Chapter 12

New Natural Herbicide Candidate for *Sicyon angulatus* Control

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Additional information is available at the end of the chapter

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1. Introduction

Most synthetic herbicides are used for controlling troublesome weed species in modern agriculture all over the world. However, consecutive use of the same herbicide brings about resistant weed problems and many countries are restricting repeated treatment in agricultural lands [1]. For these and environmental reasons, new herbicide discovery and subsequent registration is very challenging. Recently, evaluating natural products of animals, plants, microorganisms and minerals for developing environmental friendly herbicides has increased [2]. Several compounds have been developed or in development as natural herbicides such as bialaphos [3], methoxyhygromycin (MHM) [4], and pelargonic acid [5]. Essential oils such as clove oil and cinnamon oil also contain allelochemicals that control a broad spectrum of weeds and can be used as natural herbicide source [6,7]. Plumbagin isolated from *Drosophyllum lusitanicum* and *Plumbago auriculata* inhibited the seed germination of lettuce and wheat [8,9]. Several classes of natural compounds including triketones, benzoquinones, naphthoquinones and anthraquinones have been reported as hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors and hence the novel classes of HPPD inhibitors could be developed based on their structural backbones [10].

Agricultural research for herbicide discovery with new target site is increasing due to the demand from farmers and multinational companies. Even so, new mode of action have not been succesfully introduced in the past 10 years [2,3]. We have recently reported : 7-keto-8-aminopelargonic acid synthase (EC 2.3.1.47, KAPAS, also known as 8-amino-7-oxononanoate synthase, AONS) and have suggested the potential KAPAS inhibitor triphenyltin [11]. KAPAS is a pyridoxal 5'-hophate dependent enzyme which catalyzes the decarboxylative condensation of L-alanine with pimeloyl-CoA in a stereospecific manner to form7-keto-8-aminopelar-

gonic acid, Coenzyme A, and carbon dioxide in the first committed step of biotin biosynthesis. Perhaps the most important role of biotin is in the carboxylation of acetyl-CoA to give malonyl-CoA, which is the first step in fatty-acid biosynthesis. Since fatty-acid synthesis is essential for the growth and development of most organisims, biotin is thus an essential nutrient for plants and animals. Plants, microorganisms, and some fungi biosynthesize their own biotin, while other organisms require trace amounts of the vitamin in their diet. Therefore, inhibition of the enzymes involved in the biotin biosynthesis pathway can cause irreparable damage to plants but be non-toxic to non-plant organisims, and for this reason, such enzymes can be useful targets for the rational design of inhibitors in the hopes of finding new herbicides [12,13].

Also, we attempted to search for KAPAS inhibitors from plant-derived natural compounds. Several naturally occurring quinones including chrysophanic acid, tanshinones, 5,8-dihydroxy-1,4-naphthoquinone, and plumbagin was selected as potent inhibitors against KAPAS. We evaluated the plumbagin showing most effective KAPAS inhibition, as a natural herbicide under greenhouse and field tests. Field tests were focused on the annual noxious weed species of *Sicyos angulatus* (burcucumber or star-cucumber) which have migrated from eastern North America and have been designated as one of the ecological disturbance plants listed by the Ministry of Environment in Korea. The alien plant *S. angulatus* was first observed in 1989 and rapidly emerged in the marginal of agricultural fields close to riparian zone where it has been rapidly spreading along rivers in Korea over the past two decades [14,15]. Invasion into the natural ecosystems by exotic species is a major global threat to biodiversity. *S. angulatus* was also listed in Federal and State Noxious Weeds, USA and its geographical distribution was published in the OEPP/EPPO Bulletin [16]. It is adapted to wet habitats: deciduous swamps, woodland floodplains, and river floodplains. It also colonizes open habitats along fencerows, roadsides, and woodland borders. *S. angulatus* is found in every state east of the Rocky Mountains and also found in Canada's eastern provinces, Mexico, the Caribbean, and Eastern Asia. It was first introduced to Europe as an ornamental plant, but has since escaped cultivation and become a weedy invasive species. Asaeda et al. [17] reported the most dominant liana species in the floodplain is *S. angulatus* and it was first sighted in Japan in 1952. Ceschin et al. [18] reported exotic species of *S. angulatus* as a new arrival alien in the Tiber River in Rome. Many reports of its invasiveness have been published in the United Kingdom [19], Norway [20], Japan [21], Korea [14], and Spain [22] etc.

In this chapter, we briefly describe the KAPAS inhibitory activity of plumbagin, which showed the most potent inhibition during the preliminary survey of many natural products. Also the herbicidal activity of plumbagin was evaluated under greenhouse conditions and field trials. Physiological responses caused by the plumbagin treatment with respect to cellular leakage, chlorophyll loss and the rescue effect with biotin supplement through tissue section experiments or seed germination are reported. Plumbagin is under examination as a LOHAS (Lifestyles of Health and Sustainability) [23] herbicide against an invasive alien vine plant species.

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2. Development for *Sicyon angulatus* control

2.1. Plumbagin preparation

The specimens of *P. auriculata* grown in the greenhouse were collected, and the air-dried root (180 g) was soaked in 2 L of acetone at room temperature for 7 days. The extract was filtered and evaporated to dryness under negative pressure. The concentrated extract (1.5 g) was suspended in 100 ml of water and re-extracted with an equal volume of dichloromethane, which afforded 1.2 g of dichloromethane soluble fraction. The dichloromethane soluble fraction was subjected to silica gel column chromatography eluted with a mixture of hexane and ethyl acetate (20:1) to give 120 mg of plumbagin as a dark yellow crystal. The spectral data of isolated plumbagin (purity > 99%), such as UV, MS and 1H NMR and ^{13}C-NMR were well accorded with the result of Bhattacharyya and Carvalho [24]. For field trial, plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) was purchased from Sigma–Aldrich, which was originally isolated from Plumbago indica (Plumbaginaceae). The purity of commercially available plumbagin was estimated over 90% by HPLC.

2.2. Plumbagin as a KAPAS inhibitor

The full-length of AtKAPAS cDNA was amplified and isolated from *Arabidopsis thaliana* cDNA and cloned into MBP fusion vector to generate the *Escherichia coli* expression construct pEMBPek-KAPAS [11]. SDS–PAGE analysis revealed that *E. coli* transformed with MBP fusion vector showed the expression of a very strongly induced fusion protein of ca. 98.2 kDa, which consisted of the AtKAPAS protein of 51.3 kDa and the maltose binding peptide MBP affinity tag of 46.9 kDa [11,32]. Pimeloyl-CoA was synthesized according to the method described previously [25]. KAPAS activity was determined according to the method described previously [12] using a linked assay by monitoring the increase in absorption of NADH at 340 nm using a Microplate Spectrophotometer (Benchmark Plus, Bio-rad, USA), thermostatically controlled at 30^{o}C. At KAPAS protein was expressed in *E. coli* at a very high level, and a significant portion of these proteins was soluble, and their affinity-purified preparations contained a single major polypeptide. The lysates from IPTG-induced *E. coli* containing pEMBPek-KAPAS as well as from *E. coli* harboring control vector MBP fusion vector were loaded onto maltose affinity column (1.1 cm x 30 cm, Millipore, USA). The AtKAPAS protein bound to MBP resin was eluted with 10 mM maltose solution. A typical assay contained 20 mM potassium phosphate (pH 7.5), 1 mM α-ketoglutarate, 0.25 mM thiamine pyrophosphate, 1 mM NAD^{+}, 3 mM $MgCl_2$, 0.1 unit of α-ketoglutarate dehydrogenase, and 2–10 μg of KAPAS (3 mg protein/ml) in a total volume of 200 μL. L-Alanine and pimeloyl-Co A were added to give the desirable final concentrations. Prior to analysis, enzyme samples were dialyzed for 2 h at 4^{o}C against 20 mM potassium phosphate (pH 7.5) containing 100 μM PLP. The KAPAS concentration in all analyses was 10 μM in 20 mM potassium phosphate (pH 7.5). 96-well microplates containing each 528 natural compounds prepared from various medicinal plants and exotic herbs were evaluated on KAPAS inhibition assay at the concentration of 1 mM. Through the consecutive experiment at lower concentration against samples showing 90% inhibition of KAPAS activity, plumbagin were selected as the most effective KAPAS inhibitor.

IC_{50} value of KAPAS inhibition by plumbagin was calculated from the regression curve prepared with the extensive assay performed with the plumbagin ranged from 0.1 to 250 μM with five replications. A reference was prepared with all components except plumbagin.

Enzyme activity was tested with the partially purified AtKAPAS protein extracted from transgenic *E. coli*. AtKAPAS protein was expressed *in E. coli* at a very high level, and a significant portion of these proteins was soluble, and their affinity-purified preparations contained a single major polypeptide. The inhibitory effect of 528 plant-derived natural compounds collected in Korea Chemical Bank, KRICT on KAPAS was evaluated using the partially purified AtKAPAS protein, *in vitro*. Less than 2% of tested compounds exhibited significant inhibitory effect on KAPAS at the concentration lower than 20 μM. Interestingly, several naturally occurring quinones including chrysophanic acid, tanshinones, 5,8-dihydroxy-1,4-naphthoquinone, and plumbagin were observed to give a potent inhibitory effect on KAPAS. Plumbagin, a natural naphthoquinone demonstrated the most effective inhibitory effect on KAPAS with an IC_{50} of 2.1 μM (Fig. 1).

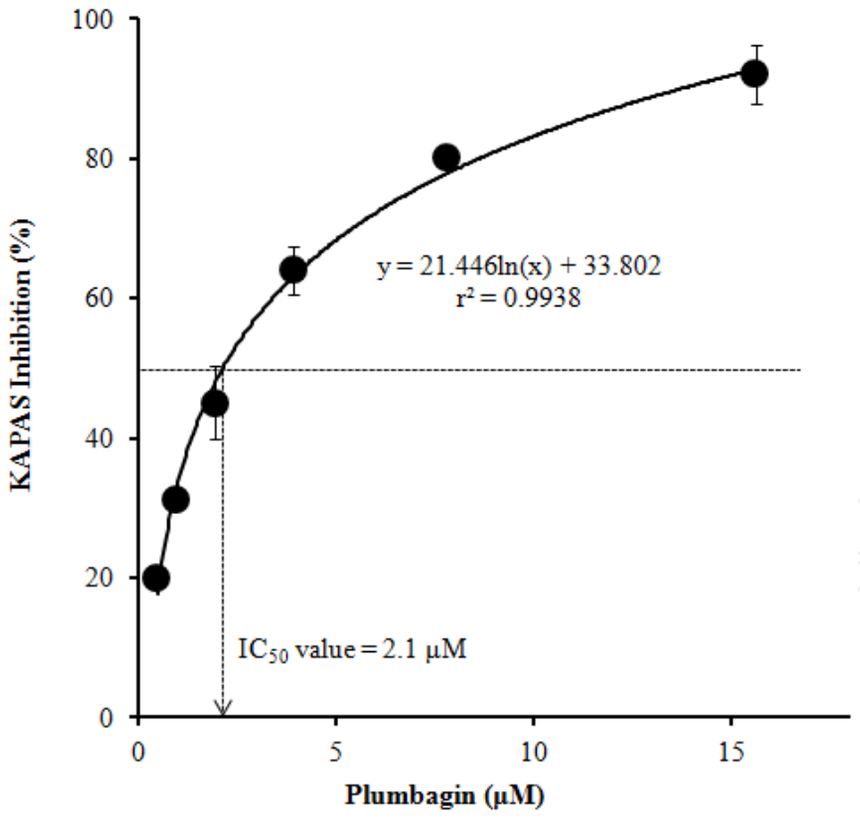

Figure 1. KAPAS inhibition by plumbagin *in vitro* assay. Vertical bars represent standard deviation. In some cases the vertical bar is obscured by the datum symbol.

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2.3. Herbicidal activity of plumbagin

2.3.1. Materials and methods

Herbicidal activity and spectrum of plumbagin were investigated against eight weed species, consisting of three grass species of *Sorghum bicolor* (sorghum), *Echinochloa crus-galli* (barnyard grass), *Digitaria sanguinalis* (large crabgrass) and five broad leaf species of *Solanum nigrum* (black nightshade), *Aeschynomene indica* (Indian joint vetch), *Abutilon avicennae* (velvetleaf), *Xanthium strumarium* (common cocklebur), *Calystegia japonica* (Japanese bindweed). Seeds of weeds for foliar application were germinated in a commercial greenhouse substrate (Boo-Nong Soil, Seoul, Korea) and watered with tap water. About five plants were grown at 30/20 ± 3°C, day/night temperature with an about 14 h photoperiod for 12 days under greenhouse. Foliar application was conducted at 12 days after sowing, the test solution was sprayed into the test pot grown with 10 ~ 15 seedlings of sorghum, barnyard grass, large crabgrass, black nightshade, Indian joint vetch and velvetleaf, and two seedling of common cocklebur and Japanese bindweed. Various concentrations of the purified plumbagin from *P. auriculata* prepared with 50% acetone solution containing 0.1% Tween-20 were sprayed onto plants with a laboratory spray gun delivering spray volume of 5 ml per pot. The control treatment recieved the same volume of spray without plumbagin. After treatment, the plants were placed in a vented cabinet to dry and returned to the same greenhouse without replication. At 5 days after treatments, visual injury of plants assessed on a scale from 0 (no injury) to 100 (complete death). A field trial was performed against 10 ~ 15 leaf-stage and 2 ~ 3 m vine length of natural *S. angulatus* habitats around riparian zones in Nam-Han River. Foliar applications were conducted with 1,000 and 2,000 μg/mL of plumbagin in 50% acetone solution containing 0.1% Tween-20 using a laboratory sprayer delivering spray volume of 300 ml/m^2 with a control treatment of the same preparation solution without plumbagin. The field trial was performed from 22th September to 6th October, 2011, and the trial contained three replicates of 1 m^2 plot size. The control value was evaluated visually at 5, 8, and 14 days after treatments. Test plots were situated directly adjacent to each other.

2.3.2. Results

Under greenhouse conditions, all eight weed species were completely controlled by the foliar application of 1,000 and 2,000 μg/mL plumbagin, while 500 μg/mL applications also showed 100% herbicidal efficacy against seven weed species with the exception of *A. avicennae* (Fig. 2). 250 μg/mL applications against eight weeds showed 60 ~ 100% control (Table 1), and especially a concentration as low as 32 μg/mL had a herbicidal efficacy of 70% on *D. sanguinalis* (data not shown). With a plumbagin treatment of eight weed species, the main herbicidal symptoms were desiccation or extensive necrosis within 2 h. The difference of symptoms caused by the plumbagin between grass species and broad leaf species was insignificant after foliar application. Field test results revealed that the natural compound plumbagin controlled alien weed *S. angulatus* completely at 2,000 μg/mL under foliar application. Visual symptoms of plant injury after plumbagin foliar application against natural *S. angulatus* were desiccation or burn down within 2 h after treatment. Control values were evaluated as 95–100% by a visual rating scale of 0–100 at 5, 8, and 14 days after treatment with 1,000 or 2,000 μg/mL. The residual activity lasted for 2 weeks without any regrowth.

Conc.	Herbicidal efficacy (%)[1)]							
(μg/mL)	SORBI	ECHCG	DIGSA	SOLNI	AESIN	ABUTH	XANSI	CAGEH
2000	100	100	100	100	100	100	100	100
1000	100	100	100	100	100	100	100	100
500	100	100	100	100	100	70	100	100
250	90	60	100	100	100	60	90	100

[1)]Herbicida1 activity was determined 7 days after treatment by visual injury. SORBI, Sorghum bicolor (sorghum); ECHCG, Echinochloa crus-galli (barnyard grass); DIGSA, Digitaria sanguinalis (large crabgrass); SOLNI, Solanum nigrum (black nightshade); AESIN, Aeschynomene indica (Indian joint vetch); ABUTH, Abutilon avicennae (velvetleai); XANSI, Xanthium strumarium (common cocklebur); CAGEH, Calystegia japonica (Japanese bindweed). ' 2,000 pg/ml. can change to 4 kg/ha.

Table 1. Herbicidal efficacy of plumbagin post-emergence foliar application against several weeds in a greenhouse condition

Figure 2. Herbicidal symptoms of post-emergence foliar application of plumbagin (μg/mL). (A) Pot test in a greenhouse condition against 8 weed species. (B) Field trials for *Sicyos angulatus* control. * 2,000 μg/mL can change to 4 kg/ha.

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2.4. Reversal study

2.4.1. Materials and methods

Seeds of *A. thaliana* were germinated on a 55 mm Polystyrene Petri-dish lined with one-layer filter paper (Advantec No. 2). One milliliter of each plumbagin solution dissolved in absolute acetone with various concentrations of 0, 25, 50 and 100 μM was dropped evenly onto the filter paper and placed in a vented cabinet to dry. After complete drying, 1 ml of distilled water with or without supplement of 0, 0.25, 0.5 and 1 mM biotin (Sigma, USA) was added, and 30 seeds were placed onto the filter paper in Petri-dish. Each Petri-dish was sealed with laboratory film and incubated in a growth chamber at 25°C, 14/10 h (Light/Dark). Germination inhibition percentages were calculated with the number of germinated *A. thaliana* seeds at 7 days after application. All treatments for each measurement were triplicates.

2.4.2. Results

The inhibited germination of *A. thaliana* seeds treated with plumbagin was significantly rescued in a dose dependent manner by biotin supplement. Germination rate of *A. thaliana* seeds at plumbagin levels of 25, 50, and 100 μM was 33.3%, 23.3%, and 16.7%, respectively. However, the inhibited germination by plumbagin was negated up to 93.3%, 86.7%, and 83.3% with the supplement of 1 mM biotin, and also it was negated up to 66.7%, 63.3%, and 60.0% with the supplement of 0.5 mM biotin, respectively (Table 2, Fig. 3). Biotin supplement apparently rescued the inhibited germination *A. thaliana* seeds caused by the treatment of plumbagin.

Plumbagin	+ Biotin (mM)			
(μM)	0	0.25	0.5	1
0	100[1)]	100	100	96.7
25	33.3	60.0	66.7	93.3
50	23.3	53.3	63.3	86.7
100	16.7	46.7	60.0	83.3

[1)]Germination rate of *A. thaliana* seed at 7 days after application.

Table 2. Reversal effect of *Arabidopsis thaliana* seed germination with biotin supplement

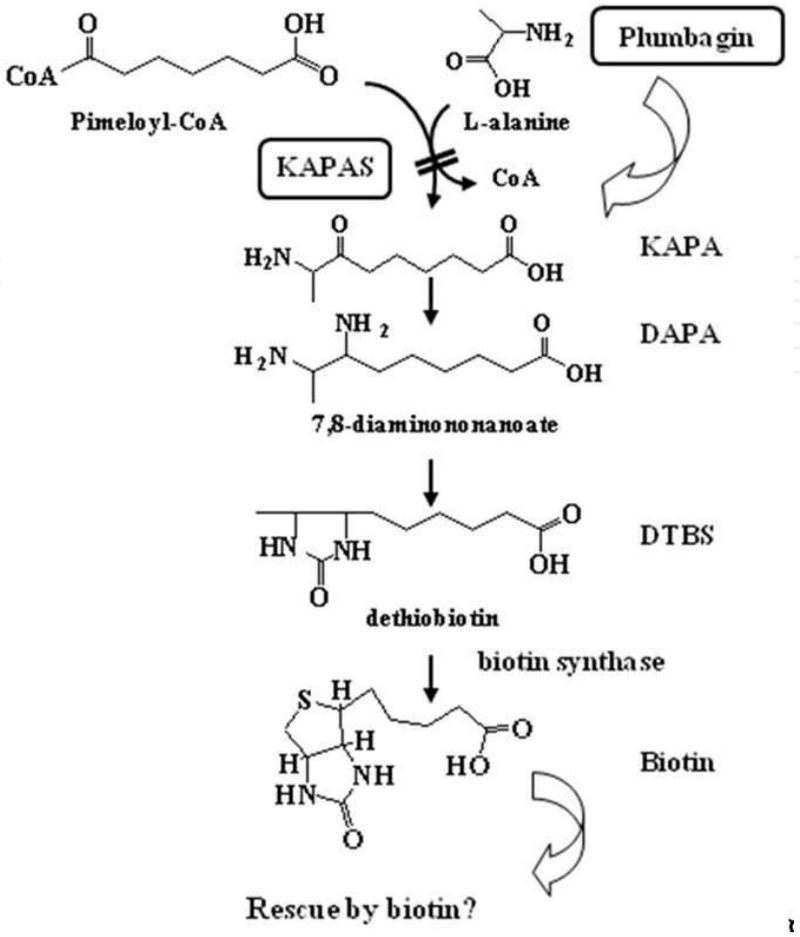

Figure 3. Proposed target site of plumbagin on KAPAS and biotin synthesis pathway in plant.

5. Summary

A new herbicide developed the lifestyle of health and sustainability (LOHAS) initiative is required to satisfy environmental and regulatory pressures. LOHAS describes an estimated $290 billion US marketplace for goods and services focused on health, the environment, social justice, personal development and sustainable living. Approximately 13–19% of the adults in the U.S. are currently considered LOHAS consumers. This is based on surveys of the U.S. adult population estimated at 215 million [23]. Also world-wide consumers demand these types of compounds as potential natural-product based herbicides. In this chapter, we attempted to develop a new herbicide from natural compounds having the new target KAPAS, and we applied this to annual noxious weed species of *S. angulatus* (burcucumber or star-cucumber). Our laboratory has performed molecular genetics dissection using anti-sense approach to identify new target AtKAPAS on the pathway of biotin biosynthesis and to characterize the phenotypic consequences of loss-of-function mutations [11]. The 528 plant-derived natural compounds stored in KRICT Chemical Bank were assessed on the inhibitory effect on KAPAS

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using the partially purified AtKAPAS protein, *in vitro*. Less than 2% of 528 compounds exhibited inhibitory effect under a concentration of 20 μM. Interestingly, several naturally occurring quinone compounds including chrysophanic acid, tanshinones, 5,8-dihydroxy-1,4-naphthoquinone, and plumbagin were observed to give a potent inhibitory effect on KAPAS. Plumbagin, a natural naphthoquinone demonstrated the most effective inhibition on KAPAS in a concentration-dependent manner, and the IC_{50} was calculated as 2.1 μM. Webster et al. [12] reported that biotin is an essential enzyme cofactor for carboxylase and transcarboxylase reactions. Abell [28] and Pillmoor et al. [29] suggested that if an enzyme is a potential target, a 60–80% inhibition of its activity leads to a severe growth. However, this requires the confirmation of potential target. For the purpose of target validation, a rescue study was carried out. Plumbagin inhibited germination of *A. thaliana* seeds but this effect was rescued by a biotin supplement. From this point of view, our results suggest that strong inhibition of KAPAS by plumbagin leads to restriction on the biotin biosynthesis in plants, ultimately the stems or leaves of plant treated with plumbagin die. Hwang et al. [11] argued that knowledge of biochemical pathways in plants is incomplete, and the next major herbicide target may lie in an unexpected area of plant metabolism; knowledge in detail how plants actually die as a result of inhibition of some known targets is still ambiguous. Also, we should note that the complete inhibition of enzyme activity at some known targets is not necessary for plant death [30]. However, it can be predicted that the herbicidal activity is somewhat connected between the reduced level of target enzyme activity and plant death. The enzyme inhibition results and rescue effect by biotin strongly suggested that the herbicidal activity by foliar treatment was due to the inhibition of KAPAS caused by the plumbagin. The natural chemical plumbagin has been shown by our research to effectively control eight weed species of *S. bicolor*, *E. crus-galli*, *D. sanguinalis*, *S. nigrum*, *A. indica*, *A. avicennae*, *X. strumarium*, *C. japonica* under non-replicated greenhouse conditions. Also, the foliar application of the natural compound plumbagin at 2,000 Mg/mL has completely controlled 10 ~ 15 leaf-stage and 2 ~ 3 m vine length natural *S. Angulatus*, with sustantial residual activity under field conditions. The residual activity lasted for 2 weeks because regrowth was not observed until then. Visual symptoms of browning and necrosis of leaf tissue after plumbagin foliar applications appear to be introduced by cellular leakage rather than the inhibition of photosynthesis since cellular leakage occurred under light and dark conditions without chlorophyll loss. It seems closely related to the membrane lipid peroxidation as a result of the biotinyl carboxylase and transcarboxylase inhibition attributable to the biotin deficiency by KAPAS inhibition. Biotin is an essential enzyme cofactor for carboxylase and transcarboxylase reactions in plant leaf, and KAPAS inhibition resulted in biotin depletion. As reviewed by Delye et al. [31] and Hwang et al. [32], these pathways in plant have been well established by acetyl-CoA carboxylase (ACCase) inhibiting herbicides, like as aryloxyphenoxypropionates and cyclohexanediones. ACCase is involved in the first step of lipid synthesis. The target site of acetyl-CoA carboxylase is a biotin-dependent enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA. The inhibition of KAPAS by plumbagin might result in the deficiency of substrate biotin to the biotinyl carboxylase in plants. However, the mechanism of action should be studied for better understanding of whole plant-compound interactions confirmative for this speculation.

In a competing mechanism, proton abstraction is involved with the attack of acetyl-CoA. When the biotin is deficient, the product, malonyl-CoA is not produced. Malonyl-CoA is a building block for new fatty acids and can inhibit the transfer of the fatty acyl group from acyl-CoA to carnitine with carnitine acyltransferase, which inhibits the beta-oxidation of fatty acids in the mitochondria. *S. angulatus* have been designated as one of the ecological disturbance plants by the Ministry of Environment in Korea. *S. angulatus* has spread across the marginal of agricultural field close to riparian zones along the rivers in Korea within the 15 years since its first appearance in 1989 (An Dong), covering more than 110 ha in 2005 [14,15]. The social and agricultural impact, risk assessment, invasion plants identification, and control management methods for alien vine plant such as *Humulus japonica* and *S. angulatus* have become a great problem in Korea. In conclusion, our results show that the herbicidal effect of plumbagin, a naturally occurring naphthoquionone, is closely associated with its inhibitory effect on KAPAS, a new target site of herbicide. Plumbagin and related 1,4-naphthoquinone compounds could be employed as a good chemical lead for an *S. angulatus* herbicide with a new mode of action (Fig. 4).

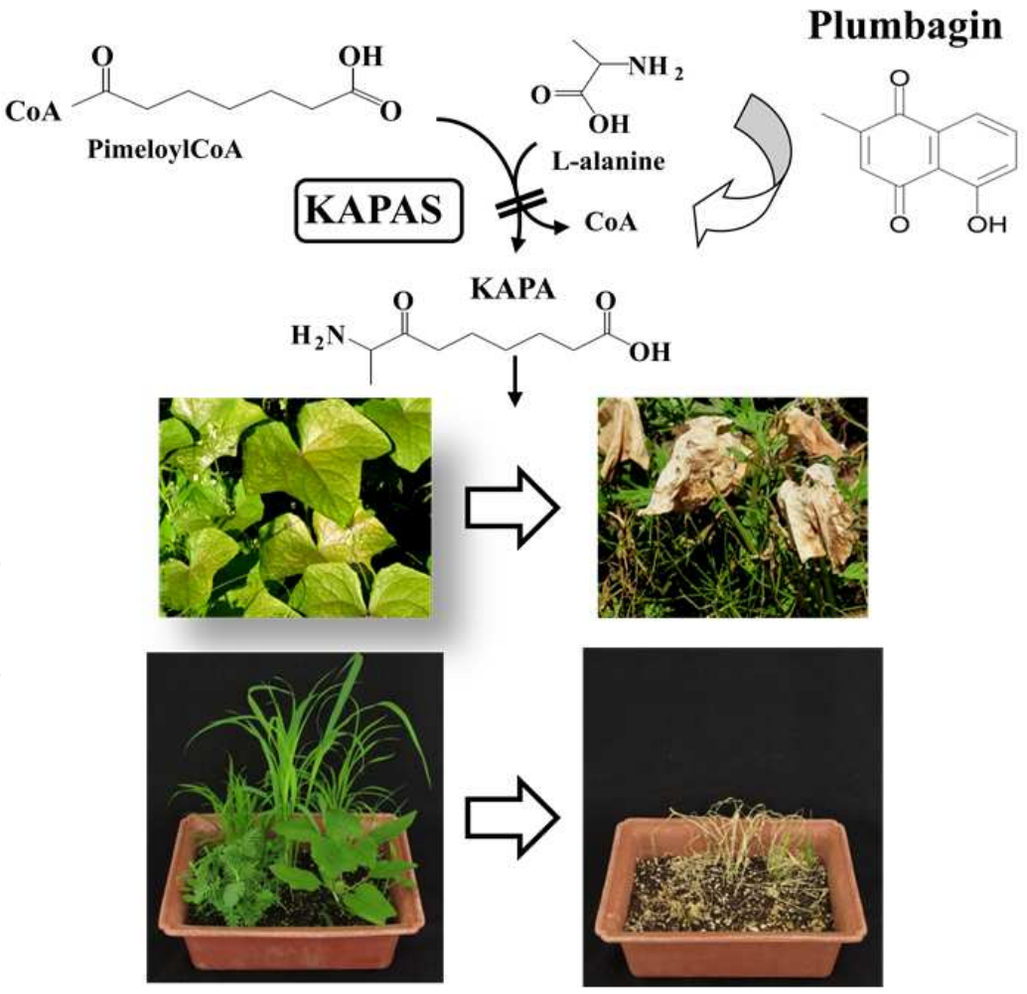

Figure 4. Proposed target site of plumbagin and herbicidal activity

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