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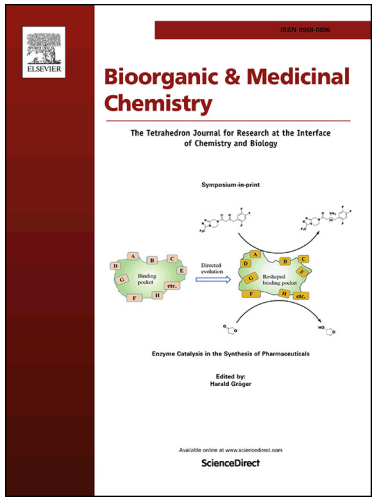

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Synthesis and inhibitory activity of mechanism-based 4-coumaroyl-CoA ligase inhibitors

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Keywords: 4-coumaroyl-CoA ligase; mechanism-based inhibitor; ANL superfamily; acylsulfamide

Abbreviations: ANL, acyl- and aryl-CoA synthetases, nonribosomal peptide synthetase adenylation domains, luciferase; CDI, 1,1′-carbonyldiimidazole; 4CL, 4-coumaroyl-CoA ligase; CoA, coenzyme A; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; MEM, 2-methoxyethoxymethyl; rt, room temperature; TMS, tetramethylsilane; Z, benzyloxycarbonyl.

Abstract

4-Coumaroyl-CoA ligase (4CL) is ubiquitous in the plant kingdom, and plays a central role in the biosynthesis of phenylpropanoids such as lignins, flavonoids, and coumarins. 4CL catalyzes the formation of the coenzyme A thioester of cinnamates such as 4-coumaric, caffeic, and ferulic acids, and the regulatory position of 4CL in the phenylpropanoid pathway renders the enzyme an attractive target that controls the composition of phenylpropanoids in plants. In this study, we designed and synthesized mechanism-based inhibitors for 4CL in order to develop useful tools for the investigation of physiological functions of 4CL and chemical agents that modulate plant growth with the ultimate goal to produce plant biomass that exhibits features that are beneficial to humans. The acylsulfamide backbone of the inhibitors in this study was adopted as a mimic of the acyladenylate intermediates in the catalytic reaction of 4CL. These acylsulfamide inhibitors and the important synthetic intermediates were fully characterized using two-dimensional NMR spectroscopy. Five 4CL proteins with distinct substrate specificity from four plant species, i.e., *Arabidopsis thaliana*, *Glycine max* (soybean), *Populus trichocarpa* (poplar), and *Petunia hybrida* (petunia), were used to evaluate the inhibitory activity, and the half-maximum inhibitory concentration (IC_{50}) of each acylsulfamide in the presence of 4-coumaric acid (100 μM) was determined as an index of inhibitory activity. The synthetic acylsulfamides used in this study inhibited the 4CLs with IC_{50} values ranging from 0.10 to 722 μM, and the IC_{50} values of the most potent inhibitors for each 4CL were 0.10 to 2.4 μM. The structure-activity relationship observed in this study revealed that both the presence and the structure of the acyl group of the synthetic inhibitors strongly affect the inhibitory activity, and indicates that 4CL recognizes the acylsulfamide inhibitors as acyladenylate mimics.

1. Introduction

4-Coumaroyl-CoA ligase (4CL, EC 6.2.1.12) plays a pivotal role in the phenylpropanoid pathway via the formation of coenzyme A (CoA) thioesters of cinnamates, i.e., key precursors for various classes of important secondary metabolites such as lignins, flavonoids, and coumarins. The 4CL proteins, which are widespread in the plant kingdom, have been obtained from angiosperm dicot families such as Apiaceae,[1] Brassicaceae,[2-4] Fabaceae,[5, 6] Lamiaceae,[7] Moraceae,[8] Rosaceae,[9, 10] Rutaceae,[11] Salicaceae,[12-14] Solanaceae,[15-17] as well as from the monocot families Poaceae,[18-20] gymnosperm Pinaceae,[21, 22] and bryophyte plants,[23, 24] and their enzymatic properties have been characterized.

In the aforementioned plants, more than two isoforms of 4CL are identified frequently. These are often considered to play distinct plant-specific physiological functions, and these functions are sometimes correlated with the localization of isoforms. In *Populus tremuloides* (aspen), for example, the expression of *Pt4CL1* is specific in lignifying xylem and associated with the lignin biosynthesis, whereas *Pt4CL2* mainly expresses in the epidermal cells of stem and leaf, and is involved in the biosynthesis of phenylpropanoids except lignins.[12] To date, 4CL proteins from dicot plants are generally categorized into two evolutionary divergent classes:[2] while class I 4CLs are regarded as key enzymes in the lignin biosynthesis, class II 4CLs concern the biosynthesis of other phenylpropanoids such as flavonoids, volatile benzenoids,[16] and coumarins that confer protection from UV, wounding, and pathological fungi, as well as the attraction of pollinators. A phylogenetic classification of 4CLs specific for monocot,[18, 20, 25, 26] gymnosperm,[22] and bryophyte plants[23] has been proposed.

As suggested by the name, almost all 4CLs recognize 4-coumaric acid as a good to excellent substrate. Caffeic and ferulic acids generally serve as good substrates, but strong preferences are sometimes observed. For example, At4CL1 and At4CL2 from *Arabidopsis thaliana* both belong to class I and catalyze the ligation of caffeic acid, although only the former also reacts with ferulic acid.[2] Moreover, only a minority of 4CL enzymes recognizes cinnamic acid as a good substrate.[9, 11]

The CoA thioester formation of cinnamic acid is catalyzed predominantly by cinnamic acid:CoA ligase, which is evolutionally distinct from 4CL.[16] To our best knowledge, only two 4CLs, i.e., Gm4CL1 from *Glycine max* (soybean)[5] and At4CL4 from *A. thaliana*,[3] can effectively catalyze the ligation of sinapic acid, and the physiological role of these sinapic acid-activating 4CLs still remains unclear. The crystal structures of 4CL clearly show that the substrate specificity mainly arises from the size of the binding pocket of the cinnamates,[27, 28] and that it is possible to broaden the reactivity of 4CL based on protein-engineering techniques.[29-31]

The regulatory position of 4CL in the biosynthesis of phenylpropanoids renders the enzyme an attractive target that controls the composition of the products of the phenylpropanoid pathway in plants. Among the phenylpropanoids lignins have attracted the most attention, and the decrease of lignin content induced by the suppression of the functions of 4CL has been reported for *A. thaliana*,[32-35] *Nicotiana tabacum* (tobacco),[36, 37] *Oryza sativa* (rice),[18] *Panicum virgatum* (switchgrass),[19] *Pinus radiate* (radiata pine),[38] *Populus* spp. (poplars),[39-42] and *Saccharum* spp. (sugarcane).[26] These results are sometimes correlated to the improvement of the biomass quality,[19, 26, 34] since the rigidity of lignins may render the digestion of biomass difficult during the production of biofuels. The decrease of the content of other phenylpropanoids such as eugenol,[7] chlorogenic acid, and tannins[42] via the suppression of the 4CL functions has also been reported. Therefore, the control of the 4CL functions potentially represents a promising approach to generate plants, which contain modified contents/compositions of a wide variety of phenylpropanoids, and thus offer properties that are beneficial to humans.

To date, almost all attempts to regulate the functions of 4CL have been conducted via genetic techniques, and chemical approaches using 4CL-specific inhibitors have not yet been developed (*vide infra*). However, the chemical regulation of enzyme functions using specific inhibitors usually offers advantages over genetic methods. One can simply start and stop the regulation of target enzymes by choosing the time and the duration of the application of the inhibitor, while the intensity of the application can be controlled via the dose of the inhibitor. Moreover, simultaneous

regulation of more than two kinds/isoforms of enzymes can be easily achieved by employing an appropriate set of inhibitors. Therefore, 4CL-specific inhibitors should be useful tools for the investigation of the physiological roles of 4CL, as well as for the regulation of the phenylpropanoid pathway in order to generate plants with properties that are beneficial to humans.

It has been reported that one substrate of a 4CL enzyme inhibits the catalytic reactions of the 4CL enzyme with other substrates. In case of the poplar 4CLs, for example, caffeic acid strongly inhibits the ligation of 4-coumaric acid and ferulic acid catalyzed by Pt4CL1 from *P. tremuloides* [43] and Ptr4CL3/5 from *P. trichocarpa*.[14] However, such inhibitions cannot suppress the pathways starting from the substrates used as inhibitors. Yogo and co-workers have reported that the herbicides propanil and swep inhibit a tobacco 4CL (Nt4CL1) expressed in *Escherichia coli*, as well as other 4CLs extracted from several plants.[44, 45] Products of the phenylpropanoid pathway such as chalcone, naringenin, and coniferin have also been reported to inhibit 4CLs.[17, 21, 46] However, these non-substrate inhibitors are not specific to 4CL, as propanil and swep are well-known inhibitors of the photosystem II,[45] while chalcone also inhibits phenylalanine ammonia lyase.[47]

In order to develop 4CL-specific inhibitors, we focused on the catalytic mechanism of 4CL. 4CL belongs to the ANL superfamily of enzymes (acyl- and aryl-CoA synthetases, nonribosomal peptide synthetase adenylation domains, and firefly luciferase).[48] The characteristic aspect of this family is that these members activate carboxylate substrates with ATP to form acyladenylate intermediates, which are used in a diverse set of second partial reactions.[49] The schematic illustration of the ligation reactions via acyladenylate intermediates catalyzed by ANL enzymes are depicted in Figure 1. The enzymes accept carboxylate substrates and ATP in the adenylate-forming conformation; the resulting acyladenylate intermediates react with CoA in the thioester-forming conformation. Due to this common catalytic mechanism, mimics of the acyladenylate intermediates are expected to act as inhibitors specific to the ANL superfamily of enzymes. In fact, acylsulfamide analogues synthesized as acyladenylate intermediate mimics, have been reported to inhibit ANL enzymes *in vivo*[50] and *in vitro*.[51] In these acylsulfamide analogues, the phosphonate moiety of the acyladenylate

intermediates is replaced with a sulfamide moiety, which is resistant to further enzymatic reactions. In the present study, we adopted this acylsulfamide strategy, and designed novel acylsulfamide analogues as mechanism-based 4CL inhibitors (Figure 1). In order to unveil the effect of substituents on the benzene ring of the cinnamate moiety against the 4CL inhibitory activity, the acyl groups of **1a**–**d** were varied to include cinnamic (**1a**), 4-coumaric (**1b**), ferulic (**1c**), and sinapic (**1d**) acid. Moreover, **1e**, which bears an octanoic acid as an acyl group, and AMP mimic **1e**, were designed to determine the presence of the cinnamate-specific recognition mechanism of 4CL. The inhibitory activity of the synthetic acylsulfamide analogues was evaluated using five distinct 4CL proteins with different substrate specificity. It is worth mentioning that the substrate specificity of each 4CL should correlate with the structure of the cinnamate binding pocket in the adenylate-forming conformation. As **1a**–**d** are expected to act as mimics of the acyladenylate intermediate, the inhibitory activity of **1a**–**d** should reflect the affinity of the 4CLs against the corresponding acyladenylate intermediates in the thioester-forming conformations.

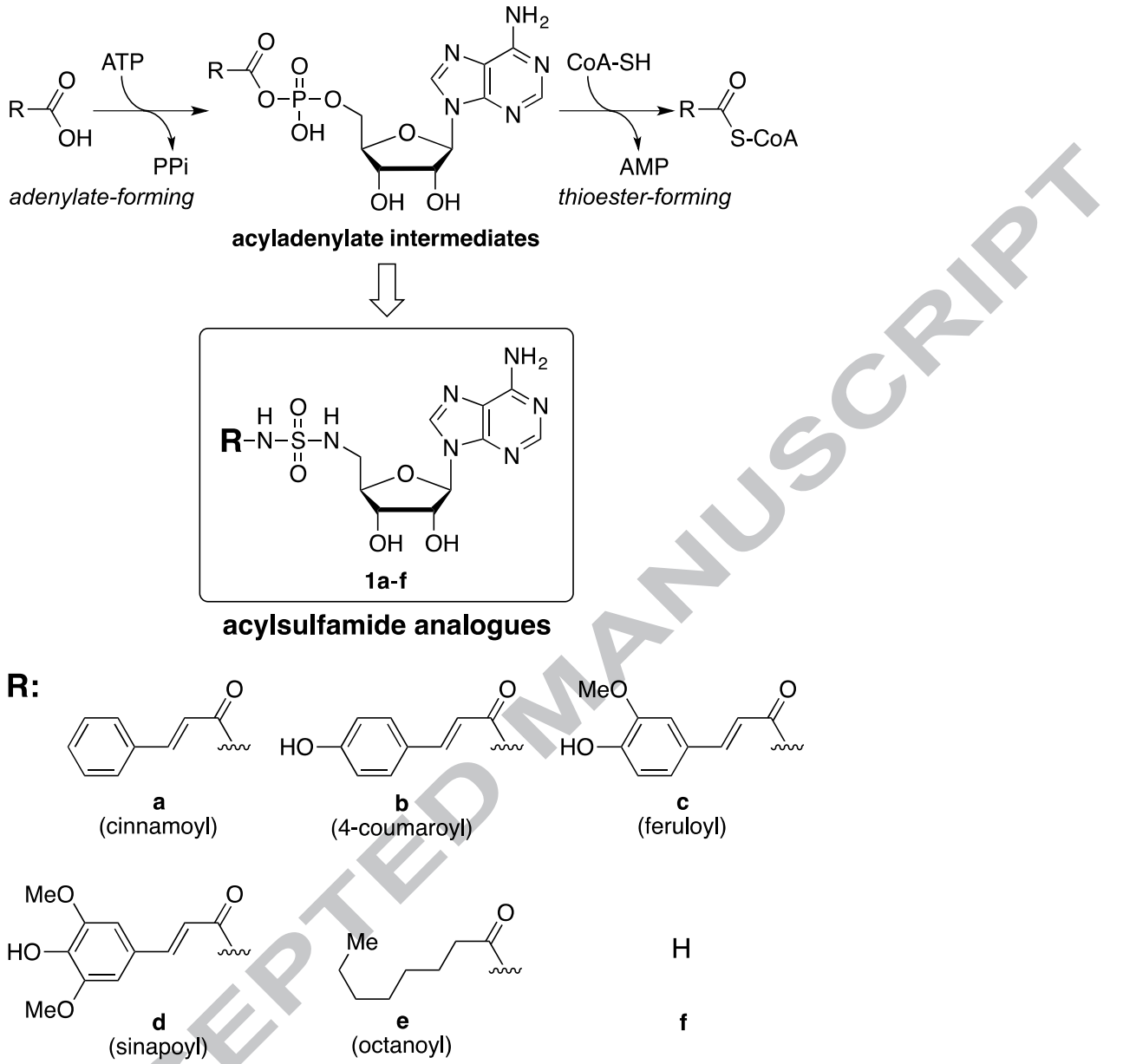


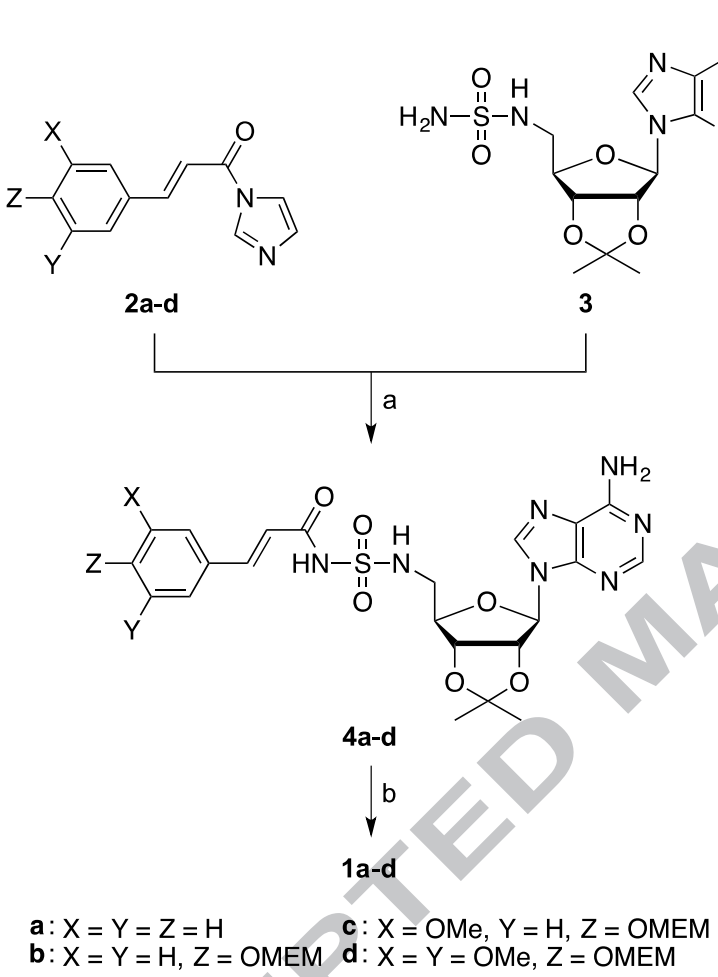
Figure 1. Schematic illustration of the ligation reactions via acyladenylate intermediates catalyzed by ANL enzymes, and structures of the acylsulfamide analogues (**1a**–**f**) used in this study.

2. Results and discussion

2.1 Synthesis

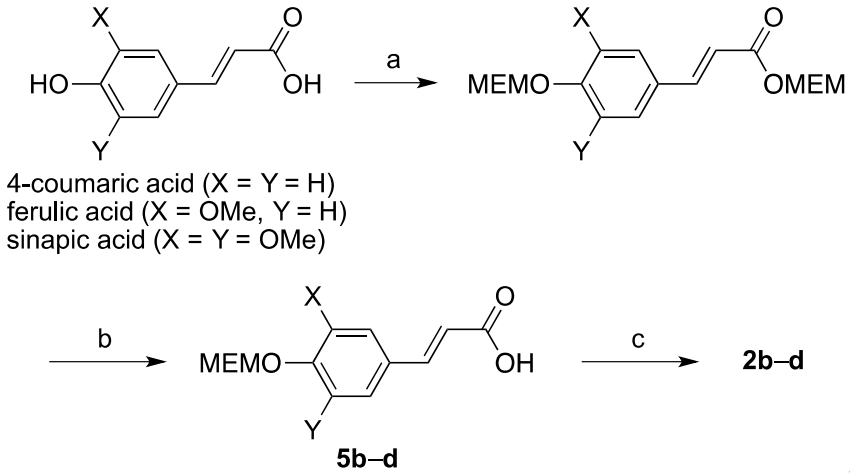
The synthetic route to **1a**–**d** is outlined in Scheme 1. The synthesis of **1a** is straightforward, i.e., 1-cinnamoylimidazole (**2a**), prepared from cinnamic acid and 1,1′-carbonyldiimidazole (CDI), was condensed with the protected AMP analogue **3** in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to furnish fully protected intermediate **4a**, which was

hydrolyzed under acidic conditions to afford **1a**.



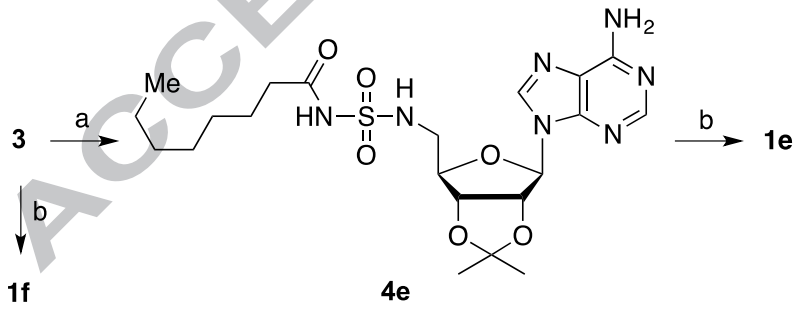
Scheme 1. Synthesis of **1a**–**d**; reagents and conditions: (a) DBU, MeCN, rt, 57–70%; (b) HCO_2H/H_2O (4:1, v/v), rt, 33–98%.

In the synthesis of **1b**–**d**, the phenolic hydroxy groups of the 4-coumaric, ferulic, and sinapic acid moieties were protected prior to condensation (Scheme 2). For that purpose, these cinnamates were treated with an excess of 2-methoxyethoxymethyl chloride (MEMCl) in the presence of ethyldiisopropylamine. The crude products, in which the carboxy groups were esterified, were converted into the carboxylic acids **5b**–**d** via basic hydrolysis prior to treatment with CDI. The thus obtained acylimidazoles (**2b**–**d**) were condensed with **3**, and subsequently subjected to acidic hydrolysis, which furnished inhibitors **1b**–**d** (Scheme 1).



Scheme 2. Synthesis of acylimidazoles **2b**–**d**; reagents and conditions: (a) MEMCl, (*i*-Pr)$_2$NEt, CH_2Cl_2, rt; (b) NaOH, MeOH/H_2O, rt, 72–84% (two steps); (c) CDI, MeCN, rt, 76%–quant.

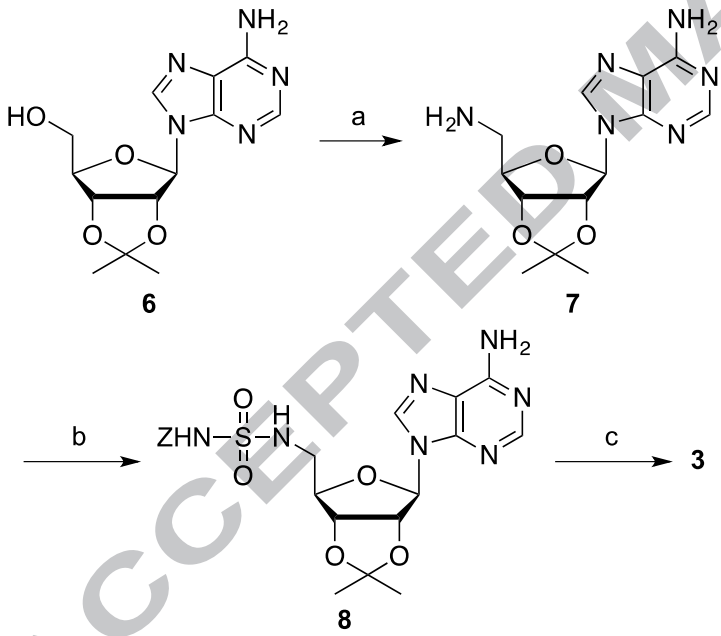
The synthesis of **1e** and **1f** is illustrated in Scheme 3. A procedure similar to that for the formation of **1a** was employed for the synthesis of **1e**, i.e., 1-octanoylimidazole[52] was condensed with **3** to generate **4e**, which was treated with aqueous formic acid to furnish **1e**. The corresponding AMP analogue (**1f**) was directly synthesized from **3** via acidic hydrolysis.



Scheme 3. Synthesis of **1e** and **1f**; reagents and conditions: (a) 1-octanoylimidazole, DBU, MeCN, rt, 79%; (b) HCO_2H/H_2O (4:1, v/v), rt, 36–97%.

As illustrated in Scheme 4, **3**, which is the key synthetic intermediate in this study, was synthesized from 2′,3′-*O*-isopropylideneadenosine (**6**). According to previous reports,[53] **6** was converted into

amine **7** in two steps. Previously, **7** was directly transformed into **3** by the treatment of sulfamoyl chloride.[54] Even though this preparation requires only a single step, chromatographic purification is required, and the reported yield (34%) is not satisfactory. In this study, **7** was initially treated with *N*-(benzyloxycarbonyl)sulfamoyl chloride, which was prepared *in situ* by the reaction between chlorosulfonyl isocyanate and benzyl alcohol, to give the protected sulfamide **8** in 82% yield. Subsequently, the benzyloxycarbonyl (Z) group on the sulfamide nitrogen atom of **8** was removed by hydrogenolysis to afford **3** in 81% yield. In this procedure, chromatographic purification was not necessary in either step, due to the high purity of the products, and the overall yield (66%) was improved by a factor of ~2 relative to the previously reported method.



Scheme 4. Synthesis of **3**; reagents and conditions: (a) ref. 53; (b) BnOH, $OCNSO_2Cl$, CH_2Cl_2, 0 ºC–rt, 82%; (c) H_2, 10% Pd-C, AcOH, rt, 81%.

2.2. Evaluation of the inhibitory activity and structure-activity relationship

The inhibitory activity of the synthetic acylsulfamide analogues was evaluated using five 4CLs with distinct substrate specificity, i.e., At4CL1 and At4CL2 from *A. thaliana*,[2] Gm4CL1 from *G. max*,[5] Ptr4CL3 from *P. trichocarpa*,[14] and Ph4CL1 from *Petunia hybrida* (petunia).[16] These 4CL proteins

were conventionally expressed in *E. coli* and purified. The 4CL activity was measured via a previously described spectrophotometric assay.[55] 4-Coumaric acid (100 µM), ATP, and CoA (250 µM each) were used as substrates. The 4CL activity was recorded in the presence of inhibitors at various concentrations, and the half-maximum inhibitory concentration (IC_{50}) determined from dose-response curves was used as an index of inhibitory activity. The corresponding results are summarized in Table 1. Recently, Li and Nair have reported the crystal structures of Nt4CL2 complexed with the acyladenylate intermediates in the thioester-forming conformations.[28] Based on their results we designed our synthetic inhibitors to mimic the acyladenylate intermediates, the inhibitory activity of the synthetic acylsulfamides should reflect the affinity of the 4CLs against the acyladenylate intermediates in the thioester-forming conformations, whereas the substrate specificity of each 4CL should correlate with the structure of the binding pocket of the cinnamates in the adenylate-forming conformation.

Table 1. Inhibitory activity of **1a**–**f** against 4CLs.

Inhibitor	Acyl group	IC_{50} (µM)[a]				
		At4CL1	At4CL2	Gm4CL1	Ptr4CL3	Ph4CL1
1a	cinnamoyl	25.1 ± 2.8	38.0 ± 4.3	69.5 ± 0.5	24.9 ± 3.3	4.8 ± 0.9
1b	4-coumaroyl	0.26 ± 0.02	2.4 ± 0.3	0.55 ± 0.01	0.87 ± 0.07	0.38 ± 0.04
1c	feruloyl	5.5 ± 0.3	34.5 ± 3.4	0.10 ± 0.01	3.9 ± 0.1	1.5 ± 0.2
1d	sinapoyl	58.2 ± 4.6	56.8 ± 3.5	0.18 ± 0.03	55.2 ± 4.1	62.2 ± 1.5
1e	octanoyl	361 ± 39	722 ± 54	249 ± 13	92.6 ± 4.2	25.1 ± 0.8
1f	–	> 1000	> 1000	> 1000	> 1000	> 1000

a) Mean values ± standard deviation (n = 3).

Against At4CL1, **1b** exhibited a strong inhibitory activity (IC_{50}: 0.26 μM), and **1c** also acted as a potent inhibitor (IC_{50}: 5.5 μM). Against At4CL2, **1b** was the most potent inhibitor (IC_{50}: 2.4 μM), while **1c** was less active. Compared to At4CL1, the sensitivity of At4CL2 against **1b** decreases by approximately one order of magnitude. These results should reflect the substrate specificity of At4CL1/2:[2] i) the preference of At4CL1 for 4-coumaric acid is ten times higher than that for ferulic acid, ii) the interaction of At4CL1 with 4-coumaric acid approximately seven times higher than that of At4CL2, and iii) At4CL2 does not react with ferulic acid. Both **1a** and **1d** weakly inhibit At4CL1/2, which uses cinnamic acid as a poor substrate and shows no activity against sinapic acid.[2] Replacement of the substrate-type acyl groups of **1a**–**d** with an octanoyl group renders **1e** almost inactive against At4CL1/2. These results indicate that the structure of the cinnamate binding pocket of At4CL1/2 in the thioester-forming conformation merely accepts the octanoyl group, and, therefore, the ligation reactions catalyzed by At4CL1/2 should be specific to cinnamates.

As expected form the wide substrate specificity of Gm4CL1, which is one of the few 4CLs that exhibits high activity against sinapic acid, **1c** (IC_{50}: 0.10 μM), **1d** (IC_{50}: 0.18 μM), and **1b** (IC_{50}: 0.55 μM) showed strong inhibitory activity. It is known that Gm4CL1 efficiently catalyzes the ligation reactions of ferulic, sinapic, and 4-coumaric acids.[5] The low inhibitory activity of **1a** against Gm4CL1 is consistent with the poor reactivity of the enzyme against cinnamic acid,[5] and these results suggest that the hydroxy groups at the 4-position of the benzene ring of **1b**–**d** play an important role in the substrate recognition of the enzyme. The ligation reactions of Gm4CL1 can be cinnamate specific, as in the cases of At4CL1/2, considering that **1e** only shows a marginal activity against Gm4CL1.

Against Ptr4CL3, **1b** (IC_{50}: 0.87 μM) and **1c** (IC_{50}: 3.9 μM) exhibit strong and potent inhibitory activity, respectively, while that of **1d** is less active. These results are consistent with the substrate specificity of Ptr4CL3, for which 4-coumaric acid is the most preferable and ferulic acid is a good substrate, while sinapic acid shows no reactivity.[14] However, no data was reported with respect to cinnamic acid, even though cinnamic acid is expected to be a poor substrate for Ptr4CL3, given that

1a does not inhibit the enzyme effectively. The inhibitory activity of **1e** against Ptr4CL3 is very weak (IC_{50}: 92.6 μM), although Ptr4CL3 is approximately 2.5–7 times more sensitive against **1e** compared to At4CL1/2 and Gm4CL1. This result suggests that the structure of the cinnamate binding pocket of Ptr4CL3 in the thioester-forming conformation is slightly different from that of At4CL1/2 and Gm4CL1.

Compared to the other 4CLs, Ph4CL1 is more sensitive against **1a** (IC_{50}: 4.8 μM), which is approximately 13-fold weaker than the most potent **1b** (IC_{50}: 0.38 μM). The difference is consistent with the substrate specificity of Ph4CL1, i.e., the catalytic activity of Ph4CL1 against 4-coumaric acid is approximately 13 times higher than that against cinnamic acid.[16] With respect to 4-coumaric and ferulic acids, a slight difference between the sensitivity toward the acylsulfamide inhibitors and the substrate specificity was observed. Ph4CL1 shows the highest reactivity against ferulic acid and the catalytic efficiency of the enzyme against 4-coumaric acid is about 60% of that against ferulic acid.[16] However, **1b** was the most potent inhibitor for Ph4CL1, followed by **1c** (IC_{50}: 1.5 μM). This difference should arise from the structural differences with respect to the cinnamate binding site of Ph4CL1 between the adenylate- and the thioester-forming conformations, and the degree of the difference between the two conformations should be higher than for other 4CLs. Among the tested 4CLs, Ph4CL1 is the most sensitive enzyme against **1e** (IC_{50}: 25.1 μM), indicating that the structure of the cinnamate binding pocket of Ph4CL1 in the thioester-forming conformation should substantially differ from that of the other 4CLs. Even though the ligation reaction catalyzed by Ph4CL1 should be specific against cinnamates, the inhibitory activity of **1e** against the enzyme is still ca. 65-fold less potent than that of **1b**. From a phylogenetic perspective,[16] Ph4CL1 is more closely related to Ptr4CL3 (designated as Pt1s07400 in the literature) than to Gm4CL1, while At4CL1/2 is even more distinct. Therefore, the differences in the cinnamate binding pocket structures could potentially arise from the evolutionary diversity of the 4CL proteins.

The presence of the acyl group of the sulfamide moiety is critical for the 4CL inhibitory activity, given that the AMP mimic **1f** was inactive against all 4CLs tested. This result indicates that 4CL

recognizes the acylsulfamide inhibitors **1a**–**e** as mimics of the acyladenylate intermediates, and the affinity of 4CL toward the acyladenylate intermediates that bear cinnamates as the acyl group is superior to that of AMP. Compared to **1f**, **1d** exhibited weak but clear inhibitory activity against At4CL1/2, Ptr4CL3, and Ph4CL1, which exhibit no catalytic activity against sinapic acid. This result also supports the notion that **1d** acts as the acyladenylate intermediate mimic, i.e., that the structures of the cinnamate binding pocket in the adenylate-forming conformations that are unable to accommodate sinapic acid could be enlarged in the thioester-forming conformations, where the binding acyladenylate intermediates react with CoA.

3. Conclusions

In this study, we designed and synthesized acylsulfamide inhibitors for 4CL as mimics of acyladenylates, which represent the key intermediates in the enzymatic reaction of 4CL. The target inhibitors and the important synthetic intermediates were fully characterized using two-dimensional NMR spectroscopy techniques. The inhibitory activity of synthetic acylsulfamides was evaluated using five 4CL proteins with distinct substrate specificity, and the highest inhibitory activity against each 4CL, represented by IC_{50} values, ranged from 0.10 to 2.4 μM. The tested 4CLs are very sensitive against the presence/structure of the acyl group of the sulfamide moiety, and inhibitors that carry better substrates as the acyl group tend to inhibit 4CL with higher potency. The structure-activity relationship established in this study indicates that 4CL recognizes the acylsulfamide inhibitors as mimics of the acyladenylate intermediates. Investigations into the biological activity of the acylsulfamide inhibitors *in vivo* are currently in progress.

4. Material and methods

4.1. Synthesis of 4CL inhibitors

4.1.1. General

Meltingpoints (mp) were measured with a Mettler-Toledo FP62 meltingpoint apparatus and are

uncorrected. NMR spectra were obtained on a JEOL JNM-AL300 (300 MHz for ^{1}H) or a Bruker AVANCEIII 600 (600 MHz for ^{1}H) spectrometers. Chemical shifts are reported in parts per million relative to tetramethylsilane as the internal standard unless otherwise noted. The ^{1}H and ^{13}C NMR signals of **4c**–**e**, **8**, and **1a**–**f** were fully assigned using two-dimensional NMR measurements based on ^{1}H-^{1}H correlation (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) techniques. These results, together with the assignment of **4a** and **4b**, are summarized in Tables S1–S3 (Supporting Information). Elemental analyses were performed on a Yanaco MT-5. High resolution mass spectrometry (HRMS) was performed on a JOEL JMS-700 spectrometer.

All reagents and solvents were used as received from commercial sources. Silica gel flash column chromatography was carried out using a Yamazen W-prep 2XY system with Hi-Flash columns (silica gel, 40 μm). Reverse-phase medium pressure column chromatography was carried out using a Büchi Sepacore chromatography system with Nacalai COSMOSIL 5C_{18}-PAQ column (φ20 mm × 250 mm). 1-Octanoylimidazole[52] and **7**[53] were prepared according to the literature methods.

4.1.2. Synthesis of protected cinnamates 5b–d

4.1.2.1. (*E*)-3-{4-[(2-Methoxyethoxy)methoxy]phenyl}acrylic acid (5b)

The following procedure is typical for **5b**–**d**. To an ice-cooled solution of 4-coumaric acid (5.06 g, 30.8 mmol) and (*i*-Pr)$_2$NEt (21.5 mL, 123 mmol) in anhydrous CH_2Cl_2 (40 mL) was added MEMCl (11.0 mL, 96.3 mmol) dropwise for 5 min. The mixture was stirred for 14 h at room temperature and refluxed for 9 h. The reaction mixture was diluted with $CHCl_3$ (60 mL) and washed with saturated aqueous $NaHCO_3$ solution (40 mL). The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give the (2-methoxyethoxy)methoxy ester of **5b** as an orange viscous oil (13.0 g). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 3.37 (3H, s), 3.40 (3H, s), 3.54–3.60 (4H, m), 3.81–3.86 (4H, m), 5.30 (2H, s), 5.46 (2H, s), 6.33 (1H, d, *J* = 16.2 Hz), 7.07 (2H, d, *J* = 8.7 Hz), 7.48 (2H, d, *J* = 8.7 Hz), 7.70 (1H, d, *J* = 16.2 Hz). This crude material was dissolved in

MeOH (60 mL) followed by addition of 10% (w/v) aqueous NaOH solution (20 mL). The reaction mixture was stirred for 1.5 h at room temperature and concentrated *in vacuo*. The residue was dissolved in water (60 mL) and washed with Et_2O (20 ml × 3). The water layer was acidified with 2M HCl and a precipitated solid was collected by suction filtration. The precipitate was washed with water and hexane respectively, and dried under reduced pressure to give **5b** (6.56 g, 84%) as a colorless solid. This was used without further purification. Mp 102–103 ºC (EtOH). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 3.38 (3H, s), 3.55–3.58 (2H, m), 3.81–3.84 (2H, m), 5.31 (2H, s), 6.33 (1H, d, *J* = 15.9 Hz), 7.07 (2H, d, *J* = 8.7 Hz), 7.50 (2H, d, *J* = 8.7 Hz), 7.74 (1H, d, *J* = 15.9 Hz). Anal. Calcd for $C_{13}H_{16}O_5$: C, 61.90; H, 6.39. Found: C, 61.89, H, 6.43.

4.1.2.2. (*E*)-3-{3-Methoxy-4-[(2-methoxyethoxy)methoxy]phenyl}acrylic acid (5c)

Yield = 84%. A colorless solid. Mp 82–84 ºC (EtOH). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 3.38 (3H, s), 3.55-3.58 (2H, m), 3.86–3.89 (2H, m), 3.92 (3H, s), 5.37 (2H, s), 6.34 (1H, d, *J* = 15.6 Hz), 7.08–7.13 (2H, m), 7.22 (1H, d, *J* = 8.1 Hz), 7.73 (1H, d, *J* = 15.6 Hz). Anal. Calcd for $C_{14}H_{18}O_6$: C, 59.57; H, 6.43. Found: C, 59.34; H, 6.48.

4.1.2.3. (*E*)-3-{3,5-Dimethoxy-4-[(2-methoxyethoxy)methoxy]phenyl}acrylic acid (5d)

Yield = 72%. A colorless solid. Mp 83–93 ºC (EtOH). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 3.37 (3H, s), 3.55–3.59 (2H, m), 3.87 (6H, s), 3.98–4.01 (2H, m), 5.25 (2H, s), 6.36 (1H, d, *J* = 15.8 Hz), 6.77 (2H, s), 7.70 (1H, d, *J* = 15.8 Hz). Anal. Calcd for $C_{15}H_{20}O_7$: C, 57.69; H, 6.45. Found: C, 57.51; H, 6.44.

4.1.3. Synthesis of acylimidazoles 2a–d.

4.1.3.1. 1-Cinnamoylimidazole (2a)

The following procedure is typical for **2a**–**d**. To a solution of cinnamic acid (201 mg, 1.36 mmol) in anhydrous MeCN (10 mL) was added CDI (267 mg, 1.65 mmol). The reaction mixture was stirred for 2.5 h at room temperature and concentrated *in vacuo*. The residue was dissolved in toluene (20 mL) and the resultant solution was washed successively with water (10 mL) and brine (10 mL). The

organic layer was dried over anhydrous $MgSO_4$ and evaporated to give **2a** (233 mg, 86%) as a colorless solid. This was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 7.04 (1H, d, *J* = 15.5 Hz), 7.12 (1H, s), 7.38–7.43 (3H, m), 7.59–7.63 (3H, m), 8.03 (1H, d, *J* = 15.5 Hz), 8.32 (1H, s).

4.1.3.2. 1-[(*E*)-3-{4-[(2-methoxyethoxy)methoxy]phenyl}acryloyl]imidazole (2b)

Yield = quantitative. A colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 3.38 (3H, s), 3.55–3.58 (2H, m), 3.82–3.85 (2H, m), 5.33 (2H, s), 6.94 (1H, d, *J* = 15.3 Hz), 7.12 (2H, d, *J* = 8.7 Hz), 7.16 (1H, dd, *J* = 1.4, 0.8 Hz), 7.61 (2H, d, *J* = 8.7 Hz), 7.63 (1H, d, *J* = 1.4 Hz), 8.03 (1H, d, *J* = 15.3 Hz), 8.32 (1H, br s).

4.1.3.3. 1-[(*E*)-3-{3-methoxy-4-[(2-methoxyethoxy)methoxy]phenyl}acryloyl]imidazole (2c)

Yield = 86%. A colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 3.38 (3H, s), 3.55–3.58 (2H, m), 3.87–3.90 (2H, m), 3.96 (3H, s), 5.40 (2H, s), 6.97 (1H, d, *J* = 15.3 Hz), 7.17–7.30 (4H, m), 7.65 (1H, s), 8.03 (1H, d, *J* = 15.3 Hz), 8.44 (1H, br s).

4.1.3.4. 1-[(*E*)-3-{3,5-dimethoxy-4-[(2-methoxyethoxy)methoxy]phenyl}acryloyl]imidazole (2d)

Yield = 76%. A pale yellowish solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 3.37 (3H, s), 3.55–3.58 (2H, m), 3.91 (6H, s), 3.99–4.02 (2H, m), 5.27 (2H, s), 6.87 (2H, s), 6.97 (1H, d, *J* = 15.2 Hz), 7.18 (1H, br s), 7.65 (1H, br s), 7.99 (1H, d, *J* = 15.2 Hz), 8.42 (1H, br s).

4.1.4. *N*-Benzyloxycarbonyl-*N′*-(2′,3′-*O*-isopropylidene-5′-deoxyadenosin-5′-yl)sulfamide (8)

To an ice-cooled solution of chlorosulfonyl isocyanate (0.95 mL, 10.9 mmol) in anhydrous CH_2Cl_2 (12.5 mL) was added benzyl alcohol (1.1 mL, 10.6 mmol) dropwise for 3 min and the resultant mixture was further stirred for 1 h at the same temperature. This solution was added to an ice-cooled mixture of **7** (3.01 g, 9.83 mmol) and Et_3N (1.5 mL, 10.8 mmol) in anhydrous CH_2Cl_2 (30 mL) dropwise for 10 min. The reaction mixture was stirred for 1 h at room temperature and the precipitate was collected by suction filtration to give **8** (4.19 g, 82%) as a colorless solid. This was used without further purification. Mp 162–164 ºC. ^{1}H NMR (600 MHz, CD_3SOCD_3) δ_H: 1.33 (3H,

s), 1.57 (3H, s), 3.20 (1H, ddd, *J* = 13.5, 6.9, 5.1 Hz), 3.30 (1H, ddd, *J* = 13.5, 5.1, 5.1 Hz), 4.36 (1H, ddd, *J* = 5.1, 5.1, 3.0 Hz), 5.01 (1H, dd, *J* = 6.3, 3.0 Hz), 5.09 (2H, s), 5.35 (1H, dd, *J* = 6.3, 3.3 Hz), 6.15 (1H, d, *J* = 3.3 Hz), 7.29–7.36 (5H, m), 7.45 (2H, br s), 8.22 (1H, s), 8.33 (1H, s), 8.77 (1H, dd, *J* = 6.9, 5.1 Hz), 11.38 (1H, br s). ^{13}C NMR (151 MHz, CD_3SOCD_3) δ_C: 25.1, 27.0, 44.7, 66.6, 81.6, 82.6, 83.4, 90.0, 113.4, 119.4, 127.8 (2C), 128.1, 128.3 (2C), 135.6, 140.1, 148.2, 151.5, 152.6, 156.2. HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{21}H_{26}N_7O_7S$, 520.1614; found, 520.1623.

4.1.5. *N*-(2′,3′-*O*-Isopropylidene-5′-deoxyadenosin-5′-yl)sulfamide (3)

Into a mixture of **8** (4.19 g, 8.07 mmol) and 10% (w/w) Pd-C (420 mg) in AcOH (30 mL) was bubbled hydrogen gas for 4 h with stirring. The catalyst was filtered off and the filtrated was concentrated under reduced pressure. The residue was recrystallized from MeOH to give the title compound (2.52 g, 81%) as a colorless solid. Mp 150 ºC dec [lit.[54] mp 150–152 ºC (EtOH)]. ^{1}H NMR (300 MHz, CD_3SOCD_3) δ_H: 1.34 (3H, s), 1.57 (3H, s), 3.01–3.28 (2H, m), 4.34–4.38 (1H, m), 5.03 (1H, dd, *J* = 6.3, 2.7 Hz), 5.37 (1H, dd, *J* = 6.3, 3.3 Hz), 6.11 (1H, d, *J* = 3.3 Hz), 6.60 (2H, s), 7.35 (2H, br s), 7.35–7.40 (1H, m), 8.17 (1H, s), 8.30 (1H, s). HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{13}H_{20}N_7O_5S$, 386.1247; found, 386.1237.

4.1.6. Condensation of acylimidazoles and sulfamide 3.

4.1.6.1. *N*-Cinnamoyl-*N′*-(2′,3′-*O*-isopropylidene-5′-deoxyadenosin-5′-yl)sulfamide (4a)

The following procedure is typical for **4a**–**d**. To an ice-cooled suspension of **2a** (100 mg, 0.504 mmol) and **3** (212 mg, 0.550 mmol) in anhydrous MeCN (10 mL) was added DBU (0.25 mL, 1.67 mmol) dropwise and the mixture was stirred for 22 h at room temperature followed by addition of AcOH (0.15 mL, 2.62 mmol). After stirring for 3 min, silica gel (2.5 g) was added and the solvent was evaporated. The residue was subjected to silica gel flash column chromatography ($CHCl_3$/MeOH = 96:4) to give **4a** (183 mg, 70%) as a colorless solid. Mp 136–139 ºC. ^{1}H NMR

(300 MHz, CD_3OD) δ_H: 1.34 (3H, s), 1.60 (3H, s), 3.40 (1H, dd, *J* = 13.7, 3.8 Hz), 3.47 (1H, dd, *J* = 13.7, 3.8 Hz), 4.48–4.52 (1H, m), 5.04 (1H, dd, *J* = 6.0, 2.1 Hz), 5.31 (1H, dd, *J* = 6.0, 4.2 Hz), 6.04 (1H, d, *J* = 4.2 Hz), 6.53 (1H, d, *J* = 15.8 Hz), 7.36–7.40 (3H, m), 7.52–7.55 (2H, m), 7.65 (1H, d, *J* = 15.8 Hz), 8.19 (1H, s), 8.40 (1H, s). ^{13}C NMR (75 MHz, CD_3OD, solvent: 49.0) δ_C: 25.5, 27.6, 46.2, 83.1, 84.2, 84.7, 93.2, 115.6, 119.1, 120.8, 129.1 (2C), 129.8 (2C), 131.5, 135.2, 141.8, 145.6, 149.5, 154.2, 157.2, 165.9. HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{22}H_{26}N_7O_6S$, 516.1665; found, 516.1665.

4.1.6.2.

***N*-(2′,3′-*O*-Isopropylidene-5′-deoxyadenosin-5′-yl)-*N*′-[(*E*)-3-{4-[(2-methoxyethoxy)methoxy]phenyl}acryloyl]sulfamide (4b)**

Yield = 61%. A colorless solid. Mp 105–125 ºC. ^{1}H NMR (300 MHz, CD_3OD) δ_H: 1.34 (3H, s), 1.60 (3H, s), 3.32 (3H, s), 3.37 (1H, dd, *J* = 13.5, 4.2 Hz), 3.44 (1H, dd, *J* = 13.5, 4.2 Hz), 3.53–3.56 (2H, m), 3.78–3.81 (2H, m), 4.46–4.50 (1H, m), 5.04 (1H, dd, *J* = 6.3, 2.7 Hz), 5.29 (2H, s), 5.31 (1H, dd, *J* = 6.3, 4.2 Hz), 6.05 (1H, d, *J* = 4.2 Hz), 6.39 (1H, d, *J* = 15.6 Hz), 7.05 (2H, d, *J* = 8.7 Hz), 7.50 (2H, d, *J* = 8.7 Hz), 7.59 (1H, d, *J* = 15.6 Hz), 8.20 (1H, s), 8.37 (1H, s). ^{13}C NMR (75 MHz, CD_3OD, solvent: 49.0) δ_C: 25.5, 27.6, 46.3, 59.1, 68.9, 72.7, 83.2, 84.3, 84.8, 93.2, 94.2, 115.6, 117.0, 117.5 (2C), 120.9, 129.1, 130.9 (2C), 141.9, 145.3, 149.6, 154.3, 157.3, 160.6, 166.3. HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{26}H_{34}N_7O_9S$, 620.2139; found, 620.2151.

4.1.6.3.

***N*-(2′,3′-*O*-Isopropylidene-5′-deoxyadenosin-5′-yl)-*N*′-[(*E*)-3-{3-methoxy-4-[(2-methoxyethoxy)methoxy]phenyl}acryloyl]sulfamide (4c)**

Yield = 57%. A pale yellow solid. Mp 99–120 ºC. ^{1}H NMR (600 MHz, CD_3OD) δ_H: 1.33 (3H, s), 1.59 (3H, s), 3.32 (3H, s), 3.39 (1H, dd, *J* = 13.2, 3.6 Hz), 3.45 (1H, dd, *J* = 13.2, 3.6 Hz), 3.53–3.55 (2H, m), 3.81–3.82 (2H, m), 3.85 (3H, s), 4.48 (1H, ddd, *J* = 3.6, 3.6, 1.8 Hz), 5.03 (1H, dd, *J* = 6.0, 1.8 Hz), 5.28 (2H, s), 5.31 (1H, dd, *J* = 6.0, 4.2 Hz), 6.04 (1H, d, *J* = 4.2 Hz), 6.40 (1H, d, *J* = 15.9 Hz), 7.09 (1H, dd, *J* = 8.4, 1.8 Hz), 7.11 (1H, d, *J* = 8.4 Hz), 7.13 (1H, d, *J* = 1.8 Hz),

7.58 (1H, d, *J* = 15.9 Hz), 8.19 (1H, s), 8.38 (1H, s). ^{13}C NMR (151 MHz, CD_3OD) δ_C: 25.6, 27.6, 46.3, 56.4, 59.1, 69.0, 72.7, 83.2, 84.3, 84.8, 93.3, 95.3, 112.3, 115.7, 117.4, 117.5, 120.9, 123.3, 130.0, 141.9, 145.5, 149.6, 150.0, 151.4, 154.3, 157.4, 166.2. HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{27}H_{36}N_7O_{10}S$, 650.2244; found, 650.2245.

4.1.6.4. *N*-(2′,3′-*O*-Isopropylidene-5′-deoxyadenosin-5′-yl)-*N*′-[(*E*)-3-{3,5-dimethoxy-4-[(2-methoxyethoxy)methoxy]phenyl}acryloyl]sulfamide (4d)

Yield = 64%. A colorless solid. Mp 117–127 ºC. ^{1}H NMR (600 MHz, CD_3OD) δ_H: 1.34 (3H, s), 1.60 (3H, s), 3.33 (3H, s), 3.38 (1H, dd, *J* = 13.8, 3.6 Hz), 3.45 (1H, dd, *J* = 13.8, 3.6 Hz), 3.53–3.55 (2H, m), 3.84 (6H, s), 3.93–3.95 (2H, m), 4.48 (1H, ddd, *J* = 3.6, 3.6, 2.4 Hz), 5.03 (1H, dd, *J* = 6.0, 2.4 Hz), 5.14 (2H, s), 5.31 (1H, dd, *J* = 6.0, 4.2 Hz), 6.05 (1H, d, *J* = 4.2 Hz), 6.43 (1H, d, *J* = 15.6 Hz), 6.85 (2H, s), 7.57 (1H, d, *J* = 15.6 Hz), 8.20 (1H, s), 8.37 (1H, s). ^{13}C NMR (151 MHz, CD_3OD) δ_C: 25.6, 27.6, 46.4, 56.6 (2C), 59.1, 69.5, 72.8, 83.3, 84.4, 84.9, 93.3, 98.0, 106.5 (2C), 115.7, 118.6, 121.0, 131.7, 137.9, 142.0, 145.7, 149.7, 154.4, 154.9 (2C), 157.5, 166.1. HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{28}H_{38}N_7O_{11}S$, 680.2350; found, 680.2344.

4.1.6.5. *N*-(2′,3′-*O*-Isopropylidene-5′-deoxyadenosin-5′-yl)-*N*′-octanoylsulfamide (4e)

To a mixture of **3** (2.00 g, 5.19 mmol) and 1-octanoylimidazole (1.11 g, 5.71 mmol) in anhydrous MeCN (100 mL) was added DBU (1.6 mL, 10.7 mmol) dropwise. The mixture was stirred for 80 min and the solvent was removed *in vacuo*. The residue was taken up in EtOAc and washed successively with 3% (w/v) citric acid/brine solution and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give a viscous oil. This material was dissolved in acetone (100 mL), and hexane (250 mL) was slowly added to the solution. After stirring for 1 h, a precipitate was collected by suction filtration to give the title compound (2.10 g, 79%) as a colorless solid. Mp 157–158 ºC. ^{1}H NMR (600 MHz, CD_3OD) δ_H: 0.86 (3H, t, *J* = 7.2 Hz), 1.19–1.30 (8H, m), 1.36 (3H, s), 1.53–1.58 (2H, m), 1.61 (3H, s), 2.18–2.26 (2H, m), 3.34 (1H, dd, *J* = 13.5, 3.9 Hz), 3.38 (1H, dd, *J* = 13.5, 3.9 Hz), 4.47 (1H, ddd, *J* = 3.9, 3.9, 2.4 Hz), 5.05 (1H, dd, *J* = 6.3, 2.4

Hz), 5.31 (1H, dd, *J* = 6.3, 4.2 Hz), 6.06 (1H, d, *J* = 4.2 Hz), 8.21 (1H, s), 8.35 (1H, s). ^{13}C NMR (151 MHz, CD_3OD) δ_C: 14.4, 23.6, 25.6, 26.0, 27.7, 30.06, 30.08, 32.8, 36.8, 46.3, 83.3, 84.5, 85.1, 93.3, 115.7, 121.0, 142.1, 149.7, 154.4, 157.5, 174.3. Anal. Calcd for $C_{21}H_{33}N_7O_6S$: C, 49.30; H, 6.50; N, 19.16. Found: C, 49.48; H, 6.45; N, 18.93.

4.1.7. Synthesis of inhibitors 1a–f

4.1.7.1. *N*-Cinnamoyl-*N′*-(5′-deoxyadenosin-5′-yl)sulfamide (1a)

The following procedure is typical for **1a**–**f**. A solution of **4a** (400 mg, 0.776 mmol) in 80% (v/v) aqueous HCO_2H solution (10 mL) was stirred for 5.5 h at room temperature followed by evaporation. The residue was purified by reverse-phase medium pressure column chromatography (H_2O/MeOH = 100:0–25:75) to give the title compound (284 mg, 77%) as a colorless solid. Mp 166 ºC dec. ^{1}H NMR (600 MHz, CD_3SOCD_3) δ_H: 3.20 (1H, ddd, *J* = 13.5, 3.6, 3.6 Hz), 3.25 (1H, ddd, *J* = 13.5, 8.4, 3.6 Hz), 4.11–4.13 (1H, m), 4.15 (1H, ddd, *J* = 3.6, 3.6, 1.8 Hz), 4.77 (1H, ddd, *J* = 6.9, 6.9, 5.4 Hz), 5.33 (1H, br d, *J* = 3.6 Hz), 5.51 (1H, br d, *J* = 6.9 Hz), 5.83 (1H, d, *J* = 6.9 Hz), 6.65 (1H, d, *J* = 15.6 Hz), 7.41–7.46 (5H, m), 7.58–7.60 (2H, m), 7.63 (1H, d, *J* = 15.6 Hz), 8.28 (1H, s), 8.30 (1H, s), 9.39 (1H, dd, *J* = 8.4, 3.6 Hz), 11.66 (1H, br s). ^{13}C NMR (151 MHz, CD_3SOCD_3) δ_C: 45.1, 71.4, 72.1, 83.7, 88.7, 119.1, 119.6, 127.9 (2C), 128.9 (2C), 130.3, 133.9, 140.7, 142.9, 148.3, 152.5, 156.2, 163.4. HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{19}H_{22}N_7O_6S$, 476.1352; found, 476.1353.

4.1.7.2. *N*-(5′-Deoxyadenosin-5′-yl)-*N′*-[(*E*)-3-(4-hydroxyphenyl)acryloyl)]sulfamide (1b)

Yield = 33%. Mp 225 ºC dec. ^{1}H NMR (600 MHz, CD_3SOCD_3) δ_H: 3.18 (1H, ddd, *J* = 13.5, 3.6, 3.6 Hz), 3.22 (1H, ddd, *J* = 13.5, 8.4, 3.6 Hz), 4.10–4.12 (1H, m), 4.14–4.15 (1H, m), 4.75–4.78 (1H, m), 5.31 (1H, br d, *J* = 3.0 Hz), 5.50 (1H, br d, *J* = 6.6 Hz), 5.82 (1H, d, *J* = 7.2 Hz), 6.43 (1H, d, *J* = 15.6 Hz), 6.81 (2H, d, *J* = 8.7 Hz), 7.42 (2H, br s), 7.43 (2H, d, *J* = 8.7 Hz), 7.53 (1H, d, *J* = 15.6 Hz), 8.27 (1H, s), 8.29 (1H, s), 9.29–9.31 (1H, m), 10.03 (1H, br s), 11.49 (1H, br s). ^{13}C NMR (151 MHz, CD_3SOCD_3) δ_C: 45.1, 71.4, 72.1, 83.7, 88.7, 115.2, 115.8 (2C), 119.6, 125.0, 129.9 (2C),

140.7, 143.1, 148.3, 152.5, 156.2. 159.7, 163.8. HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{19}H_{22}N_7O_7S$, 492.1301; found, 492.1302.

4.1.7.3. *N*-(5′-Deoxyadenosin-5′-yl)-*N*′-[(*E*)-3-(4-hydroxy-3-methoxyphenyl)acryloyl]sulfamide (1c)

Yield = 52%. A pale green solid. Mp 214 ºC dec. ^{1}H NMR (600 MHz, CD_3SOCD_3) δ_H: 3.17 (1H, ddd, *J* = 13.5, 3.6, 3.6 Hz), 3.23 (1H, ddd, *J* = 13.5, 9.0, 3.6 Hz), 3.80 (3H, s), 4.11–4.13 (1H, m), 4.15 (1H, ddd, *J* = 3.6, 3.6, 1.8 Hz), 4.77 (1H, ddd, *J* = 6.9, 6.9, 4.8 Hz), 5.29 (1H, d, *J* = 3.6 Hz), 5.48 (1H, d, *J* = 6.9 Hz), 5.83 (1H, d, *J* = 6.9 Hz), 6.46 (1H, d, *J* = 15.6 Hz), 6.81 (1H, d, *J* = 8.4 Hz), 7.04 (1H, dd, *J* = 8.4, 1.8 Hz), 7.14 (1H, d, *J* = 1.8 Hz), 7.40 (2H, br s), 7.52 (1H, d, *J* = 15.6 Hz), 8.27 (1H, s), 8.28 (1H, s), 9.30 (1H, br s), 9.61 (1H, s), 11.47 (1H, br s). ^{13}C NMR (151 MHz, CD_3SOCD_3) δ_C: 45.1, 55.4, 71.4, 72.1, 83.8, 88.8, 111.3, 115.6 (2C), 119.7, 122.2, 125.5, 140.7, 143.3, 147.7, 148.3, 149.2, 152.5, 156.3, 163.9. HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{20}H_{24}N_7O_8S$, 522.1407; found, 522.1421.

4.1.7.4.

***N*-(5′-Deoxyadenosin-5′-yl)-*N*′-[(*E*)-3-(4-hydroxy-3,5-dimethoxyphenyl)acryloyl]]sulfamide (1d)**

Yield = 98% (without purification). A pale yellow solid. Mp 144 ºC dec. ^{1}H NMR (600 MHz, CD_3SOCD_3) δ_H: 3.19 (1H, ddd, *J* = 12.6, 3.0, 3.0 Hz), 3.25 (1H, ddd, *J* = 12.6, 9.0, 3.0 Hz), 3.80 (6H, s), 4.14 (1H, dd, *J* = 4.8, 1.8 Hz), 4.18 (1H, ddd, *J* = 3.0, 3.0, 1.8 Hz), 4.79 (1H, dd, *J* = 6.9, 4.8 Hz), 5.84 (1H, d, *J* = 6.9 Hz), 6.50 (1H, d, *J* = 15.6 Hz), 6.89 (2H, s), 7.40 (2H, br s), 7.54 (1H, d, *J* = 15.6 Hz), 8.27 (1H, s), 8.31 (1H, s), 9.01 (1H, br s), 9.38 (1H, dd, *J* = 9.0, 3.0 Hz), 11.41 (1H, br s). ^{13}C NMR (151 MHz, CD_3SOCD_3) δ_C: 45.1, 55.9 (2C), 71.5, 72.2, 83.8, 88.9, 105.8 (2C), 116.0, 119.7, 124.3, 138.3, 140.7, 143.7, 147.9 (2C), 148.3, 152.6, 156.3, 163.8. HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{21}H_{26}N_7O_9S$, 552.1513; found, 522.1515.

4.1.7.5. *N*-(5′-Deoxyadenosin-5′-yl)-*N*′-octanoylsulfamide (1e)

Yield = 36% (recrystallized from hot EtOAc). A colorless solid. Mp 172 ºC. ^{1}H NMR (600 MHz,

CD_3SOCD_3) δ_H: 0.83 (3H, t, *J* = 6.9 Hz), 1.17–1.26 (8H, m), 1.46–1.51 (2H, m), 2.17–2.25 (2H, m), 3.15–3.22 (2H, m), 4.12 (1H, br s), 4.15 (1H, ddd, *J* = 3.6, 3.6, 1.8 Hz), 4.75–4.78 (1H, m), 5.32 (1H, br d, *J* = 1.8 Hz), 5.51 (1H, br d, *J* = 6.6 Hz), 5.84 (1H, d, *J* = 7.2 Hz), 7.43 (2H, br s), 8.25 (1H, s), 8.29 (1H, s), 9.16 (1H, dd, *J* = 6.0, 6.0 Hz), 11.37 (1H, br s). ^{13}C NMR (151 MHz, CD_3SOCD_3) δ_C: 13.8, 22.0, 24.3, 28.27, 28.32, 31.0, 35.1, 45.0, 71.4, 72.2, 83.7, 88.8, 119.7, 140.7, 148.4, 152.4, 156.2, 171.6. HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{18}H_{30}N_7O_6S$, 472.1978; found, 472.1973.

4.1.7.6. *N*-(5′-Deoxyadenosin-5′-yl)sulfamide (1f)

Yield = 97% (without purification). A colorless solid. Mp 136–151 ºC (lit.[54] mp 136–139 ºC). ^{1}H NMR (600 MHz, CD_3SOCD_3) δ_H: 3.20 (1H, ddd, *J* = 12.9, 8.7, 3.9 Hz), 3.27 (1H, ddd, *J* = 12.9, 3.9, 3.9 Hz), 4.18 (1H, ddd, *J* = 3.9, 3.9, 2.1 Hz), 4.20 (1H, dd, *J* = 5.1, 2.1 Hz), 4.77 (1H, dd, *J* = 6.9, 5.1 Hz), 5.87 (1H, d, *J* = 6.9 Hz), 6.63 (2H, br s), 7.40 (2H, br s), 7.81 (1H, dd, *J* = 8.7, 3.9 Hz), 8.20 (1H, s), 8.30 (1H, s). ^{13}C NMR (151 MHz, CD_3SOCD_3) δ_C: 44.8, 71.2, 72.3, 83.9, 88.6, 119.6, 140.6, 148.6, 152.3, 156.2. HRMS-FAB (*m*/*z*): $[M+H]^+$ calcd for $C_{10}H_{16}N_7O_5S$, 346.0934; found, 346.0926.

4.2. Expression of 4CL proteins in *E. coli* and its purification.

Full length cDNAs of the *4CL* genes from *A. thaliana*, *G. max*, *P. trichocarpa*, and *P. hybrida* were amplified with polymerase chain reactions using the designed primers, based on the published sequences on the website of NCBI (https://www.ncbi.nlm.nih.gov). The amplified DNAs were cloned into the multi-cloning site in pET28a for *At4CL1*, *At4CL2*, *Ph4CL1*, and *Ptr4CL3* or pCold I for *Gm4CL1*. Resulting constructs were transformed into *E. coli* Rosetta2 (DE3) pLysS. The recombinant 4CL proteins were induced by adding isopropyl-β-D-thiogalactopyranoside to 0.5 mM. Harvesting and proteins purification by affinity chromatography with COSMOGEL His-Accept (Nacalai) were performed using previously described methods.[56]

4.3. 4CL activity assay.

The 4CL activity was measured using a spectrophotometric assay as described by Knobloch and Hahlbrock.[55] 4CL reaction mixtures contained 0.1 mM 4-coumaric acid, 0.25 mM CoA, 2.5 mM ATP, 2.5 mM $MgCl_2$, 0.2 M Tris-HCl at pH 8.0, and test compounds (0.5% v/v dimethyl sulfoxide, final). The blank as background contained the same components but without CoA. The purified 4CL proteins were added to start the enzymatic reaction and its activity was measured as the increase in absorbance at the absorption maximum (333 nm) of 4-coumaroyl CoA ester. The extinction coefficient of the ester was used to calculate enzyme activities.[32] Half-maximum inhibitory concentration (IC_{50}) were determined from fitting the data to a sigmoidal dose-response model against percentage inhibition.

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Legends for figures, schemes, and tables

Figure 1. Schematic illustration of the ligation reactions via acyladenylate intermediates catalyzed by ANL enzymes, and structures of the acylsulfamide analogues (**1a**–**f**) used in this study.

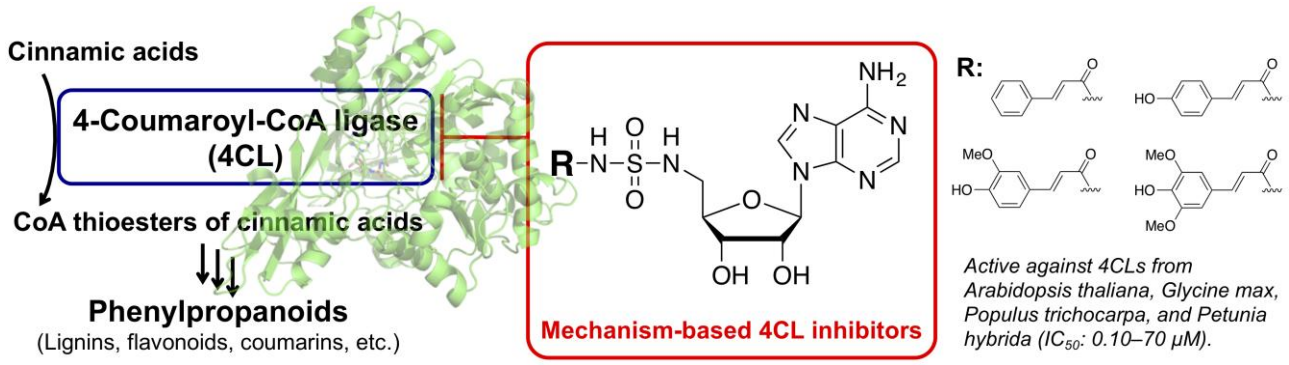
Scheme 1. Synthesis of **1a**–**d**; reagents and conditions: (a) DBU, MeCN, rt, 57–70%; (b) HCO_2H/H_2O (4:1, v/v), rt, 33–98%.

Scheme 2. Synthesis of acylimidazoles **2b**–**d**; reagents and conditions: (a) MEMCl, $(i\text{-Pr})_2NEt$, CH_2Cl_2, rt; (b) NaOH, $MeOH/H_2O$, rt, 72–84% (two steps); (c) CDI, MeCN, rt, 76%–quant.

Scheme 3. Synthesis of **1e** and **1f**; reagents and conditions: (a) 1-octanoylimidazole, DBU, MeCN, rt, 79%; (b) HCO_2H/H_2O (4:1, v/v), rt, 36–97%.

Scheme 4. Synthesis of **3**; reagents and conditions: (a) ref. 53; (b) BnOH, $OCNSO_2Cl$, CH_2Cl_2, 0 ºC–rt, 82%; (c) H_2, 10% Pd-C, AcOH, rt, 81%.

Table 1. Inhibitory activity of **1a**–**f** against 4CLs.



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