Synthesis and Structure-activity Relationships of Amastatin Analogues, Inhibitors of Aminopeptidase A

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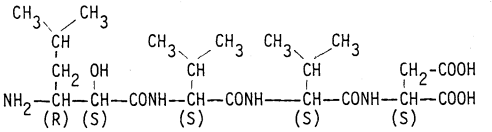
Stereoisomers and analogues of amastatin, [(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl]-L-Val-L-Val-L-Asp, were synthesized and their inhibitory activities towards aminopeptidase A (AP-A) and other arylamidases tested. Among the four stereoisomers of a new amino acid residue in amastatin, the 2*S* stereoisomers exhibited strong activity. In a series of compounds in which the C-terminal amino acid of amastatin was substituted by other amino acids, the one containing Asp or Glu showed the strongest activity towards AP-A. In a series of compounds in which the second or third residue from the amino terminal of amastatin was substituted by other amino acids, the one containing hydrophobic amino acids showed strong activity. In the study of the relationship of the length of the peptide chain and inhibitory activity, the activity towards AP-A was seen to increase until the length of the peptide reached that of a tetrapeptide.

Amastatin, which had been isolated from the culture filtrate of *Streptomyces* sp. ME98-M3, inhibits AP-A and Leu-AP but not AP-B. Amastatin is a competitive inhibitor of aminopeptidases with a Ki value of 1.0×10^{-6} M for human serum AP-A and 1.6×10^{-6} M for pig kidney Leu-AP.[1]



The structure of amastatin is [(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl]-L-Val-L-Val-L-Asp.[2] We synthesized stereoisomers of amastatin and related compounds and studied structure–activity relationships.

Synthesis

The method employed for the synthesis of (2*S*,3*R*)X and (2*R*,3*R*)X was as described previously.[2] In the same way, (2*R*,3*S*)X and (2*S*,3*S*)X were prepared from L-leucine. Other analogues were prepared using *Z*-glycine as a starting material in the same way. Resolution of the diastereoisomeric mixture of (2*S*,3*R*)X and (2*R*,3*R*)X was achieved by Dowex-50W column chromatography.[2] (2*R*,3*S*)X and (2*S*,3*S*)X were separated by the same method.

N-Benzyloxycarbonyl-X and its analogues

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Abbreviations: AP-A, aminopeptidase A (EC 3.4.11.7); AP-B, aminopeptidase B (EC 3.4.11.6); Leu-AP, leucine aminopeptidase (EC 3.4.11.1); Gly-Pro-AP, glycyl-proline dipeptidyl aminopeptidase (EC 3.4.14.1); Gly-Pro-Leu-AP, glycyl-prolyl-leucine tripeptidyl aminopeptidase; Gly-His-Lys-AP, glycyl-histidyl-lysine tripeptidyl aminopeptidase; NA, β-naphthylamine; Z, benzyloxycarbonyl; CBZ-S, benzyl *S*-(4,6-dimethylpyrimidin-2-yl) thiocarbonate; DMP, 3,5-dimethylpyrazole; DCC, dicyclohexylcarbodiimide; Boc, *tert*-butyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; Bzl, benzyl; Tos, tosyl; DCHA, dicyclohexylamine; DCU, dicyclohexylurea; THF, tetrahydrofuran; TFA, trifluoroacetic acid; X, 3-amino-2-hydroxy-5-methylhexanoic acid.

were coupled to peptide benzyl esters with DCC in the presence of HOBt. The protecting group was removed by hydrogenation with palladium-charcoal to yield amastatin analogues.

Structure-activity relationships

Arylamidases employed for testing of inhibitory activity of amastatin analogues were AP-A, AP-B, Leu-AP, Gly-Pro-AP, Gly-His-Lys-AP and Gly-Pro-Leu-AP, as shown in Table I.

Amastatin is a strong inhibitor of AP-A with a 50% inhibition concentration (IC_{50} value) of 1.1×10^{-6} M. There are three kinds of functional groups, NH_2, OH and COOH, in the amastatin molecule. The N-*t*-butyloxycarbonyl derivative and the dimethyl ester of amastatin had weak inhibitory activity (IC_{50} 2.6×10^{-5} and 3.4×10^{-5} M, respectively). These results indicated that the free amino group and the free carboxyl group are important. The role of the hydroxyl group is suggested by the activity of different stereoisomers as will be discussed later. (2*S*,3*R*)X itself showed no inhibitory activity.

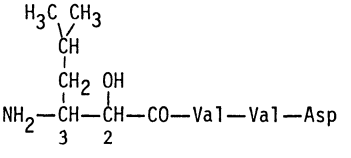
Bryce and Rabin[3] proposed the reaction mechanism for Leu-AP. We speculated that the inhibition mechanism of amastatin from their report was as shown in Scheme I. Amastatin shows strong inhibition of Leu-AP as well as AP-A. Binding of amastatin to the

TABLE I. ARYLAMIDASES EMPLOYED FOR TESTING INHIBITORY ACTIVITY OF AMASTATIN AND ITS ANALOGUES

Arylamidase	Enzyme source	Substrate (β-naphthylamide)
Aminopeptidase A (AP-A)	Human serum	Glu
Aminopeptidase B (AP-B)	Rat liver	Arg
Leucine aminopeptidase (Leu-AP)	Pig kidney	Leu
Gly-Pro dipeptidyl-aminopeptidase (Gly-Pro-AP)	Rat kidney	Gly-Pro
Gly-Pro-Leu tripeptidyl-aminopeptidase (Gly-Pro-Leu-AP)	Membrane fraction of rat liver	Gly-Pro-Leu
Gly-His-Lys tripeptidyl-aminopeptidase* (Gly-His-Lys-AP)	FM3A cell**	Gly-His-Lys

* Released from FM3A cells by thermolysin.
** Established from a mammary tumor of mouse strain C3H-He.

TABLE II. STEREOISOMERS OF X IN AMASTATIN



Compound	Stereochemistry		IC_{50} (μM)					
	2	3	AP-A	AP-B	Leu-AP	Gly-Pro-AP	Gly-Pro-Leu-AP	Gly-His-Lys-AP
Ia	*S*	*R*	1.1	>500	1.1	>500	0.1	0.6
Ib	*R*	*R*	180	>500	>500	>500	74	173
Ic	*R*	*S*	53	>500	44	>500	13	42
Id	*S*	*S*	10	>500	10	>500	1.3	4.2

active site of these enzymes is suggested by the competitive nature of the inhibition. The N-terminal amino group of the substrate corresponds to the amino group at C_3 of (2*S*,3*R*)X of amastatin, but the configuration of the N-terminal amino acid of the natural substrate is *S* while the configuration at C_3 of X is *R*. To clarify the importance of the configuration of X, the four stereoisomers were prepared and tested for inhibition of arylamidases. The results shown in Table II indicate that the configuration at C_2, but not C_3, is a very important factor for the manifestation of the activity. The fact that the *S* configuration at C_2 is essential for the enzyme inhibiting activity suggests that in the enzymatic reaction the amino and hydroxyl groups chelate to metal of the enzyme more strongly than substrate. The metal contained in AP-A[4] is speculated to be Zn^{2+} because microvillar peptidases[6,7] including Leu-AP, AP-B and aminopeptidase M are Zn^{2+}-enzymes.

Amastatin-related compounds in which the C-terminal L-aspartic acid moiety is substituted by other amino acids are shown in Table III. The compound containing L-aspartic acid (natural one) showed the strongest activity and those containing neutral or basic amino acids tended to show less activity towards AP-A. It is noteworthy that X-Val-Val-Pro (**8**) strongly inhibits Gly-Pro-Leu-AP and Gly-His-Lys-AP.

Amastatin-related compounds in which the second or third residue from the amino terminal of amastatin is substituted by other amino acids are shown in Table IV. Among the compounds, those containing hydrophobic amino acids, such as valine or leucine, showed strong activity towards AP-A. In order to inhibit Leu-AP strongly, hydrophobic amino

Table III. Substitution of C-Terminal Acid of Amastatin

Compound	IC_{50} (μM)					
	AP-A	AP-B	Leu-AP	Gly-Pro-AP	Gly-Pro-Leu-AP	Gly-His-Lys-AP
1a X-Val-Val-Asp	1.1	>500	1.1	>500	0.1	0.6
2 X-Val-Val-Glu	4.1	>500	4.5	>500	0.2	1.4
3 X-Val-Val-Thr	5.9	>500	7.6	>500	0.2	3.3
4 X-Val-Val-Val	9.4	>500	3.9	>500	0.1	2.0
5 X-Val-Val-Phe	14	>500	4.3	>500	0.1	2.2
6 X-Val-Val-Lys	43	>500	66	>500	0.8	38
7 X-Val-Val-Arg	26	>500	39	>500	0.2	35
8 X-Val-Val-Pro	76	>500	5.9	>500	0.13	0.13

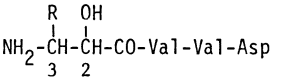
Table IV. Substitution of the Second or Third Residue from the Amino End of Amastatin

Compound	IC_{50} (μM)					
	AP-A	AP-B	Leu-AP	Gly-Pro-AP	Gly-Pro-Leu-AP	Gly-His-Lys-AP
9 X-Val-Glu-Asp	15.9	>500	0.2	>500	30	198
10 X-Val-Thr-Asp	2.1	>500	2.1	>500	1.8	4.2
11 X-Val-Leu-Asp	0.6	>500	1.4	>500	0.4	0.3
12 X-Val-Lys-Asp	20	>500	7.0	>500	2.0	2.8
13 X-Glu-Val-Asp	4.0	>500	79	>500	12	40
14 X-Thr-Val-Asp	0.3	>500	5.2	>500	0.4	2.1
15 X-Leu-Val-Asp	10	>500	0.6	>500	5.1	1.6
16 X-Lys-Val-Asp	3.0	>500	218	>500	0.5	0.8

acids, such as L-Leu and L-Val, as the second residue from the amino end are better than hydrophilic amino acids. Gly-Pro-Leu-AP and Gly-His-Lys-AP were inhibited weakly by the synthetic peptides (**13**, **9**) which had L-Glu as the second or third residue from the amino end of amastatin.

The activity of amastatin-related compounds in which the *iso*-butyl group of (2*S*,3*R*)X is substituted by hydrogen and benzyl groups is shown in Table V. The derivative with a substituted benzyl group show-

TABLE V. SUBSTITUTION OF THE *iso*-BUTYL GROUP OF X IN AMASTATIN

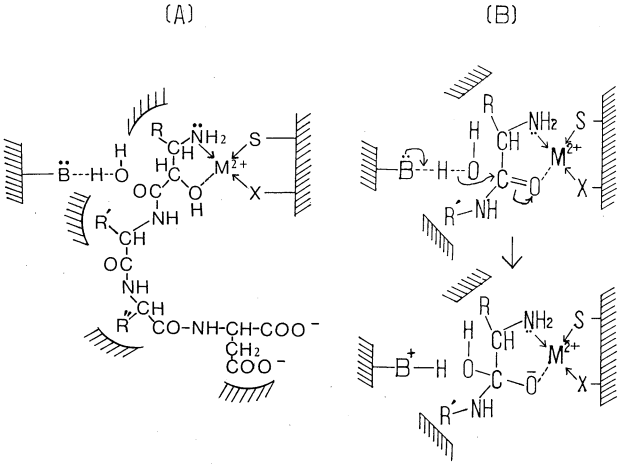


Compound	*R*	Stereochemistry 2	Stereochemistry 3	IC_{50} (μM) AP-A	AP-B	Leu-AP	Gly-Pro-AP	Gly-Pro-Leu-AP	Gly-His-Lys-AP
1a	*i*-Bu	*S*	*R*	1.1	>500	1.1	>500	0.1	0.6
17	H	*RS*	—	>500	>500	>500	>500	180	>500
18	Bzl	*S*	*R*	13.2	>500	0.1	>500	0.02	0.06

i-Bu, *iso*-butyl group; Bzl, benzyl group.

TABLE VI. LENGTH OF PEPTIDE CHAIN IN AMASTATIN

Compound	IC_{50} (μM) AP-A	AP-B	Leu-AP	Gly-Pro-AP	Gly-Pro-Leu-AP	Gly-His-Lys-AP
27a X	>1000	>1000	>1000	>1000	>1000	>1000
19 X-Asp	>1000	>1000	105	>1000	>1000	>1000
20 X-Val	865	104	1.8	>1000	46	26
21 X-Val-Asp	32	>1000	0.8	>500	19	37
22 X-Val-Val	10	600	2.5	>500	0.6	32
1a X-Val-Val-Asp	1.1	>1000	1.1	>500	0.1	0.6
23 X-Val-Val-Val-Asp	31	>1000	7.3	>500	0.1	0.2



SCHEME 1.

ed strong activity, but the derivative with substituted hydrogen was inactive for all tested arylamidases.

The study of the relationship of the length of the peptide chain and activity makes it clear that a tetrapeptide has the strongest activity towards AP-A. These data are shown in Table VI. These results indicate that the distance between terminal amino and carboxyl groups is important for the inhibitory activity towards AP-A. However, inhibitors of each aminopeptidase differ in the suitable length of the peptide chain for strong inhibitory activity.

Tables II~VI show that amastatin and its analogues inhibit many arylamidases other than Gly-Pro-AP. AP-A, AP-B and Leu-AP which were inhibited by amastatin are metalloenzymes,[5] while Gly-Pro-AP is a serine-enzyme.[6] It was speculated, as shown in Scheme 1, that amastatin and its analogues formed amastatin–Zn^{2+}-enzyme complexes in the active centers of AP-A, AP-B and Leu-AP. On the other hand, amastatin is thought to be unable to bind to Gly-Pro-AP because of a lack of M^{2+} in the active center.

EXPERIMENTAL

Melting points were determined with a Sibayama melting point apparatus. Optical rotations were measured with a photoelectric precision polarimeter (Carl Zeiss Co., Ltd., West Germany) at 578 nm. TLC was done on Merck precoated silica gel 60 F_{254} plates. D-Leu, Boc-Val, Boc-Lys (Z), Boc-Asp (OBzl), Boc-Glu (OBzl), Boc-Thr (Bzl), Boc-Leu·H_2O, Asp (OBzl)-OBzl·Tos, Thr (Bzl)-OBzl·hemioxalate, Phe-OBzl·Tos, Arg (NO_2)-OBzl·2Tos, Pro-OBzl·HCl, Z-Leu 1/2 piperazine salt, Z-Gly, DCC, HOBt and TFA were purchased from the Protein Research Foundation, Japan. Val-OBzl·Tos was purchased from Sigma Co., Ltd., U.S.A. CBZ-S reagent and DMP were purchased from Kokusan Chemical Co., Ltd., and Wako Pure Chemical Industries, Ltd., Japan, respectively. Dowex-50W ×4 (200~400 mesh) and Avicel were purchased from Dow Chemical Co., Ltd., and Funakoshi Pharmaceutical Co., Ltd., Japan, respectively.

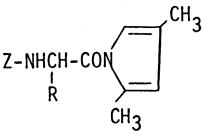
AP-A, AP-B, Gly-Pro-AP, Gly-Pro-Leu-AP and Gly-His-Lys-AP were purified according to the methods of Nagatsu *et al.*,[11] Hopsu *et al.*,[9] Walter *et al.*[10] and Aoyagi *et al.*,[11] respectively. Leu-AP was purchased from Miles Laboratories, U.S.A. Amino acid β-naphthylamides, *i.e.*, Glu·NA, Arg·NA, Leu·NA, Gly-Pro·NA and Gly-Pro-Leu·NA, were purchased from Bachem Fein Chemicalieren AG, Switzerland. Gly-His-Lys·NA was synthesized by us. [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-Val-L-Val-L-Asp was supplied kindly by Nippon Kayaku Co., Ltd., Japan.

Determination of the inhibitory activity. All the enzyme sources and substrates which were employed for testing of the inhibitory activity of amastatin and amastatin analogues are shown in Table I. The assay method for the activity of AP-A and Leu-AP is described in ref. 1. The activity of the other arylamidases was assayed by the same method as for AP-A and Leu-AP, that is, the assay system contained the following components in a total volume of 1.0 ml in a test tube (1.5×10 cm): 0.25 ml of 2 mM substrate solution, 0.5 ml of an appropriate buffer solution, an appropriate amount of enzyme, and water or amastatin solution. The buffer solutions employed for the assay of the activity of AP-B and Gly-Pro-AP were 0.1 M Tris-HCl (pH 7.5) and Tris-malate (pH 7.2), respectively. The buffer solution employed for the assay of the activity of Gly-Pro-Leu-AP and Gly-His-Lys-AP was Hank's solution (8.0 g NaCl, 0.4 g KCl, 0.14 g $CaCl_2$, 0.1 g $MgCl_2$, 0.06 g $NaHPO_4$, 0.06 g KH_2PO_4, 0.1 g $MgSO_4$ and 1.0 g glucose in 1 liter adjusted to pH 7.2 by addition of $NaHCO_3$). After 3 min preincubation at 37°C, the reaction was initiated by addition of the enzyme solution. Exactly 30 min later, the reaction was stopped by adding 1 ml of a solution of Fast Garnet GBC (1 mg/ml) in 1 M acetate buffer (pH 4.2), containing 10% Tween 20. After 20 min at room temperature, absorbance was measured at 525 nm. The reaction was also carried out without addition of the enzyme solution and the result was taken as the blank. The concentration (μg/ml or M) of amastatin or amastatin analogues required for 50% inhibition (IC_{50}) was calculated.

General synthetic procedure for Z-amino acid 3,5-dimethylpyrazolide. Z-Amino acid (10 mmol) and DMP (12 mmol) were treated with DCC (10 mmol) in CH_2Cl_2 (200 ml) at 0°C for 1 hr and then at room temperature overnight. After removal of DCU, the solvent was evaporated. The solid residue was dissolved in CH_2Cl_2 (100 ml) and crystallized from EtOAc–petroleum ether. The Z-amino acid-DMP products (**24~26**) thus obtained are summarized in Table VII.

Synthetic procedure for X and its analogues. To a suspension of $LiAlH_4$ (20 mmol) in THF (200 ml) was added a solution of Z-amino acid-DMP (10 mmol) in THF (200 ml) over a period of 1 hr, keeping the temperature at −20° to −25°C. After removal of $Al(OH)_3$ by centrifugation, the supernatant was obtained. The solvent was evaporated. The residue was dissolved in Et_2O, washed with H_2O, and then evaporated. To the oily residue was added an ice-cold solution of $NaHSO_3$ (7~8 mmol) and the mixture was stirred at room temperature overnight. To the resulting suspension of $NaHSO_3$ adduct was added

TABLE VII. Z-AMINO ACID 3,5-DIMETHYLPYRAZOLE

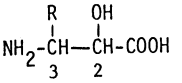


Compound	*R*	Stereo-chemistry	Yield (%)	mp (°C)	$[\alpha]_D^{20\sim27}$ in AcOH ($c=1$)	Formula
24a	*i*-Bu	*R*	65	59~60	−12.0°	$C_{19}H_{25}N_3O_3$
24b	*i*-Bu	*S*	80	63~64	+12.0°	$C_{19}H_{25}N_3O_3$
25	H	—	92	65~66	0°	$C_{15}H_{17}N_3O_3$
26	Bzl	*R*	93.5	139	−73.0°	$C_{22}H_{23}N_3O_3$

TABLE VIII. X AND ITS STEREOISOMERS

Compound	Stereochemistry		Yield (%)	mp (°C)	$[\alpha]_D^{20\sim27}$ in AcOH ($c=0.5$)
	2	3			
27a	*S*	*R*	15	188~189	−28.0°
27b	*R*	*R*	13	195~197	+34.0°
27c	*R*	*S*	6.7	225~227	+ 8.0°
27d	*S*	*S*	6.2	280~282	− 8.0°

TABLE IX. X ANALOGUES



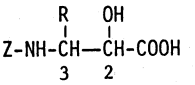
Compound	*R*	Stereochemistry		Yield (%)	mp (°C)	$[\alpha]_D^{20\sim25}$ in AcOH	Formula
		2	3				
28	H	*SR*	—	28	245~246	+10.0°	$C_3H_7NO_3$
29	Bzl	*S*	*R*	30	219~222	+29.5°	$C_{10}H_{13}NO_3$

TABLE X. Z–X AND ITS STEREOISOMERS

Compound	Stereochemistry		Yield (%)	mp (°C)	$[\alpha]_D^{20\sim25}$ in MeOH ($c=1$)
	2	3			
30a	*S*	*R*	Quantitative	78~79	+42.0°
30b	*R*	*R*	Quantitative	116~118	+24.0°
30c	*R*	*S*	Quantitative	54~56	−17.0°
30d	*S*	*S*	Quantitative	94~96	−7.0°

EtOAc (200 ml) and NaCN aqueous solution (7~8 mmol in 50 ml) and the reaction mixture was stirred for 3~4 hr at room temperature. The EtOAc phase was washed with H_2O and then evaporated to give the cyanohydrin as an oil. This was hydrolyzed in dioxane–concentrated HCl (1 : 1, 100 ml) by refluxing (12 hr). The hydrolyzate was dried after washing with EtOAc. The aqueous reaction mixture was adsorbed on a Dowex-50W (H^+) column followed by elution with 1 N NH_4OH. The eluate was dried and then washed with acetone giving a colorless powder of a mixture of (2*R*,3*R*)X and (2*S*,3*R*)X. The powder was dissolved in 0.1 M pyridine–formic acid buffer (pH 3.1)

Table XI. Z–X Analogues



Compound	R	Stereochemistry 2	Stereochemistry 3	Yield (%)	mp (°C)	$[\alpha]_D^{20\sim25}$ in AcOH	Formula
31	H	*RS*	—	70	102~103	0	$C_{11}H_{13}NO_5$
32	Bzl	*S*	*R*	67	154~155	+85.5°	$C_{18}H_{19}NO_5$

Table XII. Amastatin Analogues

Compound	mp (°C)	$[\alpha]_D^{20\sim25}$ in AcOH (c=0.5)	Compound	mp (°C)	$[\alpha]_D^{20\sim25}$ in AcOH (c=0.5)
1a (2*S*,3*R*)X-Val-Val-Asp	202~205	−42.0°	**11** X-Val-Leu-Asp	264~266	−41.7°
1b (2*R*,3*R*)X-Val-Val-Asp	205~207	−20.0°	**12** X-Val-Lys-Asp	240~241	−22.9°
1c (2*R*,3*S*)X-Val-Val-Asp	182~184	−20.0°	**13** X-Glu-Val-Asp	165~166	−27.2°
1d (2*S*,3*S*)X-Val-Val-Asp	220~222	−26.0°	**14** X-Thr-Val-Asp	180~181	−39.1°
2 *X-Val-Val-Glu	195~196	−26.0°	**15** X-Leu-Val-Asp	249~250	−30.2°
3 X-Val-Val-Thr	229~230	−53.0°	**16** X-Lys-Val-Asp	170~171	−28.6°
4 X-Val-Val-Val	180~181	−63.0°	**19** X-Asp	129~130	−15.2°
5 X-Val-Val-Phe	165~166	−18.8°	**20** X-Val	145~147	−19.0°
6 X-Val-Val-Lys	224~225	−51.2°	**21** X-Val-Asp	135~137	−14.0°
7 X-Val-Val-Arg	98~99	−38.6°	**22** X-Val-Val	148~150	−31.0°
8 X-Val-Val-Pro	176~178	−88.6°	**23** X-Val-Val-Val-Asp	208~210	−44.0°
9 X-Val-Glu-Asp	159~161	−29.4°	**17** Y**-Val-Val-Asp	190~191	−28.0°
10 X-Val-Thr-Asp	196~198	−19.9°			

* (2*S*,3*R*)X.
** 3-Amino-2-hydroxy-propionic acid.

and applied to a Dowex-50W column equilibrated with the same buffer. (2*S*,3*R*)X was eluted later than its diastereoisomer. When the separation was incomplete, further purification was done by cellulose column chromatography using EtOAc–pyridine–AcOH–H_2O (7:1:1:1, v/v). (2*R*,3*R*)X and (2*S*,3*R*)X were crystallized from *n*-PrOH–H_2O.

(2*R*,3*S*)X (**27c**) and (2*S*,3*S*)X (**27d**) were synthesized from L-Leu in the same manner as (2*S*,3*R*)X (**27a**) and (2*R*,3*R*)X (**27b**) were from D-Leu. The physicochemical properties of **27a**~**27d** are summarized in Table VIII.

3-Amino-2-hydroxy-propionic acid (**28**) was synthesized from Z-Gly in the same manner. The method of synthesis of (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoic acid (**29**) was as described by Nishizawa *et al.*[12] (Table IX).

The yield and properties of the Z–X stereoisomers (**30a**~**30d**) and Z–X analogues (**31**~**32**) are summarized in Table X and Table XI, respectively.

General synthetic procedure for amastatin and related compounds. Boc-Val-Asp(OBzl)-OBzl, Boc-Val-Glu(OBzl)-OBzl, Boc-Val-Thr(Bzl), Boc-Val-Val-OBzl, Boc-Val-Phe-OBzl, Boc-Val-Lys(Z)-OBzl, Boc-Val-Arg(NO_2)-OBzl and Boc-Val-Pro-OBzl were synthesized by coupling of N-Boc amino acids (0.1 mmol) to amino acid benzyl esters (0.1 mmol) with DCC (0.12 mmol) in the presence of HOBt (0.12 mmol) and Et_3N (0.12 mmol). After the Boc group of the dipeptide was cleaved by TFA, these N-terminal deblocked peptides were coupled to Boc-Val with DCC in the presence of HOBt and Et_3N. Thus Boc-Val-Val-Asp(OBzl)-OBzl, Boc-Val-Val-Glu(OBzl)-OBzl, Boc-Val-Val-Thr(OBzl)-OBzl, Boc-Val-Val-Val-OBzl, Boc-Val-Val-Phe-OBzl, Boc-Val-Val-Lys(Z)-OBzl, Boc-Val-Val-Arg(NO_2)-OBzl and Boc-Val-Val-Pro-OBzl were synthesized. Boc-Val-Val-Val-Asp(OBzl)-OBzl was obtained by coupling of Boc-Val-Val-OH (0.1 mmol) and Val-Asp(OBzl)-OBzl (0.1 mmol) with DCC (0.12 mmol) in the presence of HOBt (0.12 mmol) and Et_3N (0.12 mmol).

Di, tri and tetra peptides which were synthesized by the method described above were purified by crystallization with benzene–petroleum ether. The peptides (0.05 mmol) were cleaved at the Boc group by TFA, and then coupled to Z–X and its analogues (0.05 mmol) with DCC (0.06 mmol) in the presence of HOBt (0.06 mmol) and Et_3N

(0 06 mmol). After the synthetic peptides were purified by crystallization with benzene–EtOAc, the protecting groups (Z and Bzl groups) of the peptides were removed by hydrogenolysis using palladium-charcoal as catalyst to yield amastatin analogues. The physicochemical properties of the synthetic peptides (1 ~ 23) are summarized in Table XII. The structure of the synthetic peptides was confirmed by elementary analysis, PMR, IR and mass spectra

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