International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN: 0974-4290
Vol.8, No.10 pp 90-95, 2015

Synthesis, Anhelmintic, Antimicrobial and Anti-inflammatory activities of Axinastatin-3

Himaja Malipeddi* and Moonjit Das

Pharmaceutical Chemistry Division, School of Advanced Sciences, VIT University, Vellore - 632014, Tamil Nadu, India

Abstract: Axinastatins are cyclic heptapeptides, produced by the Western Pacific marine sponge *Axinella* species. Axinastatin-3, Cyclo[Asn-Pro-Phe-Ile-Leu-Pro-Val] was synthesized by solution phase peptide synthesis using dicyclohexylcarbodiimide as the coupling reagent and triethylamine as the base. The structure of the synthesized heptapeptide was confirmed by IR, ^{1}H NMR, FABMASS and elemental analysis. Axinastatin-3 was evaluated for anthelmintic activity against common earthworms. It was also screened for antimicrobial activity against four strains of bacteria and two strains of fungi and *in vitro* anti-inflammatory activity by membrane stabilizing method. Axinastatin-3 showed good anthelmintic activity as compared to the standard drug, mebendazole. It possessed potent antibacterial activity against both the Gram negative and Gram positive bacteria. It also showed potent anti-inflammatory activity as compared to the standard, ibuprofen.

Keywords: Axinastatin-3, solution phase peptide synthesis, dicyclohexylcarbodiimide, anthelmintic, antimicrobial, anti-inflammatory.

Introduction and Experimental

Peptides have emerged as an important class of organic compounds in the late nineteenth century with the discovery of pituitary hormones. Peptides function as hormones, enzymes, enzyme-inhibitors or substrates, growth promoters or inhibitors, neurotransmitters and immunomodulators.[1,2] Most of the cyclic peptides are found to exhibit antifungal, antibacterial, cytotoxic, antineoplastic, insecticidal, anti-inflammatory, antitumor anthelmintic, tyrosinase inhibitory and melanin-production inhibitory activities.[3-6]

Axinastatin-3 is a cyclic heptapeptide, possessing a relatively simple chemical structure consisting of one valine, one phenylalanine, two proline, one leucine, one isoleucine and one asparagine units (Figure 1). It is produced by the Western Pacific marine sponge *Axinella* species. It was first isolated by Pettit et al. in 1994.[7] They did the structural elucidation with high resolution FABMS and tandem MS/MS techniques augmented by high field (400 & 500 MHz), 2D-NMR spectral analysis. The absolute configurations was established by a combination of hydrolysis, derivatization and chiral gas chromatographic methods. Axinastatin-3 exhibited strong cytotoxicity against human ovarian, lung and colon cell lines.

To carry out the synthesis of Axinastatin-3, Cyclo[Asn-Pro-Phe-Ile-Leu-Pro-Val], was disconnected into three dipeptide units and a single amino acid unit. The dipeptides were prepared from the respective protected amino acids. The amino group was protected with tertiary Butyloxycarbonyl (Boc-) group and the carboxyl group was protected by converting it into methyl

ester. The Boc-amino acids were coupled with the amino acid methyl ester hydrochlorides by using dicyclohexylcarbodiimide (DCC) as the coupling agent and triethylamine (TEA) as the base.

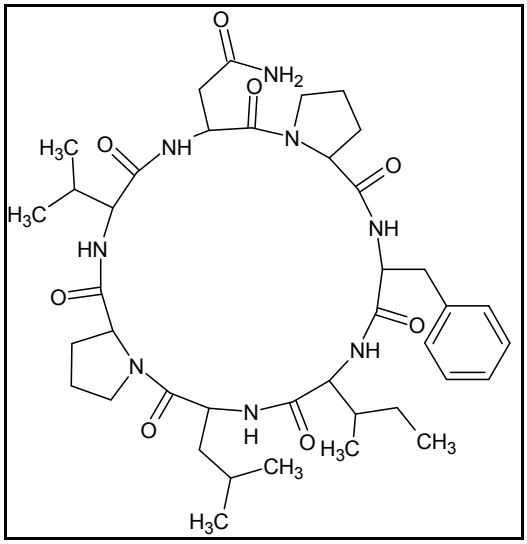


Figure 1. Structure of Axinastatin-3.

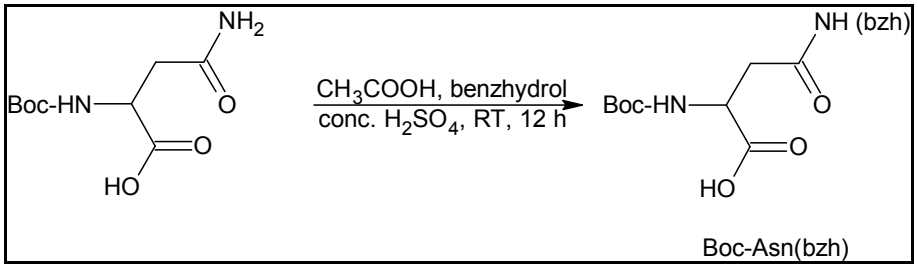
Materials

Amino acids, di-tert-butylpyrocarbonate, trifluoroacetic acid and triethylamine were obtained from Spectrochem Ltd. Mumbai. DCC, DCM, diethyl ether, methanol and chloroform was obtained from AVRA. Melting points were determined by capillary method and were uncorrected. IR spectra were recorded on Perkin-Elmer IR spectrometer using a thin film supported on KBr pellets for solids and chloroform as a solvent for semisolids. The values are reported as ν_{max} (cm^{-1}). ^1H NMR spectra were recorded on Bruker JOEL (400MHz) NMR spectrometer. The spectra were obtained in $CDCl_3$ and the chemical shift values are reported as values in ppm relative to TMS ($\delta = 0$) as internal standard. FABMASS spectra were recorded on a Joel Sx 102/DA-6000 mass spectrometer using xenon as the carrier gas. The spectra were recorded at room temperature, m-nitrobenzyl alcohol was used as the matrix. The protection of amino group, carboxyl group and side chain and their deprotection were done by standard procedures.[8-9]

Preparation of Dipeptides

Amino acid methyl ester hydrochloride (10 mmol) was dissolved in dichloromethane (DCM) (20 mL). To this, triethylamine (TEA) (4 mL, 28.7 mmol) was added at 0°C and the reaction mixture was stirred for 15 mins. Boc-amino acid (10 mmol) in DCM (20 mL) and DCC (10 mmol) were added with stirring. After 36 h, the reaction mixture was filtered and the residue was washed with DCM (30 mL) and added to the filtrate. The filtrate was washed with 5% $NaHCO_3$ (20 mL), 5% HCl (20 mL) and distilled H_2O (20 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated in a vacuum. The residue was purified by recrystallization from $CHCl_3$. Boc-Pro-Phe-OMe, Boc-Ile-Leu-OMe and Boc-Pro-Val-OMe was prepared in this manner.

Protection of the Side chain function

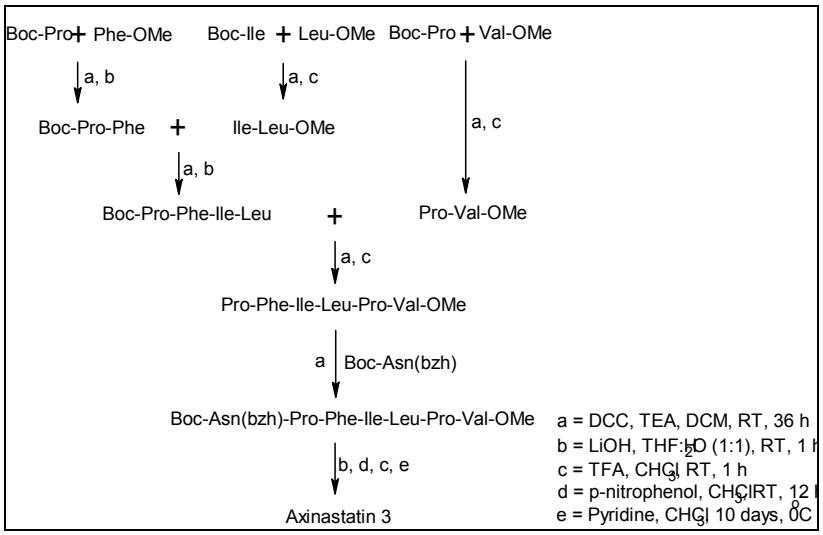


Scheme 1. Protection of side chain of Asparagine.

The carboxamide side function of Boc-Asparagine was protected by introducing the benzhydryl group (bzh) by Bodanszky[9] method (Scheme 1).

Preparation of Axinastatin-3

The ester group of Boc-Pro-Phe-OMe was removed with LiOH and the Boc- group of Boc-Ile-Leu-OMe was removed by trifluoroacetic acid (TFA). The deprotected dipeptides were coupled using DCC and TEA to get the tetrapeptide Boc-Pro-Phe-Ile-Leu-OMe. The ester group of the tetrapeptide and the Boc- group of the dipeptide Boc-Pro-Val-OMe were removed coupled to give the hexapeptide Boc-Pro-Phe-Ile-Leu-Pro-Val-OMe. After the removal of the Boc- group of the hexapeptide unit, it was coupled with Boc-Asn(bzh)-OH to obtain the heptapeptide Boc-Asn(bzh)-Pro-Phe-Ile-Leu-Pro-Val-OMe. The ester group of the heptapeptide was removed and p-nitrophenyl group (pnp) was introduced**.** The Boc- and the bzh- groups were removed by TFA and the linear fragment was cyclized by adding pyridine and keeping the whole contents at 0°C for ten days to get the Axinastatin-3 (Scheme 2).



Scheme 2. Synthetic scheme of Axinastatin-3.

Anthelmintic Activity

Anthelmintic activity study of Axinastatin-3 was carried out against earthworms (*Eudrilus eugeniae*).[10] Suspension of Axinastatin-3 was prepared by triturating it with 15% Tween 80 and distilled water and the mixture was stirred for 30 mins using a magnetic stirrer. The suspension was diluted to contain 100 mg and 200 mg in 20 mL of the test sample. The standard drug, mebendazole was also prepared in a similar way. Earthworms were placed in three petri dishes containing 20 mL of each sample, control (20 mL suspension of distilled water and 15% Tween 80) and standard drug, respectively at RT. The time needed for the paralysis and death of the earthworms were noted. The death time was determined by placing the earthworms in warm water at 50°C, which stimulated the movement if the earthworms were alive.

Antimicrobial activity

For antimicrobial activity study of the heptapeptide, Axinastatin-3, four strains of bacteria and two strain of fungal cultures were used. The bacterial isolates included two Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*). The fungal cultures were *Aspergillus niger* and *Candida albicans.*

The antimicrobial activity of Axinastatin-3 was tested at 50 µg/mL, using agar well diffusion assay method.[11] The bacterial test organisms were grown in nutrient broth at 37°C for 24 h and then spread on Muller Hinton Agar (MHA) plates. For the fungal cultures, spore suspensions was grown in Sabor Dextrose Broth (SDB) at 25°C for 48 h and then spread on Potato Dextrose Agar (PDA) plates. Dimethyl formamide was used

as negative control and disks containing streptomycin (10µg/disk) and griseofulvin (25µg/disk) as positive controls. All the tests were performed in triplicates. The antimicrobial activity was evaluated based on the diameter of zone of inhibition.

In vitro Anti-inflammatory Activity[12]

Preparation of erythrocyte suspension

Blood (10 mL) was collected from a healthy individual (blood group B positive) in a tube containing EDTA. The blood was centrifuged at 3000 rpm for 10 mins in a centrifuge. Plasma was discarded and the pellet was washed with 0.9% saline solution for three times. The volume of the blood was reconstituted as 20% v/v suspension with 10 mM sodium phosphate buffer solution (pH 7.4).

Heat induced haemolysis

The membrane stabilizing activity of Axinastatin-3 was measured by heat induced haemolysis of human blood. The reaction mixture contained solutions of Axinastatin-3 (50-200 µg/mL) in different tubes. 1 mL of 20% RBCs suspensions was added to all the tubes. Instead of the compound only 10 mM sodium phosphate buffer solution was added to the control tube. The tubes containing the mixture were incubated at 56^{o}C for 30 minutes in a water bath. The reaction mixture was centrifuged at 3000 rpm for 10 minutes and the absorbance of the supernatants was measured at 560 nm using Hitachi U-2910 Spectrophotometer. Ibuprofen (50-200 µg/mL) was used as the reference standard. Membrane stabilizing activity (in %) was calculated by the following formula:

$$\text{Inhibition (\%)} = 100 \times \frac{A_t}{A_c}$$

Where, A_t = Absorbance of test sample and A_c = Absorbance of control.

Results and Discussion

Physical Data and Spectral Analysis

Axinastatin-3: Yield 58%; pale white solid; m.p. 294-297^{o}C; IR spectrum (ν/cm^{-1}): 3325 (br. s), 3025 (s), 2935 (s), 2855 (s), 1730 (s), 1700 (s), 1685 (br. s), 1660 (br. s), 1645 (s), 1615 (s), 1535(s), 1445 (s), 1395 (s), 1315 (s), 1245 (s), 1165 (br. s), 1095 (s), 1055 (s), 895 (s) cm^{-1}; ^{1}H NMR spectrum (δ, ppm): 8.35 (3H, br. s), 7.4 (4H, br. s), 7.3-7.1 (5H, m), 4.8-4.6 (1H, m), 4.5-4.2 (2H, m), 4.2-4.0 (4H, m), 3.65-3.2(6H, m), 2.2- 1.5 (14H, m), 1.3-1.1 (3H, m), 1.0 (18H, d, J = 6.0 Hz); FABMASS: m/z $(M + H)^{+}$ = 781; Elemental Analysis: M. F. = $C_{40}H_{61}N_8O_8$, M. W. = 780, Found (Cal) %C: 61.5 (61.46), %H: 7.8 (7.81), %N: 14.32 (14.34).

Anthelmintic activity

Axinastatin-3 have shown moderate anthelmintic activity as compared to the standard drug, mebendazole. Table 1 illustrates the anthelmintic activity of sample and standards.

Table 1. Anthelmintic activity of Axinastatin-3.

Sl. No.	Compound	Conc. of the compound (mg)	Mean paralysing time (mins) ± S.E	Mean death time (mins) ± S.E
1	Control	100	–	–
		200	–	–
2	Mebendazole	100	17.44 ± 0.09	54.4 ± 1.08
		200	12.14 ± 1.02	31.04 ± 1.09
3	Axinastatin-3	100	66.24 ± 2.01	98.16 ± 2.03
		200	57.58 ± 2.05	88.24 ± 2.16

Antimicrobial Activity

Evaluation of antimicrobial activity was done for Axinastatin-3 (Table 2) and it was found to possess antibacterial activity against both Gram negative (*P. aeruginosa* and *E. coli*) as well as Gram positive (*B. subtilis* and *S. aureus*) bacteria. However, it did not show antifungal activity against *C. albicans* and *A. niger*.

Table 2. Antimicrobial activity of Axinastatin-3.

Sl. No.	Compound	Diameter of zone of inhibition (in mm).					
		P. aer	***E. coli***	***B. sub***	***S. aur***	***C. alb***	***A. niger***
1	Axinastatin-3	9.1±0.25	14.2±0.5	18.3±0.6	18.1±0.2	-	-
2	Streptomycin	16.2±0.2	16.1±0.5	12.0±1	12.2±0.8	-	-
3	Griseofulvin	-	-	-	-	20.1±0.7	18.2±1

Values are expressed as mean ± S. E of the three replicates. Zone of inhibition not include the diameter of the well (7 mm).

In vitro Anti-inflammatory Activity

Axinastatin-3 exhibited membrane stabilization effect by inhibiting hypotonic induced lysis of erythrocyte membrane. From the obtained results (Table 3) it was concluded that Axinastatin-3 has a significant membrane stabilizing activity which was comparable to the standard ibuprofen. The maximum inhibition of 80.3±0.60% was observed with 200 µg/mL of Axinastatin-3. Whereas, diclofenac sodium, showed the maximum inhibition of 89.4±0.63% at 200 µg/mL.

Table 3. *In vitro* anti-inflammatory activity of Axinastatin-3.

Concentration (µg/mL)	Standard (ibuprofen)	Axinastatin-3
50	71.9±0.86	63.4±1.01
100	82.4±0.59	72.1±0.79
200	89.4±0.63	80.3±0.60

Values are expressed as mean ± S. E of the three replicates.

Conclusion:

Axinastatin-3 was easily synthesized under normal laboratory conditions by solution phase peptide synthesis. The structure of the cyclic peptide was confirmed by IR, ^{1}H NMR, FABMASS and elemental analysis. The compound was screened for anthelmintic activity and it showed moderate to good activity as compared to the standard drug, mebendazole. It was also screened for antimicrobial activity against four strains of bacteria and two strains of fungi and it showed potent antibacterial activity against both Gram negative and Gram positive bacteria. It also showed potent anti-inflammatory activity as compared to the standard, ibuprofen.

Acknowledgements

We are grateful to the VIT-SIF Lab, School of Advanced Sciences, VIT University, Vellore, for the support of this research.

References

1. Victor J.H., Fahad A. and Wieslaw K., Emerging approaches in the molecular design of receptor-selective peptide ligand, conformational, topographical and dynamic considerations, Biochem. J., 1990, 268, 249.
2. David S., Lenka Z. and Milos B., High performance liquid chromatography and nuclear magnetic resonance study of linear tetrapeptides and octapeptides containing *N*-methylated amino acid residues, J. Chromatogr, A, 2007, 1160, 128.

3. Harish K., Boja P. and Belagali S., Characterization of biologically active cyclic peptides, Ind J Hetero Chem., 1997, 12, 149.
4. Inman W. and Crews P., Isolation of cyclic depsipepticle, J Am Chem Soc., 1989, 111, 2822.
5. Sakai Y., Yoshida T., Sujita T., Ochiai K. and Agatsuma T., Isolation of cyclic hexadepsipeptide antitumor antibiotic GE 3, J Antibiot., 1997, 50, 659.
6. Das M. and Himaja M., Synthesis, antioxidant and anthelmintic activity of the linear tetrapeptide L-(Leu-Pro-Gly)-D-Ala (LPGA), Int. J. Pharm. Pharm. Sci., 2013, 5(Suppl 3), 713.
7. Petit G.R., Herald C.L., Boyd M.R., Leet J.E., Dufresne C., Dennis L.D., Schmidt J.M., Cerny R.L., Hooper J.N.A. and Rutzler KC., Isolation and structure of axinastatins 2 and 3 from a Western Caroline Island marine sponge. J. Med. Chem., 1994, 37(8), 1165.
8. Dahiya R. and Gautam H., Total synthesis and antimicrobial activity of a natural cycloheptapaptide of marine origin, Marine Drugs, 2010, 8, 2384.
9. Bodanszky M. and Bodanszky A., Practice of peptide synthesis, New York: Springer-Verlag, 1984, p.78.
10. Garg L.C. and Atal C.K., Evaluation of Anthelmintic activity, Ind. J. Pharmacol., 1969, 32, 104.
11. Rahman M.M., Mosaddik M.A., Wahed M.I.I. and Haque ME., Antimicrobial activity and cytotoxicity of *Trapa Bispinosa*, Fitoterapia, 2010, 71, 704-706.
12. Shinde U.A., Phadke A.S., Nari A.M., Mungantiwar A.A., Dikshit V.J. and Saraf M.N., Membrane stabilization activity - A possible mechanism of action for the anti-inflammatory activity of *Cedrusdeodara* wood oil, Fitoterapia, 1999, 70, 251.

*****

Table 1. Anthelmintic activity of Axinastatin-3.

Sl. No.	Compound	Conc. of the compound (mg)	Mean paralysing time (mins) ± S.E	Mean death time (mins) ± S.E
1	Control	100	–	–
		200	–	–
2	Mebendazole	100	17.44 ± 0.09	54.4 ± 1.08
		200	12.14 ± 1.02	31.04 ± 1.09
3	Axinastatin-3	100	66.24 ± 2.01	98.16 ± 2.03
		200	57.58 ± 2.05	88.24 ± 2.16

Table 2. Antimicrobial activity of Axinastatin-3.

Sl. No.	Compound	Diameter of zone of inhibition (in mm).					
		P. aer	***E. coli***	***B. sub***	***S. aur***	***C. alb***	***A. niger***
1	Axinastatin-3	9.1±0.25	14.2±0.5	18.3±0.6	18.1±0.2	-	-
2	Streptomycin	16.2±0.2	16.1±0.5	12.0±1	12.2±0.8	-	-
3	Griseofulvin	-	-	-	-	20.1±0.7	18.2±1

Values are expressed as mean ± S. E of the three replicates. Zone of inhibition not include the diameter of the well (7 mm).

Table 3. *In vitro* anti-inflammatory activity of Axinastatin-3.

Concentration (µg/mL)	Standard (ibuprofen)	Axinastatin-3
50	71.9±0.86	63.4±1.01
100	82.4±0.59	72.1±0.79
200	89.4±0.63	80.3±0.60

Values are expressed as mean ± S. E of the three replicates.

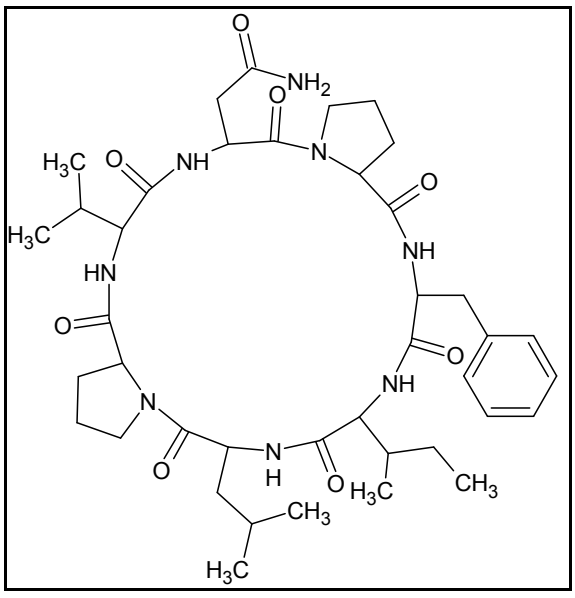
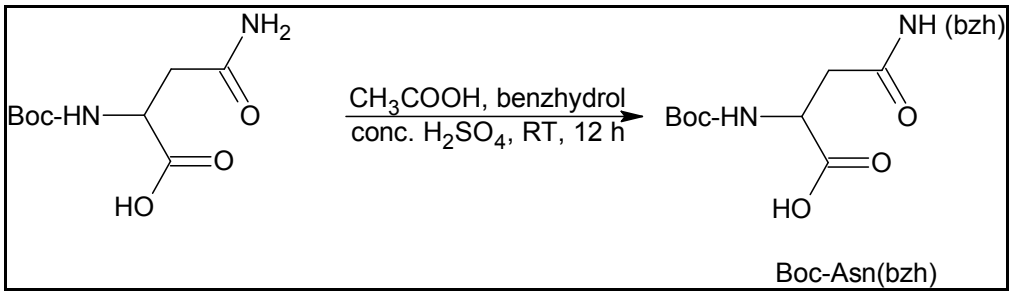
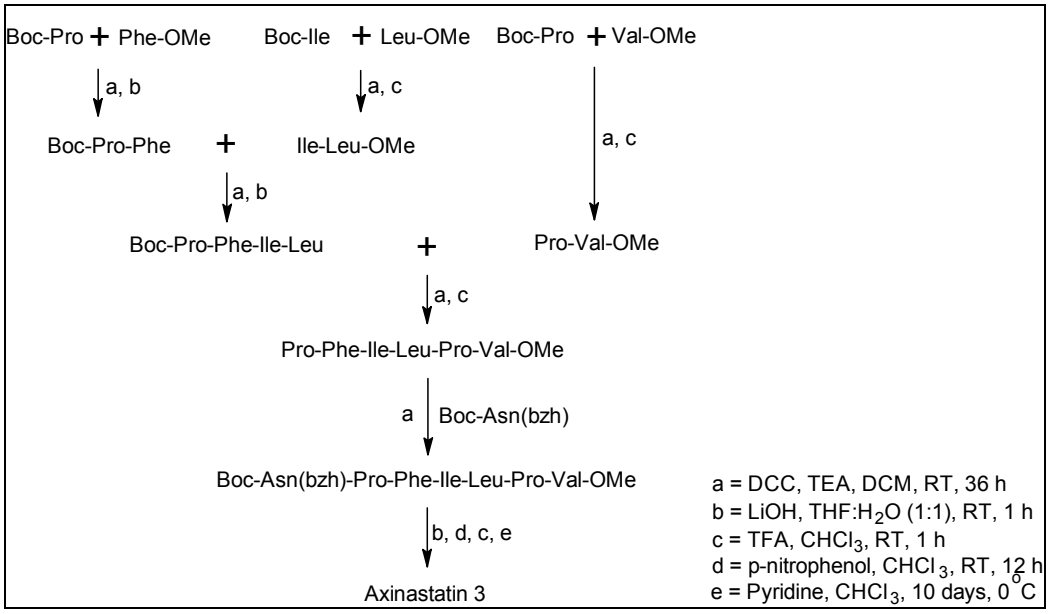


Figure 1. Structure of Axinastatin-3.



Scheme 1. Protection of side chain of Asparagine.



Scheme 2. Synthetic scheme of Axinastatin-3.