β	1.86 (2H, m)	1.83 (2H, m)
H_γ	1.05 (t, 7.4)	1.00 (t, 7.4)
NH	10.07 (d, 2.6)	9.94 (d, 3.9)

Measurements were performed in pyridine-d_5 at 400 MHz. Multiplicity and coupling constants (*J*/Hz) are shown in parentheses.



5, **7**, **10** **12**, **13**, **14**

Chart 2. Compound **12** Was Derived from **5**, **13** from **7**, and **14** from **10**



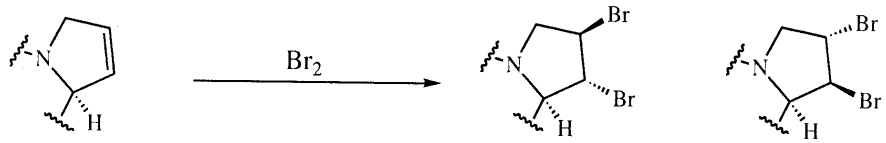
14

Fig. 2. Coupling Correlations around Pro Residues of **14**; Numbers Show Coupling Constants

Table 4. ^{1}H-NMR Chemical Shifts (ppm) for Derivatives (**12**—**14**)

Proton	**12**	**13**	**14**
Pro[1]			
H_α	5.82 (d, 1.0)	5.12 (s)	5.13 (d, 1.7)
H_β	5.19 (dd, 1.0, 3.8)	5.42 (d, 3.1)	5.25 (dd, 1.7, 3.6)
$H_{\gamma 1}$	4.93 (dt, 3.8, 7.9)	4.87 (ddd, 3.1, 7.4, 8.7)	4.87 (ddd, 3.6, 7.1, 8.1)
$H_{\delta 1}$	4.35 (2H, m)	4.28 (dd, 7.4, 11.4)	4.27 (dd, 7.1, 11.6)
$H_{\delta 2}$		4.40 (dd, 8.7, 11.4)	4.35 (dd, 8.1, 11.6)
Abu[2](*allo* Thr[2])			
H_α	5.13 (m)	5.23 (t, 9.8)	5.08 (m)
$H_{\beta 1}$	2.14 (2H, m)	4.98 (m)	2.14 (2H, m)
H_γ	1.01 (t, 7.3)	1.61 (d, 6.1)	1.01 (t, 7.3)
NH	8.88 (d, 8.3)	9.57 (d, 9.8)	8.96 (d, 8.8)
Ser[3]			
H_α	4.68 (m)	4.75 (m)	4.72 (m)
$H_{\beta 1}$	4.47 (dd, 5.4, 11.0)	4.22 (dd, 3.9, 10.8)	4.41 (dd, 4.6, 11.0)
$H_{\beta 2}$	4.55 (dd, 6.3, 11.0)	4.42 (dd, 4.8, 10.8)	4.51 (dd, 6.0, 11.0)
NH	9.29 (d, 6.1)	10.04 (d, 4.9)	9.00 (d, 6.0)
β-Phe[4]			
$H_{\alpha 1}$	2.85 (dd, 9.5, 13.6)	2.59 (t, 12.6)	2.74 (dd, 9.9, 13.3)
$H_{\alpha 2}$	3.12 (dd, 4.9, 13.6)	3.16 (dd, 4.8, 12.6)	3.08 (dd, 4.8, 13.3)
H_β	5.56 (ddd, 4.9, 6.1, 9.5)	5.53 (ddd, 4.8, 5.7, 12.6)	5.55 (ddd, 4.8, 6.7, 9.9)
H_δ			
H_ε	7.21—7.46 (m)	7.10—7.34 (m)	7.12—7.43 (m)
H_ζ			
NH	9.06 (d, 6.1)	8.33 (d, 5.7)	8.95 (d, 6.7)
allo Thr[5](Abu[5])			
H_α	5.25 (dd, 6.1, 7.7)	5.02 (m)	4.92 (m)
$H_{\beta 1}$	4.48 (m)	1.82 (2H, m)	1.76 (m)
$H_{\beta 2}$			1.91 (m)
H_γ	1.53 (d, 6.2)	1.00 (t, 7.4)	0.98 (t, 7.5)
NH	9.67 (d, 6.1)	9.90 (d, 5.6)	9.77 (d, 5.0)

Measurements were performed in pyridine-d_5 at 400 MHz. Multiplicity and coupling constants (J/Hz) are shown in parentheses.



5, **7**, **10** **15**, **16**, **17** **18**

Chart 3. Compound **15** Was Derived from **5** and **16** from **7**, and **17** and **18** from **10**

The effectiveness was evaluated in terms of the tumor growth ratio [GR(%)=(test group packed cell volume/control group packed cell volume)×100]. When administered for 5 d at a dose of 0.5 mg/kg/d (**1**), 0.5 mg/kg/d (**2**) and 5.0 mg/kg/d (**3**), astins A, B and C gave the GR values of 40%, 26% and 45%, respectively, whereas the other natural astins (astins D—J) and the derivatives of dichlorinated proline residues of **1**—**3** shown above did not inhibit the tumor growth at 10.0 mg/kg/d.

We previously suggested,[9] that astins A and C, with weaker activity than astin B took different backbone conformations from that of astin B, and that therefore the backbone conformations of astins were believed to affect the antitumor activity of astins. However, the presence of *cis* dichlorinated proline residues was then concluded to be a more important structural motif for astins to show antitumor activity on S-180A.

Hepatic Microsomal Biotransformation in Rats We were interested in studying the relationship between the metabolic disposition of astins and the antitumor activity of the metabolites. Therefore, we attempted to understand the astin metabolism by clarifying the structures of the metabolites *in vitro*. To isolate the metabolites of astin C (**3**), a major component in this series of cyclic pentapeptides, the biotransformation was examined by means of rat hepatic microsome. An important characteristic of cytochrome P450 is that it oxidizes a wide variety of xenobiotics in various types of reaction by the reductive activation of molecular oxygen.[13] Astin C was incubated aerobically with rat liver microsomes in the presence of an NADP-generating system (Chart 4). After removal of protein with acetonitrile, the butanol extract of the incubation mixture showed the presence of a metabolite in HPLC performed with 55% aqueous methanol with 0.05% trifluoroacetic acid (TFA) using octadecyl silica (ODS) column, which was identical with the acyclic pentapeptide, astin J,[4] without antitumor activity. It was reported earlier that acyclic astins were chemically derived from cyclic astins under basic conditions.[4] Astin J could be obtained by 2 N NaOH treatment of astin C in THF. This decyclization process was thought to be produced by dechlorination and aromatization from Pro(Cl_2) to pyrrole under basic conditions, following the cleavage of the amide bond in Pro. The metabolic process

Table 5. ^{1}H-NMR Chemical Shifts (ppm) for Derivatives (**15** and **16**)

Proton	**15**	**16**
Pro(Br)$_2$[1]		
H_α	5.17 (s)	4.77 (s)
H_β	5.42 (s)	5.28 (s)
H_γ	4.97 (d, 5.2)	5.00 (d, 5.4)
$H_{\delta1}$	3.81 (d, 14.8)	3.80 (d, 14.9)
$H_{\delta2}$	4.44 (dd, 5.2, 14.8)	4.43 (dd, 5.4, 14.9)
Abu[2](*allo* Thr[2])		
H_α	4.39 (dd, 4.5, 9.6)	4.27 (m)
$H_{\beta1}$	1.89 (m)	4.27 (m)
$H_{\beta2}$	2.08 (m)	5.81 (d, 5.8, OH)
H_γ	0.97 (t, 7.3)	1.23 (d, 5.5)
NH	8.75 (d, 9.6)	8.85 (d, 9.7)
Ser[3]		
H_α	3.88 (m)	3.86 (m)
$H_{\beta1}$	3.67 (m)	3.64 (2H, m)
$H_{\beta2}$	3.72 (m)	
	4.98 (br d, 8.3, OH)	
NH	7.70 (d, 5.8)	8.88 (d, 4.8)
β-Phe[4]		
$H_{\alpha1}$	2.12 (t, 12.6)	2.12 (t, 12.4)
$H_{\alpha2}$	2.89 (dd, 4.6, 12.6)	2.87 (dd, 4.5, 12.4)
H_β	4.92 (ddd, 4.6, 7.0, 12.6)	4.90 (ddd, 4.5, 6.7, 12.4)
H_δ		
H_ε	7.22—7.33 (5H, m)	7.22—7.32 (5H, m)
H_ζ		
NH	7.39 (d, 7.0)	7.40 (d, 6.7)
allo Thr[5](Abu[5])		
H_α	4.27 (dd, 4.5, 9.0)	4.27 (m)
$H_{\beta1}$	3.77 (m)	1.64 (2H, m)
$H_{\beta2}$	5.44 (d, 4.6, OH)	
H_γ	1.20 (d, 6.0)	1.02 (t, 7.4)
NH	8.57 (d, 4.5)	8.81 (d, 3.3)

Measurements were performed in DMSO-d_6 at 400 MHz. Multiplicity and coupling constants (J/Hz) are shown in parentheses.

Table 6. ^{1}H-NMR Chemical Shifts (ppm) for Derivatives (**17** and **18**)

Proton	**17**	**18**
Pro(Br)$_2$[1]		
H_α	4.82 (s)	4.98 (d, 5.9)
H_β	5.27 (s)	5.32 (d, 5.9)
H_γ	5.00 (d, 5.6)	4.96 (d, 3.6)
$H_{\delta1}$	3.82 (d, 14.8)	4.17 (dd, 3.6, 13.7)
$H_{\delta2}$	4.45 (dd, 5.6, 14.8)	4.29 (d, 13.7)
Abu[2]		
H_α	4.38 (m)	4.34 (m)
$H_{\beta1}$	1.88 (m)	1.55 (m)
$H_{\beta2}$	2.09 (m)	1.72 (m)
H_γ	0.97 (t, 7.3)	0.95 (t, 7.3)
NH	8.62 (d, 9.5)	8.17 (d, 9.0)
Ser[3]		
H_α	3.87 (m)	3.79 (m)
$H_{\beta1}$	3.64 (m)	3.70 (2H, m)
$H_{\beta2}$	3.73 (m)	
		4.94 (t, 5.5, OH)
NH	7.73 (d, 5.8)	7.94 (d, 5.6)
β-Phe[4]		
$H_{\alpha1}$	2.16 (t, 12.6)	2.30 (dd, 11.2, 13.4)
$H_{\alpha2}$	2.89 (dd, 4.5, 12.6)	2.78 (dd, 4.7, 13.4)
H_β	4.94 (ddd, 4.5, 6.9, 12.6)	4.91 (ddd, 4.7, 6.7, 11.2)
H_δ		
H_ε	7.23—7.32 (5H, m)	7.21—7.32 (5H, m)
H_ζ		
NH	7.47 (d, 6.9)	7.75 (d, 6.7)
Abu[5]		
H_α	4.21 (m)	4.36 (m)
$H_{\beta1}$	1.64 (2H, m)	1.78 (m)
$H_{\beta2}$		2.01 (m)
H_γ	1.00 (t, 7.3)	0.95 (t, 7.3)
NH	8.73 (d, 3.5)	8.42 (d, 5.0)

Measurements were performed in DMSO-d_6 at 400 MHz. Multiplicity and coupling constants (J/Hz) are shown in parentheses.

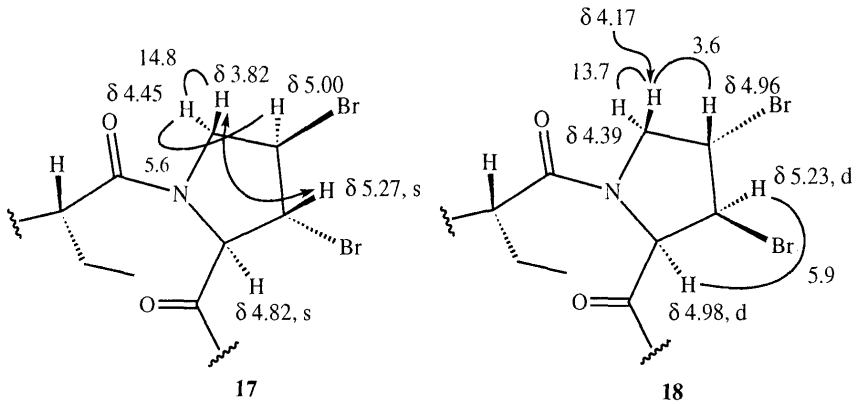


Fig. 3. Coupling Correlations around Pro Residues of **17** and **18**; Arrows Show Long-Range Coupling and Numbers Show Coupling Constants

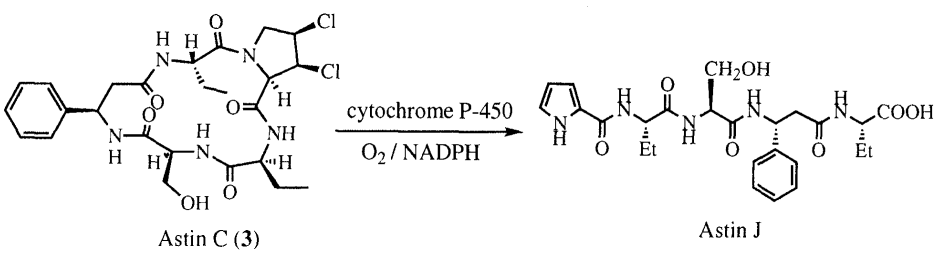


Chart 4. Hepatic Microsomal Biotransformation from Astin C (**3**) to Astin J

of astin C to astin J was believed to be detoxification and the importance of cyclic nature and the presence of *cis*-dichlorinated proline residue in showing antitumor activity was also suggested.

Experimental

General Details Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 spectrometer and the $[\alpha]_D$ values are given in 10^{-1} deg·cm^2 g^{-1}. FAB and high resolution mass spectra were taken with a VG Autospec spectrometer. IR spectrum was recorded on a Perkin Elmer 1710 spectrophotometer. HPLC was performed with an Inertsil PREP-ODS column (20 mm i.d. × 250 mm, GL Science Inc.) packed with 10 μm ODS. TLC was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck) and the spots were detected by spraying Dragendorff reagent[14] and iodine. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker spectrometers (AM400 and AM500) at 303 K and processed on a Bruker data station with an Aspect 3000 computer. Phase sensitive nuclear Overhauser effect spectroscopy (NOESY) experiments were made with a mixing time of 0.6 s. The NMR coupling constants (J) are given in Hz.

Materials Astins A, B and C were prepared according to the previous procedure.[6]

Dechlorination of Astin A (1) Solution of **1** (20 mg, 0.034 mmol) and α,α'-azobisisobutylonitrile (4.0 mg) in tetrahydrofuran (4 ml) and *n*-tributyltinhydride (60 μl, 0.206 mmol) were stirred at 100 °C. After 24 h, the reaction mixture was concentrated to dryness and the reversed phase HPLC of the residue using 25% MeOH in H_2O as eluent gave proline derivative, **4** in 38% yield and dehydro derivative, **5** in 29% yield, respectively.

Compound 4 Colorless needles, mp 263—265 °C, $[\alpha]_D$ −121.7° (c=0.29, MeOH). MS m/z: 517 $(M)^+$ [Found: M^+, 517.2353. $C_{25}H_{35}N_5O_7$ requires, 517.2353]. IR ν_{max}^{KBr} cm^{-1}: 3300 (NH), 1630 (amide C=O), 1535, 1505, 1430. ^{13}C-NMR δ_C (100 MHz, pyridine-d_5): 10.61 (q), 21.60 (q), 22.94 (t), 25.98 (t), 31.81 (t), 42.16 (t), 47.25 (t), 52.30 (d), 55.51 (d), 59.49 (d), 60.07 (d), 60.95 (t), 62.14 (d), 68.40 (d), 126.78 (d) × 2, 127.12 (d), 128.78 (d) × 2, 143.20 (s), 170.27 (s), 171.67 (s), 172.21 (s), 172.55 (s), 172.73 (s).

Compound 5 Colorless powder, $[\alpha]_D$ −203.7° (c=0.21, MeOH). MS m/z: 516 $(M+H)^+$ [Found: $(M+H)^+$, 516.2476. $C_{25}H_{34}N_5O_7$ requires, 516.2458]. IR ν_{max}^{KBr} cm^{-1}: 3300 (NH), 1640 (amide C=O), 1525, 1435, 1210. ^{13}C-NMR δ_C (100 MHz, pyridine-d_5): 10.42 (q), 21.67 (q), 26.50 (t), 41.87 (t), 52.17 (d), 54.62 (t), 55.08 (d), 59.03 (d), 60.17 (d), 60.87 (t), 68.96 (t), 69.63 (d), 126.97 (d) × 2, 127.09 (d) × 2, 128.27 (d), 128.80 (d) × 2, 143.15 (s), 170.14 (s), 170.85 (s), 171.36 (s), 172.54 (s), 172.83 (s).

Dechlorination of Astin B (2) A solution of **2** (20 mg, 0.034 mmol) and α,α'-azobisisobutylonitrile (4.0 mg) in tetrahydrofuran (4 ml) and *n*-tributyltinhydride (60 μl, 0.206 mmol) was stirred at 100 °C. After 12 h, the reaction mixture was concentrated to dryness and the reversed phase HPLC of the residue using 18% CH_3CN in H_2O as eluent gave proline derivative, **6** in 36% yield, dehydro derivative, **7** in 25% yield and monochlorinated derivative, **8** in 14 % yield, respectively.

Compound 6 Colorless needles, mp 234—236 °C, $[\alpha]_D$ −122.6° (c=0.51, MeOH). MS m/z: 518 $(M+H)^+$ [Found: $(M+H)^+$, 518.2651. $C_{25}H_{36}N_5O_7$ requires, 518.2615]. IR ν_{max}^{KBr} cm^{-1}: 3250 (NH), 1630 (amide C=O), 1520, 1450, 1325. ^{13}C-NMR δ_C (100 MHz, pyridine-d_5): 10.37 (q), 22.59 (q), 22.59 (t), 24.35 (t), 31.49 (t), 43.24 (t), 47.21 (t), 52.97 (d), 55.13 (d), 59.03 (d) × 2, 61.50 (t), 61.89 (d), 67.68 (d), 126.23 (d) × 2, 127.16 (d), 128.71 (d) × 2, 143.42 (s), 170.88 (s), 171.39 (s), 172.95 (s), 172.20 (s), 172.41 (s).

Compound 7 Colorless needles, mp 247—249 °C, $[\alpha]_D$ −180.4° (c=0.73, MeOH). MS m/z: 516 $(M+H)^+$ [Found: $(M+H)^+$, 516.2497. $C_{25}H_{34}N_5O_7$ requires, 516.2458]. IR ν_{max}^{KBr} cm^{-1}: 3300 (NH), 1640 (amide C=O) and 1540. ^{13}C-NMR δ_C (100 MHz, pyridine-d_5): 10.53 (q), 22.47 (q), 24.12 (t), 43.23 (t), 52.76 (d), 54.71 (t), 54.99 (d), 58.68 (d), 59.24 (d), 61.39 (t), 67.71 (d), 68.44 (d), 126.23 (d) × 2, 126.96 (d), 127.14 (d), 128.24 (d), 128.71 (d) × 2, 143.46 (s), 170.63 (s), 170.83 (s), 171.72 (s), 171.81 (s), 172.47 (s).

Compound 8 Colorless needles, mp 241—243 °C, $[\alpha]_D$ −83.0° (c=0.45, MeOH). MS m/z: 552 $(M+H)^+$ [Found: $(M+H)^+$, 552.2231. $C_{25}H_{35}N_5O_7Cl$ requires, 552.2225]. IR ν_{max}^{KBr} cm^{-1}: 3300 (NH), 1638 (amide C=O), 1550, 1540, 1520, 1510, 1458, 1438. ^{13}C-NMR δ_C (100 MHz, pyridine-d_5): 10.55 (q), 23.13 (q), 24.11 (t), 40.58 (t), 43.71 (t), 53.08 (d), 55.14 (d), 56.62 (d), 57.96 (t), 58.98 (d), 59.04 (d), 60.53 (d), 61.56 (t), 67.83 (d), 126.19 (d) × 2, 127.20 (d), 128.71 (d) × 2, 143.37 (s), 171.09 (s), 171.83 (s), 172.20 (s), 172.52 (s), 172.68 (s).

Dechlorination of Astin C (3) A solution of **3** (20 mg, 0.034 mmol) and α,α'-azobisisobutylonitrile (4.0 mg) in tetrahydrofuran (4 ml) and *n*-tributyltinhydride (60 μl, 0.206 mmol) was stirred at 100 °C. After 6 h, the reaction mixture was concentrated to dryness and the reversed phase HPLC of the residue using 23% CH_3CN in H_2O as eluent gave proline derivative, **9** in 31% yield, dehydro derivative, **10** in 39% yield and monochlorinated derivative, **11** in 10% yield, respectively.

Compound 9 Colorless needles, mp 289—291 °C, $[\alpha]_D$ −107.9° (c=1.14, MeOH). MS m/z: 502 $(M+H)^+$ [Found: $(M+H)^+$, 502.2711. $C_{25}H_{36}N_5O_6$ requires, 502.2666]. IR ν_{max}^{KBr} cm^{-1} 3325 (NH), 3080, 2990, 2950, 1645 (amide C=O), 1520, 1435. ^{13}C-NMR δ_C (100 MHz, pyridine-d_5): 10.26 (q), 10.84 (q), 22.51 (t), 24.66 (t), 25.11 (t), 31.79 (t), 42.45 (t), 47.10 (t), 52.44 (d), 54.39 (d), 55.54 (d), 59.80 (d), 61.02 (t), 61.88 (d), 126.51 (d) × 2, 127.12 (d), 128.72 (d) × 2, 143.13 (s), 170.36 (s), 171.50 (s), 171. 98 (s), 172.06 (s), 172.41 (s).

Compound 10 Colorless needles, mp 247—249 °C, $[\alpha]_D$ −218.2° (c=0.29, MeOH). MS m/z: 500 $(M+H)^+$ [Found: $(M+H)^+$, 500.2522. $C_{25}H_{34}N_5O_6$ requires, 500.2509]. ^{13}C-NMR δ_C (100 MHz, pyridine-d_5): 10.43 (q), 10.69 (q), 24.73 (t), 25.80 (t), 42.15 (t), 52.30 (d), 53.94 (d), 54.68 (t), 55.29 (d), 59.96 (d), 60.89 (t), 69.07 (d), 126.78 (d) × 2, 127.13 (d), 127.52 (d), 128.05 (d) × 2, 128.78 (d), 143.13 (s), 170.27 (s), 170.47 (s), 171.73 (s), 171.78 (s), 172.56 (s).

Compound 11 Colorless needles, mp 180—181 °C, $[\alpha]_D$ −92.4° (c=0.24, MeOH). MS m/z: 536 $(M+H)^+$ [Found: $(M+H)^+$, 536.2264. $C_{25}H_{35}N_5O_6Cl$ requires, 536.2276]. ^{13}C-NMR δ_C (100 MHz, pyridine-d_5): 10.49 (q), 11.30 (q), 24.34 (t), 24.54 (t), 40.93 (t), 43.33 (t), 52.75 (d), 54.80 (d), 55.54 (d), 56.52 (d), 57.82 (t), 59.94 (d), 60.60 (d), 61.17 (t), 126.37 (d) × 2, 127.23 (d), 128.75 (d) × 2, 143.23 (s), 170.73 (s), 171.71 (s), 172.40 (s) × 2, 172.44 (s).

Hydroxylation of Compound 5 A solution of **5** (30.4 mg, 0.059 mmol) and pyridine (6 drops) in dioxane (3 ml) and 2.5% (w/v) osmium tetraoxide *n*-BuOH solution (1200 μl, 0.118 mmol) was stirred at room temperature. After 12 h, 10% sodium hydrogensulfite solution (3 ml) was added to the mixture which was left standing for 20 min. The reaction mixture was subjected to HP-20 column chromatography, eluted with water and then methanol. The methanol eluted fraction was concentrated to dryness and the reversed phase HPLC of the residue using 15% CH_3CN in H_2O containing 0.05% trifluoroacetic acid as eluent gave a dihydroxy derivative, **12** in 32% yield.

Compound 12 Colorless needles, mp 182—184 °C, $[\alpha]_D$ −76.2° (c=0.21, MeOH). MS m/z: 550 $(M+H)^+$. ^{13}C-NMR δ_C (100 MHz, pyridine-d_5): 10.70 (q), 21.06 (q), 25.69 (t), 42.35 (t), 51.25 (t), 52.27 (d), 55.49 (d), 58.73 (d), 59.98 (d), 61.02 (t), 68.10 (d), 69.36 (d), 70.34 (d), 77.26 (d), 126.73 (d) × 2, 127.12 (d), 128.75 (d) × 2, 143.22 (s), 170.44 (s), 170.83 (s), 171.72 (s), 172.46 (s), 172.63 (s).

Hydroxylation of Compound 7 A solution of **7** (10.0 mg, 0.0194 mmol) and pyridine (2 drops) in dioxane (1 ml) and 2.5% (w/v) osmium tetraoxide *n*-BuOH solution (400 μl, 0.039 mmol) was stirred at room temperature. After 12 h, 10% sodium hydrogensulfite solution (1 ml) was added to the mixture which was left standing for 20 min. The reaction mixture was subjected to HP-20 column chromatography, eluted with water and then methanol. The methanol eluted fraction was concentrated to dryness and the reversed phase HPLC of the residue using 15% CH_3CN in H_2O containing 0.05% trifluoroacetic acid as eluent gave a dihydroxy derivative, **13** in 61% yield.

Compound 13 Colorless needles, mp 157—160 °C, $[\alpha]_D$ −66.2° (c=0.13, MeOH). MS m/z: 550 $(M+H)^+$. ^{13}C-NMR δ_C (100 MHz, pyridine-d_5): 10.58 (q), 22.63 (q), 24.68 (t), 43.31 (t), 51.02 (t), 53.00 (d), 54.47 (d), 58.85 (d), 59.07 (d), 61.47 (t), 67.62 (d), 69.03 (d), 70.15 (d), 76.77 (d), 126.23 (d) × 2, 127.19 (d), 128.73 (d) × 2, 143.42 (s), 170.67 (s), 170.92 (s), 171.88 (s), 172.29 (s), 172.42 (s).

Hydroxylation of Compound 10 A solution of **10** (12.0 mg, 0.0240 mmol) and pyridine (2 drops) in dioxane (1 ml) and 2.5% (w/v) osmium tetraoxide *n*-BuOH solution (247 μl, 0.024 mmol) was stirred at room temperature. After 12 h, 10% sodium hydrogensulfite solution (1 ml) was added to the mixture which was left standing for 20 min. The reaction mixture was subjected to HP-20 column chromatography, eluted with water and then methanol. The methanol eluted fraction was concentrated to dryness and the reversed phase HPLC of the residue using 20% CH_3CN in H_2O containing 0.05% trifluoroacetic acid as eluent gave a dihydroxy derivative, **14** in 72% yield.

Compound 14 Colorless needles, mp 185—187 °C, $[\alpha]_D$ −84.1° (c=0.18, MeOH). MS m/z: 534 $(M+H)^+$. ^{13}C-NMR δ_C (100 MHz, pyridine-d_5): 10.42 (q), 10.87 (q), 25.06 (t), 25.24 (t), 42.45 (t), 51.13 (t), 52.40 (d), 53.60 (d), 55.65 (d), 59.76 (d), 61.06 (t), 69.09 (d), 70.17 (d), 77.11 (d), 126.57 (d)×2, 127.14 (d), 128.76 (d)×2, 143.16 (s), 170.41 (s), 170.60 (s), 171.88 (s), 172.41 (d)×2.

Bromination of Compound 5 A solution of **5** (29.4 mg, 0.057 mmol) in dioxane (5 ml) and bromine (10 μl) was stirred at room temperature. After 12 h, the reaction mixture was concentrated to dryness and the reversed phase HPLC of the residue using 25% CH_3CN in H_2O as eluent gave a 3α, 4β-dibromoproline derivative, **15** in 19% yield.

Compound 15 Colorless needles, mp 193—194 °C, $[\alpha]_D$ −58.9° (c=0.15, MeOH). MS m/z: 674 $(M+H)^+$. ^{13}C-NMR δ_C (100 MHz, DMSO-d_6): 10.97 (q), 21.04 (q), 22.84 (t), 42.78 (t), 48.88 (d), 51.12 (d), 54.31 (d), 54.70 (d), 55.49 (t), 58.85 (d), 59.21 (d), 60.19 (t), 66.50 (d), 68.21 (d), 125.54 (d)×2, 126.71 (d), 128.23 (d)×2, 142.55 (s), 168.55 (s), 169.12 (s), 170.62 (s), 171.03 (s), 172.47 (s).

Bromination of Compound 7 A solution of **7** (17.6 mg, 0.034 mmol) in dioxane (5 ml) and bromine (10 μl) was stirred at room temperature. After 12 h, the reaction mixture was concentrated to dryness and the reversed phase HPLC of the residue using 30% CH_3CN in H_2O as eluent gave 3α,4β-dibromoproline derivative, **16** in 34% yield.

Compound 16 Colorless needles, mp 190—192 °C, $[\alpha]_D$ −62.4° (c=0.20, MeOH). MS m/z: 674 $(M+H)^+$. ^{13}C-NMR δ_C (100 MHz, DMSO-d_6): 10.33 (q), 22.18 (q), 23.14 (t), 42.73 (t), 47.87 (t), 51.59 (d), 53.60 (d), 54.41 (d), 55.34 (t), 57.54 (d), 57.96 (d), 60.33 (t), 66.00 (d), 69.27 (d), 125.57 (d)×2, 126.67 (d), 128.18 (d)×2, 142.69 (s), 167.64 (s), 169.15 (s), 170.06 (s), 171.46 (s), 171.63 (s).

Bromination of Compound 10 A solution of **10** (10.0 mg, 0.02 mmol) in dioxane (5 ml) and bromine (10 μl) was stirred at room temperature. After 12 h, the reaction mixture was concentrated to dryness and the reversed phase HPLC of the residue using 38% CH_3CN in H_2O as eluent gave a 3α,4β-dibromoproline derivative, **17** in 67% yield and a 3β,4α-dibromoproline derivative, **18** in 13% yield, respectively.

Compound 17 Colorless needles, mp 168—171 °C, $[\alpha]_D$ −89.4° (c=0.40, MeOH). MS m/z: 658 $(M+H)^+$ [Found: $(M+H)^+$, 658.0824. $C_{25}H_{34}N_5O_6Br_2$ requires, 658.0876]. ^{13}C-NMR δ_C (100 MHz, DMSO-d_6): 10.22 (q), 10.93 (q), 22.97 (t), 23.32 (t), 42.48 (t), 47.98 (d), 51.16 (d), 53.45 (d), 54.58 (d)×2, 55.31 (t), 59.13 (d), 60.16 (t), 69.28 (d), 125.56 (d)×2, 126.72 (d), 128.24 (d)×2, 142.54 (s), 167.51 (s), 169.06 (s), 170.58 (s), 171.23 (s), 171.64 (s).

Compound 18 Colorless needles, mp 225—227 °C, $[\alpha]_D$ −31.4° (c=0.14, MeOH). MS m/z: 658 $(M+H)^+$. ^{13}C-NMR δ_C (100 MHz, DMSO-d_6): 10.11 (q), 10.81 (q), 23.55 (t), 23.63 (t), 41.92 (t), 50.85 (d), 51.07 (d), 52.78 (d), 53.86 (t), 54.40 (d), 55.46 (d), 59.09 (d), 59.79 (t), 64.35 (d), 125.76 (d)×2, 126.58 (d), 128.16 (d)×2, 142.16 (s), 167.13 (s), 169.06 (s), 170.44 (s), 170.75 (s), 172.11 (s).

Assay of Antitumor Activity on Sarcoma 180 Ascites 5 week old ICR male mice weighing 23—25 g, supplied by Clea Japan Co., Ltd., were used in groups of 6 animals. Sarcoma 180 ascites, provided by the National Cancer Center Research Institute and maintained in successive generations by us, was implanted i.p. at 1×10^6 cells/body. A test drug was administered i.p. for 5 d starting on the day following the implantation. The effectiveness was evaluated by the total packed cell volume method[18]: growth ratio (GR%)=(packed cell volume (PCV) of test groups/PCV of control groups)×100.

Drug Treatment A 0.5% solution of carboxymethylcellulose (CMC) in isotonic sodium chloride was used as a vehicle for the injection of test drugs. Control mice received equal volumes of normal saline containing 0.5% CMC. The results were evaluated according to the standard methods described above.

Incubation Conditions and Extraction of a Metabolite of Astin C Astin C (0.5 mg) dissolved in 30 μl of dimethylsulfoxide was incubated with 200 μl of the S9-fraction and 800 μl of cofactor solution for S-9 Mix ($MgCl_2$ 8 μmol/ml, KCl 33 μmol/ml, glucose-6-phosphate 5 μmol/ml. NADPH 4 μmol/ml, NADH 4 μmol/ml, sodium phosphate buffer PH 7.4 100 μmol/ml) at 37 °C for 24 h. After the usual excision of protein with 800 μl of acetonitrile, the reaction mixture was subjected to HPLC under the following conditions: column (4 mm i.d.×250 mm ODS, GL Science Inc.), solvent (55% methanol with 0.05% TFA), flow rate (1 ml/min), UV detector (220 nm). The t_R values of astins J and C were 14.18 and 16.58, respectively).

Alkaline Catalyzed Cleavage of Astin C (3) Astin C (10 mg) was treated with 2 N NaOH in tetrahydrofuran overnight. The reaction mixture neutralized with dil. HCl was subjected to Diaion HP-20 column chromatography eluted with water and then methanol. The fraction eluted with methanol was concentrated to give astin J quantitatively.

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