

1 **Structure of *Allium* lachrymatory factor synthase**

2 **elucidates catalysis on sulfenic acid substrate**

3
4 Takatoshi Arakawa ^{a 1 2}, Yuta Sato ^{a 1}, Jumpei Takabe ^{a 1}, Noriya Masamura ^b,
5 Masahiro Kato ^b, Morihiro Aoyagi ^b, Takahiro Kamoi ^b, Nobuaki Tsuge ^b,
6 Shinsuke Imai ^b, Shinya Fushinobu ^a

7
8 ^aGraduate School of Agricultural and Life Sciences, The University of Tokyo,
9 Tokyo, 113-8657, Japan. ^b Basic Research Division, Central Research &
10 Development Institute, House Foods Group Inc., Yotsukaido, 284-0033, Japan.

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17

18 **Abstract**

19 Natural lachrymatory effects are invoked by small volatile *S*-oxide
20 compounds. They are produced through alkene sulfenic acids by the action of
21 lachrymatory factor synthase (LFS). Here we present the crystal structures of
22 onion LFS (*AcLFS*) revealed in solute-free and two solute-stabilized forms.
23 Each structure adopts a single seven-stranded helix-grip fold possessing an
24 internal pocket. Mutagenesis analysis localized the active site to a layer near the
25 bottom of the pocket, which is adjacent to the deduced key residues Arg71,
26 Glu88, and Tyr114. Solute molecules visible on the active site have suggested
27 that *AcLFS* accepts various small alcohol compounds as well as its natural
28 substrate, and they inhibit this substrate according to their chemistry. Structural
29 homologs have been found in the SRPBCC superfamily, and comparison of the
30 active sites has demonstrated that the electrostatic potential unique to *AcLFS*
31 could work in capturing the substrate in its specific state. Finally, we propose a
32 rational catalytic mechanism based on intramolecular proton shuttling in which
33 the microenvironment of *AcLFS* can bypass the canonical [1,4]-sigmatropic
34 rearrangement principle established by microwave studies. Beyond revealing

35 how *AcLFS* generates the lachrymatory compound, this study provides insights

36 into the molecular machinery dealing with highly labile organosulfur species.

37

38 **Significance statement**

39 Crushing of onion liberates a volatile compound, *syn*-propanethial *S*-oxide
40 (PTSO), which causes lachrymatory effect on humans. We present the crystal
41 structures of onion LFS (*AcLFS*), the enzyme responsible for natural production
42 of PTSO. *AcLFS* features a barrel-like fold, and mutagenic and inhibitory
43 analyses revealed that the key residues are present in the central pocket,
44 harboring highly concentrated aromatic residues plus a dyad motif. The
45 architecture of *AcLFS* is widespread among proteins with various biological
46 functions, such as abscisic acid receptors and polyketide cyclases, and
47 comparisons with these homologs indicate that unique steric and electronic
48 properties maintain the pocket as a reaction compartment. We propose the
49 molecular mechanism behind PTSO generation and shed light on biological
50 decomposition of short-lived sulfur species.

51

52 **Introduction**

53 Organosulfur compounds are often accumulated in vegetables, and they
54 have improved the quality of human life in the form of particular sensory
55 experiences as well as nutritional and health benefits (1-5). Pungent smells
56 evoked by the *Allium* plants stem from cytosolic (+)-*S*-alk(en)yl cysteine
57 sulfoxides (CSOs), which are produced abundantly from γ -glutamyl-*S*-alk(en)yl
58 cysteines (6). CSOs are odorless precursors, but disruption stresses such as
59 injury, biting and cutting allow them to contact vacuolar C-S lyase (alliinase; EC
60 4.4.1.4), after which spontaneous decomposition and polymerization occur (Fig.
61 1). The population of mostly volatile end products [*e.g.*, dialk(en)yl
62 thiosulfinates (5), cepathiolanes (6), cepaenes (7), polysulfides (8), and
63 zwieberanes (9)] contributes to determining alliaceous smells and flavors (7, 8).

64 In addition, crushing of bulb onion (*Allium cepa*) and other limited plants
65 induce weeping. *syn*-Propanethial *S*-oxide (PTSO) (3) is a prototype of
66 lachrymatory agents (9) and generated by the conjunct catalysis initiated by the
67 alliinase-mediated cleavage of *trans*-*S*-1-propenyl L-cysteine sulfoxide

68 (*trans*-1-PRENCISO/isoalliin) (1) (7). Lachrymatory factor synthase (LFS; EC
69 5.3.-.-) serves on the subsequent conversion to PTSO, and thus branches the
70 common spontaneous degradation pathway of CSOs (10) (Fig. 1). Onion LFS
71 (AcLFS, 169 aa) is assumed to be an irreversible isomerase against
72 (*E*)-1-propene-1-sulfenic acid (1-PSA) (2), but molecular machinery of AcLFS
73 remains unveiled, only implicating that the two residues (R71 and E88)
74 associate in this catalysis (11). Before the discovery of LFS, Block and
75 colleagues have established a canonical theory of PTSO formation through
76 1-PSA, termed [1,4]-sigmatropic rearrangement (Fig. 5A), employing flash
77 vacuum pyrolysis (FVP) (12). This non-enzymatic, intramolecular pericyclic
78 reaction-based principle proceeds under vacuum and heat, while AcLFS
79 mediates an equivalent reaction under ambient conditions and without the
80 assistance of any cofactors.

81 LFS is a unique enzyme because it acts on a the short-lived intermediate.
82 Sulfenic acid (R-SOH) is one of the reactive sulfur species (RSS), which is
83 completely sensitive to thiol and sulfenyl groups as well as oxidants due to its
84 bifacial preference as a nucleophile and an electrophile (13, 14). Sulfenic acids

85 appear as a protein modification of cysteine residues (15-17) and function in
86 specific regulated activities (18-24). However, they have rarely been identified
87 as substrates due to them being too labile to be detected, with an estimated
88 half-life of several milliseconds (25) and not more than a minute for methane
89 sulfenic acid generated in the vacuum gas phase (26). In organisms lacking LFS
90 genes, for example, garlic (*Allium sativa*), free sulfenic acids are condensed
91 immediately, and thus are just destined to produce dialk(en)yl thiosulfinates (2,
92 6). Gene silencing of onion LFS derives increases of (5) and (9) isomers,
93 displaying a metabolite composition similar to that of garlic (8, 27).

94 Here, we present the crystal structures of AcLFS. They enable us to
95 elucidate the natural mechanism by which coincident and spontaneous RSS
96 reactions are selectively modified by this enzyme.

97

98 **Results**

99 *Overall structure and confirmation of the active site*

100 Crystal structures of *AcLFS* were determined at a resolution of 1.7 to 2.1 Å
101 (Table S1). In the present models, the N-terminal region (1–22), which is not
102 required for the activity (11), was not determined due to conformational disorder.
103 *AcLFS* (23–169) formed a single globular domain with dimensions of 40×40×43
104 Å, consisting of a seven-stranded antiparallel β-sheet (S1–S7), a long α-helix
105 (H3), two flanking helices (H1, H2), and connecting loops (Fig. 2A). The
106 molecular architecture in which the β-sheet bends so as to wrap H3 is known as
107 a helix-grip fold (28).

108 There are five cysteine residues on the molecular periphery of *AcLFS* (Fig.
109 S1A). Inactivation of the free thiols by an excess amount of iodoacetamide
110 (IAA) caused no significant change in PTSO generation (Fig. 2D). Hence, the
111 catalysis does not involve any cysteine residues, which is also supported by the
112 result that the activity of C107A remained at the same level as that of intact
113 *AcLFS*.

114 *AcLFS* possesses an elongated pocket extending 18 Å from the molecular
115 surface. This pocket can be divided into three layers, termed Gate, Wall, and
116 Floor (Fig. 2*B*). Wall and Floor are pocket-lining apparatuses provided by side
117 chains of S1, S3–7, and H2–3, and consist mainly of aromatic and aliphatic
118 residues, respectively (Fig. 2*C*, S4). A series of mutagenesis analyses proved
119 that the active site resides in the Wall region. Specifically, variants of Wall
120 residues (F84A, Y102A, F104A, Y114A, W133A, W155A) exhibited equivalent
121 reductions in activities to less than 10%, while those of Floor (L47A, M51A,
122 L54A, V73A, M118A) and a part of Gate (C107A, T109A, W112Q, M143A)
123 maintained the activities roughly at the level of the wild type (Fig. 2*D*). Bulky
124 side chains of the Wall residues appear to be necessary for the catalysis since
125 substitutions that lead to maintenance of the aromatic rings (*e.g.*, Y102F and
126 F104Y) partially retained the activities compared with their alanine mutants.

127 R71 and E88, which are suspected of being involved in catalysis (11), were
128 positioned proximally at the boundary of Wall and Floor. E88A and E88Q
129 comparably caused severe decreases in the activities to less than 1% of that of
130 the wild type. We could not measure the activity of R71A due to protein

131 instability. Instead, a double mutant, R71A/E88A, was examined and showed
132 that the activity declined more than that of E88A. Although R71 influenced the
133 activity, it was unlikely to contact 1-PSA directly, since it is located slightly
134 away from the pocket surface (Fig. 2C, 3A). It should be noted that E88D
135 showed a low level of activity like E88A, and R71K maintained activity at
136 nearly half that of the wild type (Fig. 2D). These findings indicate that the
137 catalysis is ruled strictly by the geometry of the two residues. The charge groups
138 of these residues were associated tightly with two hydrogen bonds (Fig. 2C).
139 Closely aligned acid and base residues can form a dyad motif in which a proton
140 is localized between the residues, and induce a decrease in pK_a of the acid
141 residue (29).

142 The hydroxyl group of Y114 is an additionally important factor, as
143 suggested by the activities of Y114A and Y114F being at the same level as those
144 of the alanine mutants of Wall residues. In turn, the activities of Y102F and
145 F104Y were restored compared with those of the alanine mutants (Fig. 2D). In
146 the condensed hydrophobic side chains, Y114, Y102, R71, and E88 were
147 arrayed to form a polar zone, in which Y114 is positioned near the entrance.

148 Therefore, its hydroxyl group is the first contacting point for solute molecules
149 (Fig. 3A). From these findings, we concluded that Y114 and E88, supported by
150 R71, are the primal key residues for the catalysis of AcLFS, and the surrounding
151 aromatic residues work in auxiliary regulation of this activity.

152

153 *Alcohols as inhibitors*

154 In this study, three AcLFS structures were determined together with
155 exogenous molecules (Table S1). The first one is glycerol, which originates
156 from cryoprotectant solution (Fig. 3A, top). A glycerol molecule is stabilized
157 near the bottom of the pocket, and two of the three hydroxyl groups are fixed by
158 hydrogen bonds to E88, Y102, and Y114. The carbon moiety orients toward
159 W133 and W155; thus, the conformation is also maintained by hydrophobic
160 interactions. When trehalose was used as a cryoprotectant instead of glycerol,
161 three water molecules were observed at the corresponding site of the glycerol
162 (Fig. 3A, low). Subsequently, 1,2-propanediol was added to the cryoprotectant
163 solution to obtain its complex structure (Fig. 3A, middle). A 1,2-propanediol
164 molecule was observed in the pocket and adopts a conformation like that for

165 glycerol, except for the lack of a hydrogen bond to Y114. Trehalose molecules
166 were seen only at minor peripheral sites; thus, the pocket is unlikely to
167 accommodate the disaccharide molecules (Fig. 3B).

168 Next, the inhibition of AcLFS by these compounds was examined.
169 1,2-Propanediol and crotyl alcohol exhibited concentration-dependent decreases
170 of PTSO production (Fig. 3C). They are deduced to behave as competitive
171 inhibitors, considering that 1,2-propanediol was stabilized at the active site. We
172 further investigated the inhibition patterns of cognate compounds, and
173 demonstrated that the efficiencies are determined by the number of hydroxyl
174 groups (Fig. S2); while linear alcohols ($C_nOH_{2n+2/2n}$; $n \leq 6$) inhibited the reaction
175 with half-inhibition concentration (IC_{50}) values from 20 to 80 mM, diol
176 compounds [ethylene glycol (**19**), 1,2-propanediol (**20**), and
177 2-methyl-2,4-pentanediol (**21**)] did so at least at 600 mM. Glycerol (**22**) did not
178 affect the activity, even at excess concentrations (Fig. 3C). These findings
179 indicate that the active site of AcLFS prefers alcohols rather than glycerol and
180 diols, and suggest that alcohols stay in the pocket with disordered conformations
181 due to perturbation of their aliphatic portions. Linear alcohols with larger

182 aliphatic chains were prone to having lower IC_{50} values, except for 1-pentanol
183 (**12**) and *cis*-pentene-1-ol, (**17**) (Fig. S2A). Concerning an exchange of solute
184 molecules with 1-PSA at the active site, the flexible linear alcohols would gain
185 less entropy than diols and glycerol, which would gain entropy due to the
186 release from the two or more hydrogen bonds (Fig. 3A). Assuming that the
187 differential entropies are the major factor behind the net free-energy changes
188 upon replacements, the diols and glycerol are feasibly stable, but poor inhibitors
189 of AcLFS.
190

191 **Discussion**

192 *Comparison with structural homologs*

193 The seven-stranded helix-grip fold is an architectural feature shared by
194 members of the recently established superfamily, START/RHO alpha
195 C/PITP/Bet v 1/CoxG/CalC (SRPBCC; cl14643) (30). LFS presents no
196 significant sequence homologies with other proteins, but a structure similarity
197 search highlighted 3 out of 23 SRPBCC subsets (Table S2, Fig. S3A).
198 Pyrabactin-resistant protein-like proteins (PYLs) [maximal score for
199 *Arabidopsis thaliana* PYL10 (31)] are intracellular receptors for a plant
200 hormone, abscisic acid (ABA). Ginseng major latex-like protein 151 (MLP151),
201 which is thought to be associated with lysophosphatidic acid regulation, is a
202 member of the PR-10/Bet v 1-type plant allergens (32, 33). ZhuI of
203 *Streptomyces* sp. R1128 (34) belongs to the aromatase/cyclase components of
204 bacterial type II polyketide synthases (PKCs) and catalyzes the C7–C12-specific
205 first ring-closing reaction toward octaketide–acyl carrier protein conjugate (35).

206 Each SRPBCC protein has a central cavity that provides a platform for

207 hydrophobic molecules, as observed in complex structures of PYLs and PKCs
208 (36, 37). Although no potent ligand of MLP151 has been identified, extra
209 non-protein electron densities were found inside the pocket in the
210 MLP151-cognate allergen proteins (38, 39). Compared with PYL10 and ZhuI,
211 *AcLFS* has a small cavity (Fig. 4D). These differences are apparently brought
212 about by displacements of L1, S3, and S4 (Fig. S3B). In PYL proteins, L1
213 corresponds to the Pro-cap region, which arises from a conformational change
214 upon the binding of ABA and constructs part of the binding site for downstream
215 regulator proteins (36, 40). On the other hand, the C α traces of *AcLFS* were
216 identical among the present solute molecule complexes; thus, no conformational
217 changes were indicated during catalysis (Fig. 3B). The displacement seems not
218 only to ensure the narrow entrance of *AcLFS*, in which bulky hydrophobic
219 residues gather on the Gate layer (Fig. S3C), but also to restrict the dimensions
220 of their original ligands and substrates (Fig. S3A).

221 Among the four proteins, conserved residues are mapped mainly on their
222 structural frameworks rather than the active sites (Fig. S4). PYL10 harbors the
223 ABA-binding site extending to peripheral L1 and L2 (Fig. 4A). ABA is

224 recognized in the stretched conformation and a Floor residue K56 acts in
225 orienting the carboxylate end (31). Three key residues of *AcLFS* are fully
226 conserved among *PYL* proteins, but R75, E90, and the hydroxyl group of Y116
227 do not directly contribute to the binding of ABA. Studies on mutagenesis and
228 molecular docking have shown that the active site of *ZhuI* exists at a deeper
229 position, around which a CAPS molecule is stabilized (34) (Fig. 4D). The
230 substrate is thought to be placed in a compact conformation, which is ready for
231 the nucleophilic attack from D146. R66 and H109 (Fig. 4B) are assumed to play
232 an essential role in binding of the polyketide substrate (Fig. S3A). On the
233 structures of *WhiE* aro/cyc and *TcmN* aro/cyc (41, 42), which are PKC isoforms
234 possessing distinct regiospecificities toward dodeca- and decaketides, two of the
235 deduced key residues needed for the corresponding catalytic reactions do not lie
236 at the same positions: Y35 and E34 in *WhiE* aro/cyc instead of D146 and H109
237 in *ZhuI* (Fig. 4B). R66 is located on S3 and conserved among these PKCs (41).
238 On S3 and S4, *ZhuI* has two other arginine residues, which are structurally not
239 aligned on the R-E dyad motif of *AcLFS* and *PYL* proteins (Fig. 4C), and the
240 side chains of R50 and R65 are pointed away from the molecule by one-frame

241 shifts. These findings indicate that the R-E dyad is a functional motif unique to
242 LFS and not shared with the structural homolog proteins.

243 Although the key residues of SRPBCC proteins are totally divergent, the
244 electrostatic landscapes in the pockets are comparable to the properties of
245 respective acceptor molecules. In *PYL10* and *ZhuI*, positive patches are
246 distributed on nearly neutral intramolecular surfaces (Fig. 4D). They fit well
247 with the carboxylate of ABA and negatively polarized acyl oxygen atoms of
248 polyketide substrates. On the other hand, *AcLFS* possesses an interior with
249 comprehensively high negative potential, which would be effective at
250 prohibiting the entry of anionic substances. In fact, a sulfate ion is visible only
251 on the outer surface of *AcLFS* (Fig. 3B). Sulfenic acid is regarded as being in
252 protonation equilibrium ($\text{R-SOH} \rightleftharpoons \text{R-SO}^- + \text{H}^+$) under physiological and
253 aqueous conditions (13, 43). Therefore, the electronic filter is likely to have an
254 advantage in isolating single 1-PSA in its protonated form, not
255 1-propene-1-sulfenate.

256

257 *Proposed catalytic mechanism*

258 Taking these findings together, a catalytic cycle of AcLFS is proposed, as
259 shown in Fig. 5B. The Wall apparatus first confines 1-PSA in a similar site of
260 glycerol and 1,2-propanediol. The hydroxyl group of Y114 forms a hydrogen
261 bond to the sulfenyl oxygen, and the adjacent hydrogen atom of the sulfenic acid
262 orients toward the R71-E88 dyad, in which E88 constitutively rests in the
263 dissociated form. The sulfenic acid is polarized and then deprotonated safely in
264 the highly negative electrostatic potential inside the molecule, which is
265 convenient for retention of a cation. The liberated proton forms a bond directly
266 through the π electron pair of the 1-propenyl group, considering that there is no
267 possible proton acceptor in the pocket. Indeed, the decrease in activity of E88D
268 to the same level as its nonpolar variants indicates that AcLFS would not
269 exchange any electron pairs with the substrate (Fig. 2D). Y114 acts as not only a
270 trap of 1-PSA but also an anchor of sulfenate anion, which is prone to leaving
271 the site due to charge repulsion. Upon release of the product, Y114 no longer
272 keeps PTSO in the pocket because of displacement around the S-linked oxygen,
273 which arises from a valence change of the sulfur atom.

274 The reaction scheme based on the intramolecular proton shuttle is
275 consistent with a previous deuterium tracer study, which showed that 1-PSA is
276 the proton source of PTSO (44). This scheme is apparently true in
277 [1,4]-sigmatropic rearrangement, but the retro ene-reaction-like process occurs
278 in a concerted fashion without yielding any intermediates (12) (Fig. 5A). *AcLFS*
279 provides a tailored compartment in order to increase the chances of correctly
280 aligned overlap of binding orbitals, which is needed for the concerted reaction.
281 Further, the compartment can precisely avoid self-condensation of 1-PSA
282 monomers, involving a similar function to protection groups equipped in stable
283 sulfenic acid compounds (45, 46). The roles in the catalysis are distinct from
284 those of regular enzymes since the active site of *AcLFS* is prepared for
285 regulating the inherently labile nature of 1-PSA, rather than activating the
286 substrates into respective transition states.

287 The present study provides a rationale for an RSS catalysis by revealing the
288 three-dimensional structures of *AcLFS*, which has a simple protein architecture.
289 This work might not only decipher the principle of *Allium*-specific lachrymator
290 generation, but also provide inspirations for multiple lines of work, particularly

291 the synthesis of transient *S*-oxide compounds, which are not abundant and thus

292 are rarely exploited.

293

294 **Materials and Methods**

295 *Construction of plasmids*

296 The sequences were amplified from a template plasmid harboring the
297 complete open reading frame of *AcLFS* (GenBank accession: AB089203).
298 Fragments were digested with NdeI/XhoI and HindIII/BamHI, and then inserted
299 into the corresponding sites of pET26b (Novagen) and pPAL7 (Bio-Rad) vectors
300 to prepare the expression plasmids pET26b-*AcLFS* and pPAL7-*AcLFS*,
301 respectively. Expression plasmids for *AcLFS* mutants were prepared following
302 the instructions of the PrimeStar mutagenesis kit (TaKaRa) using
303 pET26b-*AcLFS* as the template.

304

305 *Expression and purification of AcLFS*

306 *AcLFS* and its variants were expressed in *Escherichia coli* BL21AI cells
307 (Life Technologies). Cells were cultured at 37°C in LB medium containing 50
308 mg/L kanamycin. After OD₆₀₀ reached 0.6, *AcLFS* was induced for 2 hours by
309 adding a final concentration of 1 mM IPTG and 0.2% L-arabinose. Harvested

310 cell pellets were suspended in solution A [50 mM Hepes-Na (pH7.5), 0.1 μ M
311 PMSF] and disrupted by sonication (Sonifier 250D, Branson); then, insoluble
312 particles were removed by centrifugation (20,000 \times g, 30 min). The supernatants
313 were loaded onto HisTrap FF crude 5 mL (GE Healthcare) equilibrated with
314 solution B [50 mM Hepes-Na (pH 7.5), 500 mM NaCl] and the eluates for
315 solution C (solution B containing 500 mM imidazole) were pooled. The samples
316 were loaded onto Superdex 200 10/300 GL (GE Healthcare) equilibrated with
317 solution D [20 mM Hepes-Na (pH 7.5), 150 mM NaCl]. Peak fractions
318 monitored by absorbance at 280 nm were collected and concentrated to around
319 20 mg/mL with the buffer exchanged by 20 mM Hepes-Na (pH 7.5) using
320 Vivaspin turbo 10K MWCO (Sartorius).

321 For the production of selenomethionine (SeMet)-derivatized AcLFS,
322 pPAL7-AcLFS was introduced into *E. coli* BL21 CodonPlus(DE3) RP-X cells.
323 These cells were cultured in LeMaster medium containing 100 mg/L ampicillin
324 and 35 mg/L chloramphenicol, and IPTG was added to the medium at a final
325 concentration of 1 mM for induction. Extraction and purification were
326 performed as per the protocols of non-derivatized AcLFS, except for using a

327 Profinity eXact column (Bio-Rad) instead of a HisTrap FF crude column, in
328 accordance with the manufacturer's instructions. In each step, purity was
329 checked by SDS-PAGE and protein concentration was estimated using BCA
330 protein assay kit (Thermo Fisher Scientific).

331

332 *Crystallography and structure determination*

333 Crystallization was performed by the sitting-drop vapor diffusion method at
334 20°C. Crystals appeared within a week from the several batches of the
335 conditions of the JCSG core I–IV and plus kits (Qiagen); then, the reservoir
336 compositions were specifically optimized to the following condition: 2.0 M
337 ammonium sulfate, 0.1 M MES-Na (pH 6.0). Each single crystals on the drops
338 was picked out on CryoLoop (Hampton Research) and directly snapped in liquid
339 nitrogen after cryo-protection, which was performed by transferring the crystal
340 gradually to the reservoir solution containing 20% (v/v) glycerol or 16%
341 D-trehalose. Immersion of the crystals in 1,2-propanediol [final 2% (v/v)] was
342 performed together with the D-trehalose treatment.

343 Diffraction experiments were carried out at synchrotron facilities of KEK

344 (Tsukuba, Japan), employing the mounting robot PAM (47) and client software
345 UGUI. The data acquisition scheme was basically set as follows: exposure of a
346 $100 \times 100 \mu\text{m}^2$ beam for 1 sec per frame and the collection of 180 (360 for the
347 SeMet dataset) frames with 1° oscillation steps. Diffraction images were
348 indexed, integrated, and scaled using the HKL2000 program suite (HKL
349 Research Inc.) or XDS (48).

350 Initial phases were determined by the single-wavelength anomalous
351 dispersion (SAD) method using the SeMet dataset, practically by using the
352 phenix.autosol module implemented in Phenix program suite (49). The resultant
353 model was built mostly via phenix.autobuild and extended to the whole of the
354 molecule manually, with viewing using the program Coot (50). Initial models
355 corresponding to the other datasets were determined by molecular replacement,
356 using the program Molrep (51) if needed. Models were refined iteratively using
357 REFMAC5 in the CCP4 program suite (52, 53), and heteroatoms found were
358 added according to $m|F_o| - D|F_c|$ maps at the posterior rounds. The crystals
359 originally belong to the $P2_12_12_1$ space group, but are prone to being adapted as
360 $P4_1$ during pretreatment processes. The twin refinement option implemented in

361 REFMAC5 was applied for the datasets indexed as the $P4_1$ space group since
362 nearly perfect twinning was found by phenix.xtriage. In addition, the
363 occupancies of all of the atoms were set to 0.8 in the REFMAC5 calculations to
364 reduce the phase bias-conducted lack of the resulting map densities around a
365 region of one of the eight molecules in an asymmetric unit. The final model was
366 verified by respective composite omit maps. Crystallographic and refinement
367 statistics are listed in Table S1. Molecular graphics were prepared using PyMOL
368 (Schrödinger LLC). Structure similarity searches were queued on Dali (54).
369 Superposition and alignments of models were carried out on Coot. Electrostatic
370 potentials were calculated using APBS (55) after charge state assignments with
371 PROPKA (56). Coordinates and structure factors were deposited in the Protein
372 Data Bank (PDB) with the accession codes of 5GTE (solute-free), 5GTF (with
373 glycerol), and 5GTG (with 1,2-propanediol).

374

375 *Product assay*

376 Liberated PTSO was isolated by reverse-phase chromatography at 25°C, as
377 described previously (11). Briefly, a 3-min-reaction mixture was subjected to

378 Pegasil ODS 4.5×250 (Senshu Kagaku) equilibrated with 0.01% trifluoroacetic
379 acid in 30% methanol solution (pH~3.3) and then developed for 12 min with a
380 flow rate of 0.6 mL/min. Absorbance at 254 nm was monitored using an
381 Alliance instrument (Waters). A total batch of 25 μ L initially consisted of 5 μ L
382 of 0.01 mg/mL *AcLFS*, 5 μ L of respective inhibitor in 5-fold concentrate and 10
383 μ L of garlic alliinase (250 U/mL), then 5 μ L of 20 mg/mL *trans*-1-PRENCISO
384 was added to start the reaction. Cysteine thiols on *AcLFS* were inactivated by
385 adding iodoacetamide at a final concentration of 1 mM in 0.01 mg/mL *AcLFS*
386 and incubating the mixture at 4°C for several hours in the dark. Chemical
387 reagents were purchased from Wako Pure Chemicals and Tokyo Chemical
388 Industry.
389

390 **Footnotes**

391 ¹ T. A., Y. S. and J. T. contributed equally to this work.

392 ² To whom correspondence should be addressed. Email:
393 arakawa@mail.ecc.u-tokyo.ac.jp.

394

395 Author contributions: S.F. conceived the research and T.A. designed
396 experiments; J.T., Y.S., and T.A. performed research; N.M., M.K., M.A, T.K.,
397 N.T., and S.I. supplied *AcLFS* gene and materials; All the authors discussed
398 data; T.A. wrote the paper with assistance from all other authors.

399

400 The authors declare no conflict of interest.

401

402

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- 556
- 557

558 **Figure legends**

559

560 Fig. 1. Chemical utilization pathways of γ -glutamyl-*S*-alkenyl cysteines via
561 *trans*-1-PRENCISO in crushed onion. Solid and dashed arrows represent
562 enzymatic and non-enzymatic processes, respectively. Brackets indicate
563 undetectable materials. Numbered chemical names are noted in the main text,
564 except for pyruvic acid (**4**).

565

566 Fig. 2. Structure and the active site of AcLFS. (A) Overall structure displayed by
567 schematic representation in spectral colors. (B) Positions of mutated residues
568 colored by impacts on the LFS activity. Spheres are γ -atoms colored in cyan
569 (none) to red (significant), based on the activities of respective alanine variants,
570 R71K and E112Q. (C) Pocket-lining residues. Views from the entrance (top) and
571 the direction of the parallel to longitudinal axis (bottom, stereoview). The side
572 chains of Wall and Floor layers (sticks) are shown with the cartoon drawing of
573 the main chain. The carbon atoms of Wall are colored as in (A) and those of
574 Floor are in gray. (D) List of variants and relative productivities. Error bars

575 represent standard deviations of at least triplicate measurements.

576

577 Fig. 3. Solute molecules inside the pocket. (A) Non-protein electron densities

578 found at the active site. $m|F_o| - D|F_c|$ omit maps (red, 3σ) are displayed. Surfaces

579 colored in gray are provided by the hydrophobic residues. Atoms suggested to

580 form hydrogen bonds are connected with dashes. (B) Superposition of a

581 protomer of each model. Traces of C α atoms are taken from the data of

582 +glycerol (white), +1,2-propanediol (pink), and solute-free (cyan). Sticks

583 represent exogenous compounds observed on the crystals. (C) Inhibition curves

584 of AcLFS in the presence of glycerol, 1,2-propanediol, and crotyl alcohol.

585

586 Fig. 4. Comparisons with structural homologs. Close-up views of (A)

587 PYL10-ABA (PDB 3r6p) and (B) ZhuI-CAPS (3tfz) complexes.

588 ABA-contacting residues of PYL10 are colored cyan. Suggested primal catalytic

589 residues of ZhuI and WhiE aro/cyc (PDB 3tvr) are colored magenta and pink.

590 Corresponding residues of AcLFS are overlaid as green sticks. (C) Superposition

591 around the R71-E88 dyad motif of AcLFS. Structure-based sequence alignment

592 is depicted below. Arrowheads indicate residues whose side chains point inward.

593 (D) Cutaway views of *AcLFS*, MLP151 (PDB 4reh), PYL10, and ZhuI from left

594 to right. Calculated electrostatic potentials are indicated on the surfaces

595 following the order of blue to red.

596

597 Fig. 5. PTSO generation principles (A) under FVP condition and (B) with the

598 assistance of *AcLFS*.

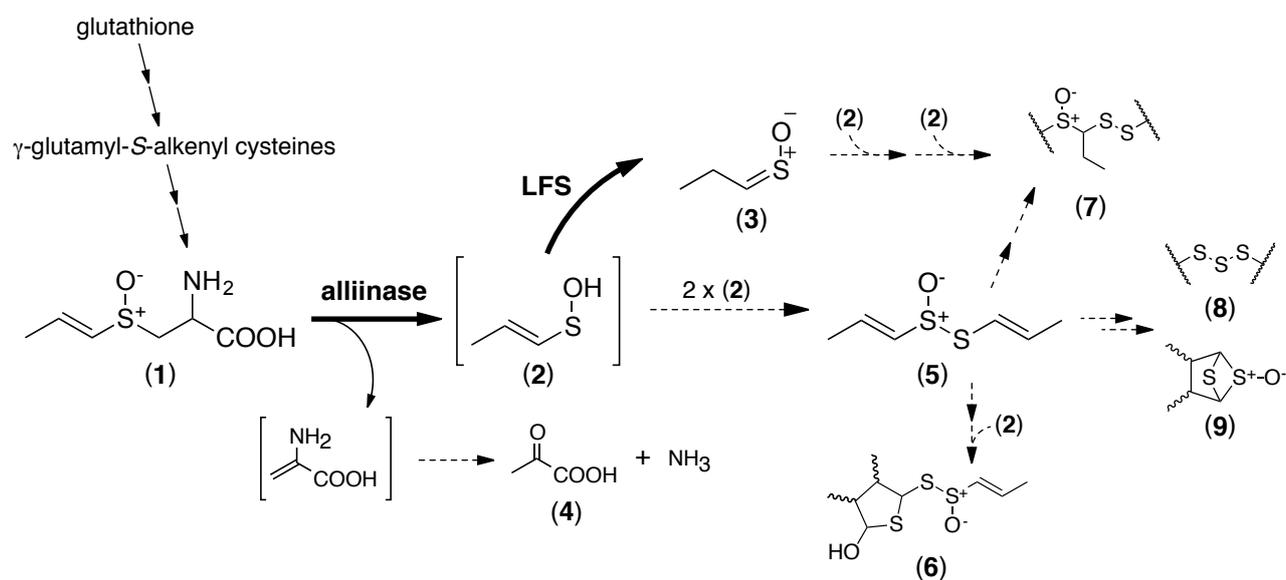


Figure 1

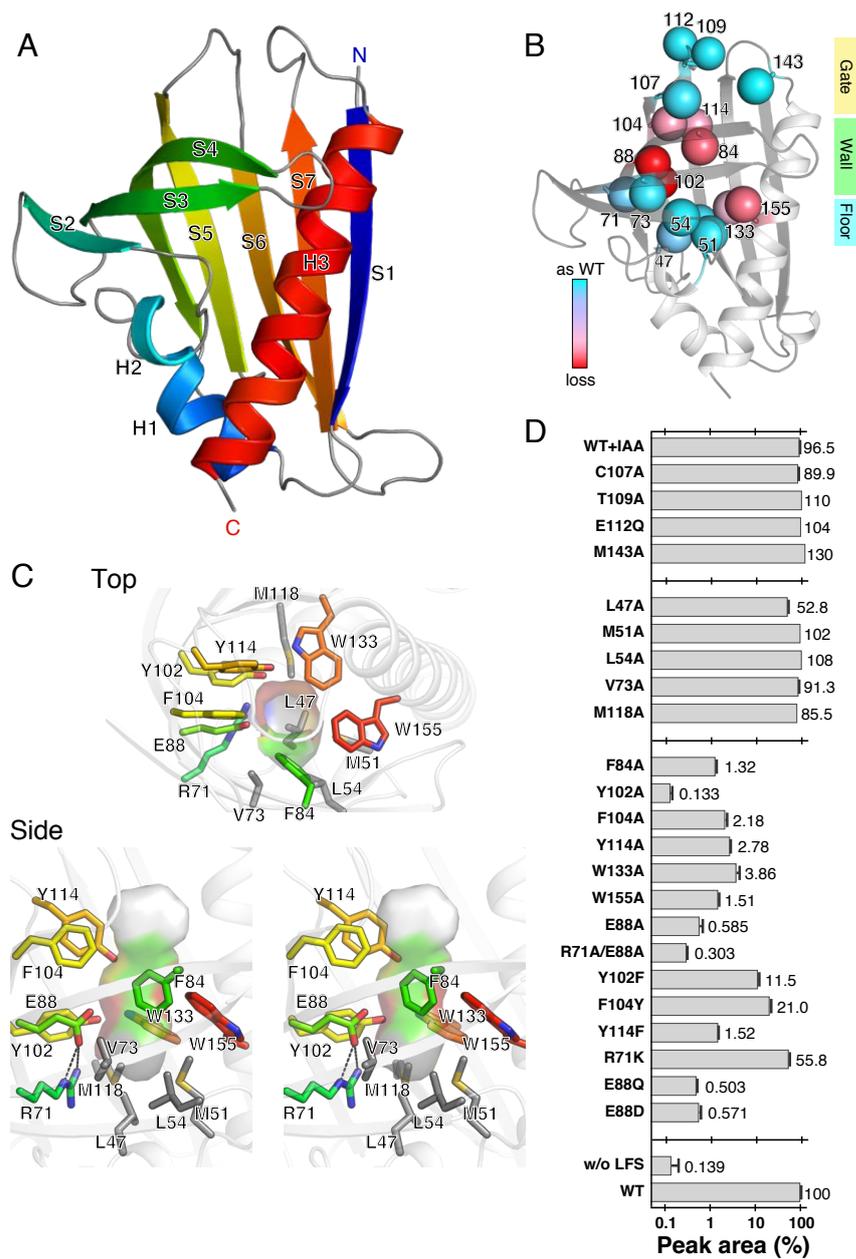


Figure 2

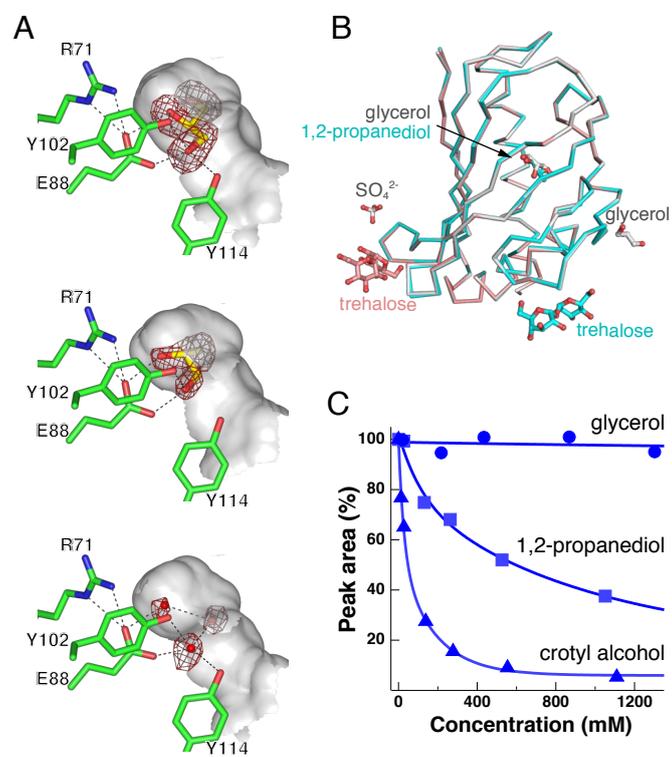


Figure 3

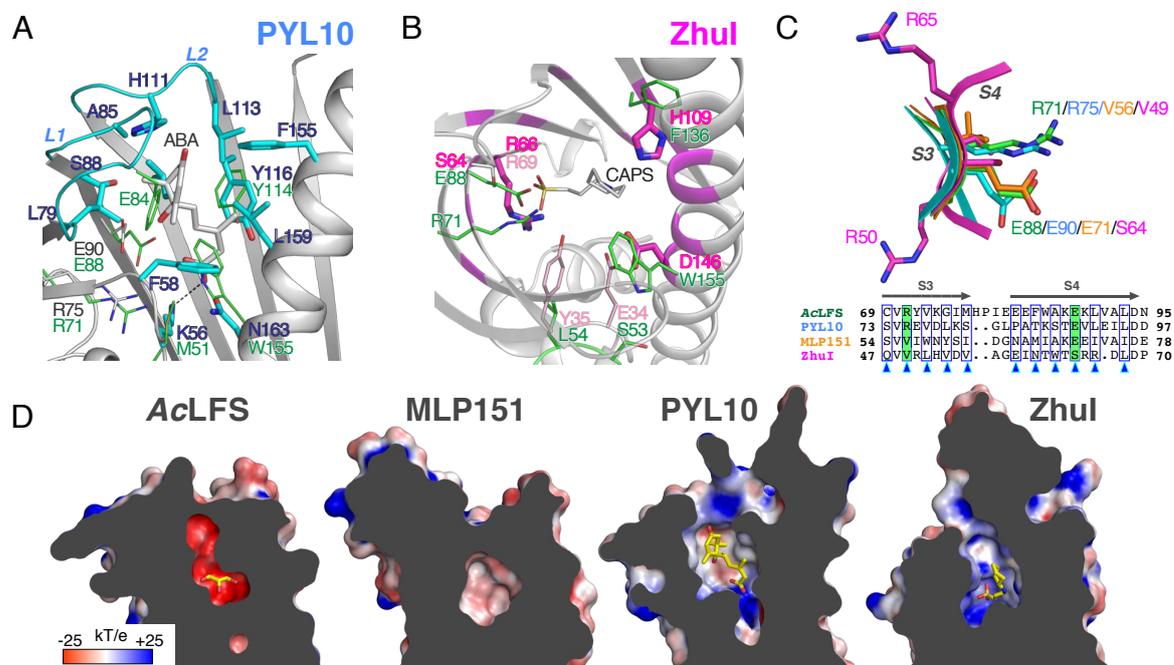


Figure 4

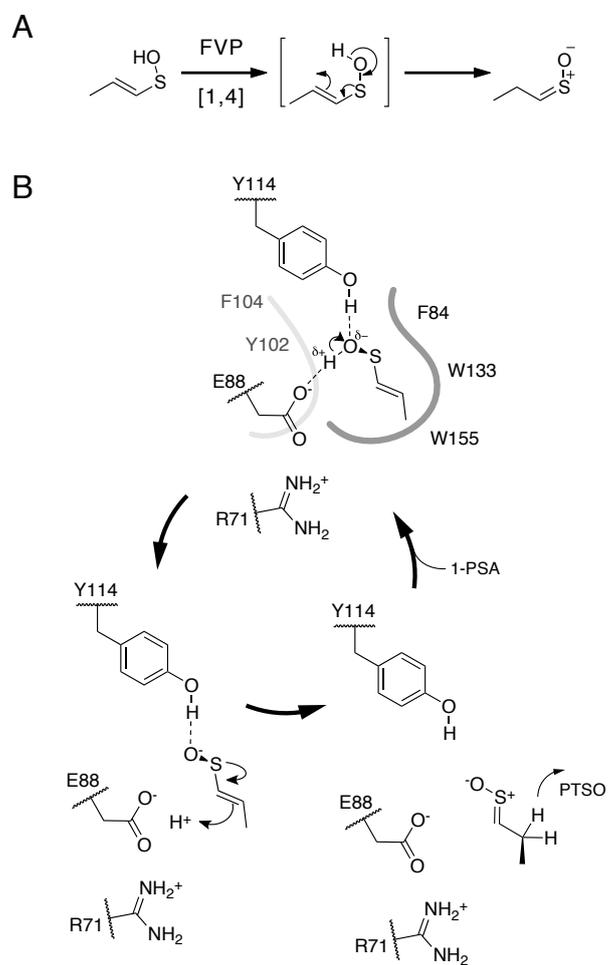


Figure 5

Supplemental information

Supplemental tables and figure legends

Table S1 Data collection and refinement statistics

	SeMet (peak)	Solute-free	+ glycerol	+1,2-propanediol
PDB ID		5GTE	5GTF	5GTG
Data collection				
Beamline	PF NE3A	PF NE3A	PF BL5A	PF NW12A
Wavelength (Å)	0.9787	1.0000	1.0000	1.0000
Space group	$P4_1$	$P2_12_12_1$	$P2_12_12_1$	$P4_1$
Cell dimensions				
a, b, c (Å)	173.1, 173.1, 64.9	65.1, 118.7, 124.5	65.0, 119.9, 125.6	172.9, 172.9, 65.0
Molecules per ASU	8	4	4	8
Resolution (Å)	50.00-2.10 (2.14-2.10) ¹	50.00-2.00 (2.07-2.00)	50.00-1.80 (1.83-1.80)	50.00-1.70 (1.76-1.70)
Unique reflections	138,410 (5,543)	66,057 (6,398)	91,533 (8,871)	204,662 (16,550)
Completeness (%)	99.8 (99.3)	99.6 (98.0)	99.7 (98.6)	97.3 (79.1)
Multiplicity	6.1 (5.5)	6.7 (6.2)	5.8 (5.2)	6.1 (4.9)
Mean $I/\sigma I$	24.6 (3.7)	15.2 (2.8)	18.9 (2.2)	19.8 (4.2)
R_{merge}	0.079 (0.459)	0.100(0.676)	0.053 (0.600)	0.062 (0.378)
$CC_{1/2}$	(0.98)	(0.80)	(0.93)	(0.86)
Refinement				
Resolution (Å)		50.00-2.00	50.00-1.80	50.00-1.70
No. of reflections		62,884	86,948	194,424
$R_{\text{work}} / R_{\text{free}}$		0.191/0.233	0.170/0.199	0.230/0.260 ²
No. of atoms				
Protein		4873	4893	9646
Solute		43	85	65
Water		352	648	584
B-factors				
Protein (Å ²)		29.7	20.3	20.1
Solute (Å ²)		55.9	40.7	22.0
Water (Å ²)		37.4	34.8	25.9

RMS deviation			
Bond lengths (Å)	0.019	0.020	0.031
Bond angles (°)	1.87	1.93	2.83
Ramachandran plot			
Favored (%)	99.0	98.7	95.0
Allowed (%)	0.8	1.2	4.1
Outlier (%)	0.17	0.17	0.85

¹ Values in parentheses are for the highest resolution shell. $R_{merge} = \sum_h \sum_i |I_{h,i} - I_h| / \sum_h \sum_i I_{h,i}$, where I_h is the mean intensity of the i observations of symmetry-related reflections of h . $R = \sum |F_{obs} - F_{calc}| / \sum F_{obs}$, where F_{calc} is the calculated protein structure factor from the atomic model (R_{free} was calculated with 5% of the reflections selected randomly).

² High R factor values because of twinning (see Materials and Methods).

Table S2 Proteins of similar structure to AcLFS¹

Name	Source	Z _{max}	Identity ²	Similarity ²	Domain family ³	PDB ID ⁴
PYL10	<i>Arabidopsis thaliana</i>	19.0	16.7% (31/186)	32.3% (60/186)	PYR_PYL_RCAR_like (cd07821)	3R6P
PYL9	<i>A. thaliana</i>	17.3	17.3% (33/191)	30.4% (58/191)	PYR_PYL_RCAR_like	3OQU
MLP151	<i>Panax ginseng</i>	17.2	18.8% (32/170)	34.1% (58/170)	Bet_v_1_like (cd07816/smart01037)	4REH
PYL2	<i>A. thaliana</i>	17.1	16.7% (32/192)	32.3% (62/192)	PYR_PYL_RCAR_like	3KB0
PYL1	<i>A. thaliana</i>	16.9	16.8% (30/179)	31.8% (57/179)	PYR_PYL_RCAR_like	3JRS
ZhuI	<i>Streptomyces</i> sp. R1128	16.6	12.1% (21/174)	24.7% (43/174)	OtcD1_ARO-CYC_like (cd08861)	3TFZ
PYL3	<i>A. thaliana</i>	16.5	17.2% (36/209)	31.6% (66/209)	PYR_PYL_RCAR_like	4DSC
WhiE aro/cyc	<i>S. coelicolor</i> A3(2)	15.7	14.1% (25/177)	27.7% (49/177)	OtcD1_ARO-CYC_like	3TVQ

¹ Sorted by a descending order of the maximum Z-scores of DaliLite v. 3.

² Taken from the output of EMBOSS Stretcher.

³ Acquired from Conserved Domain Database (CDD).

⁴ Representative entry (ligand complex if available) is shown.

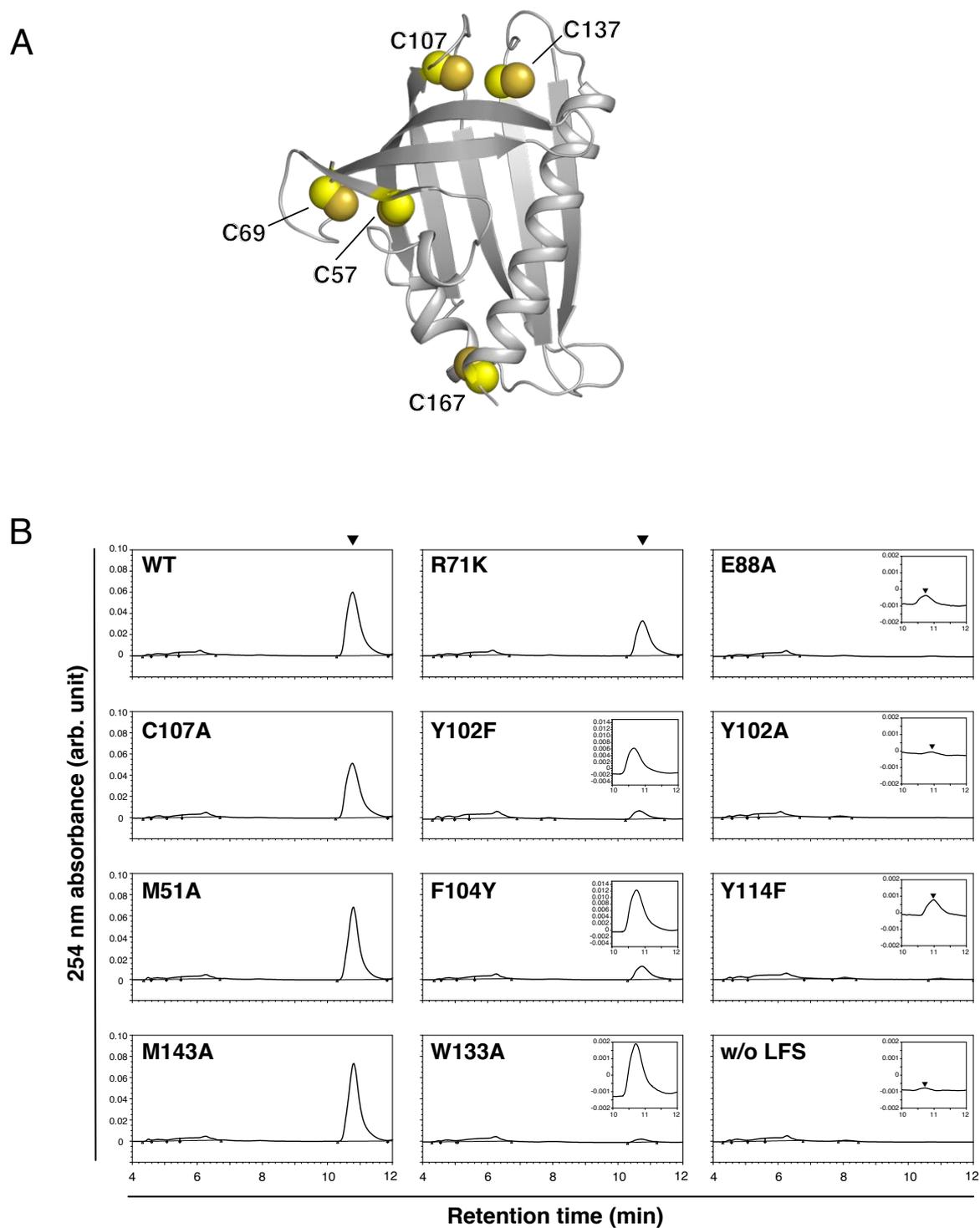


Figure S1

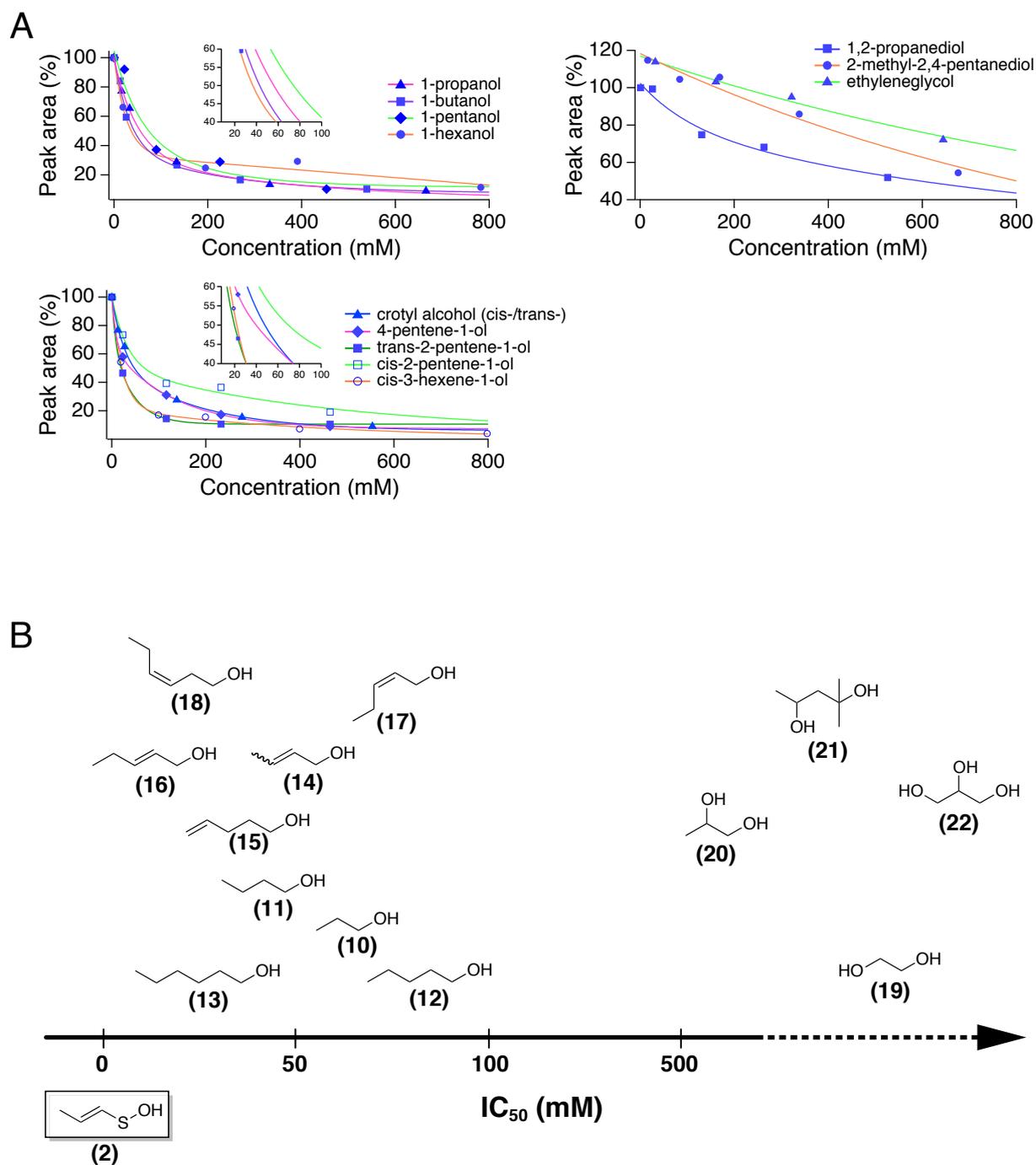


Figure S2

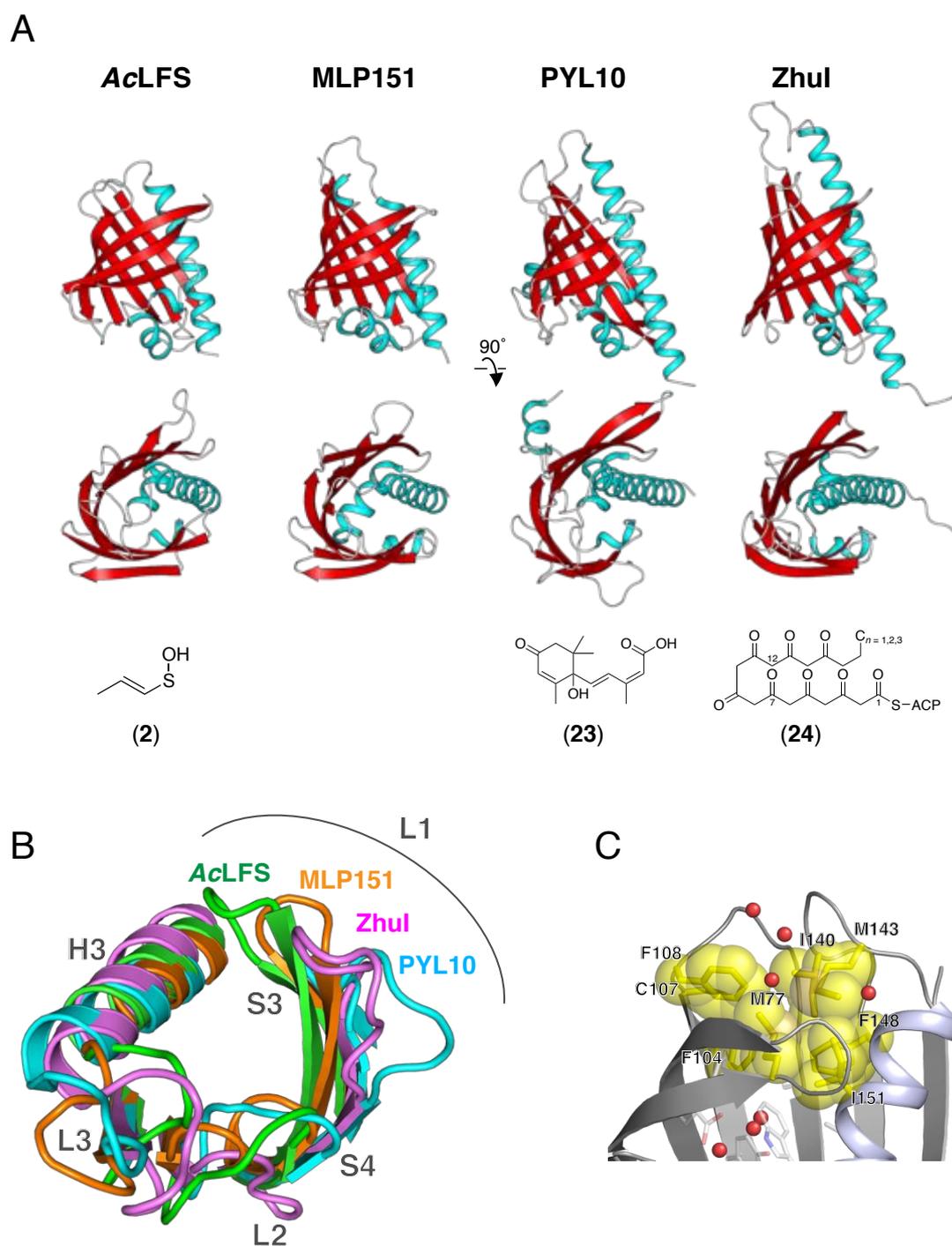


Figure S3

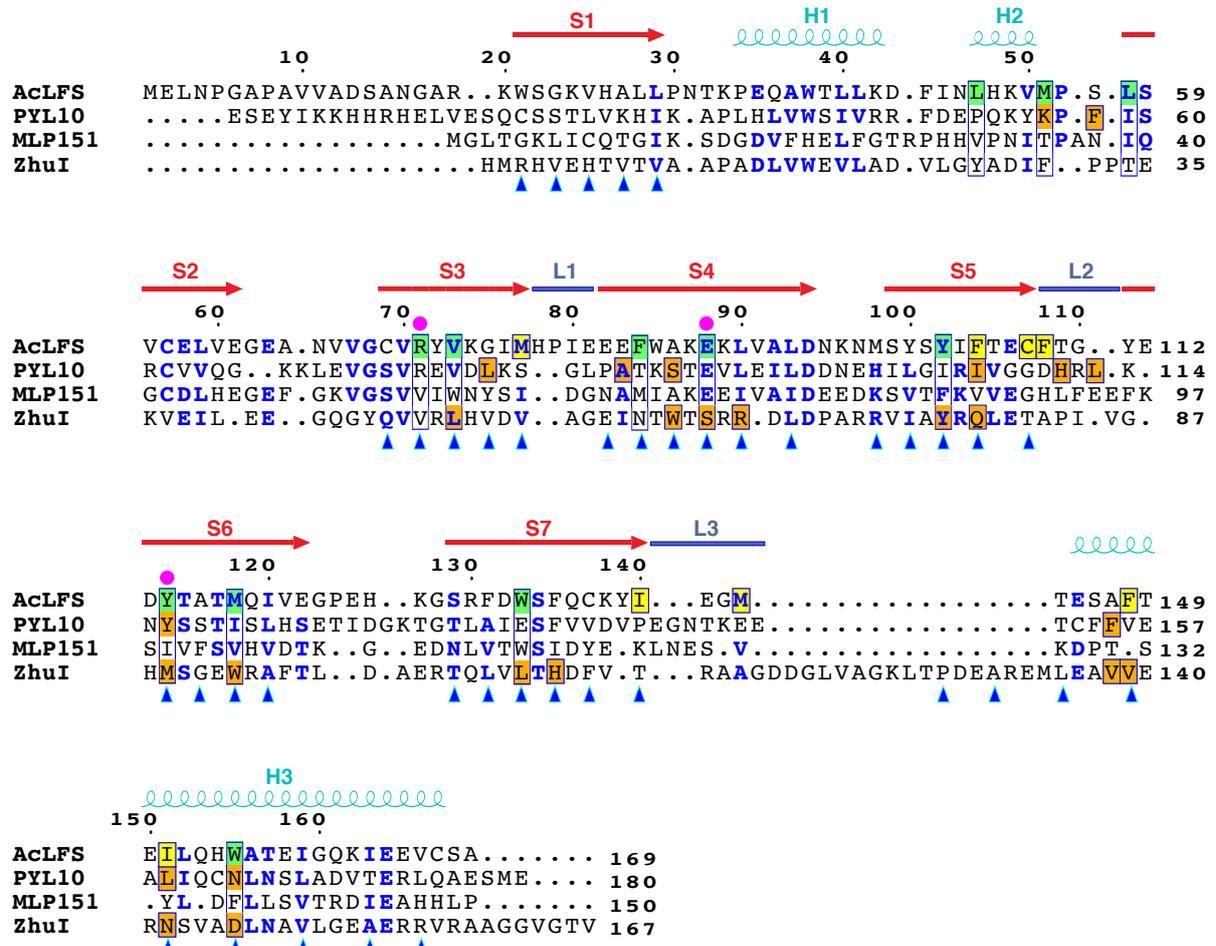


Figure S4

Figure legends

Figure S1 (A) Distribution of cysteine residues on *AcLFS* structure. (B) chromatograms of the selected measurements of reaction products. Arrowheads indicate the retention times of PTSO.

Figure S2 Inhibitions by organic hydroxide compounds. (A) Inhibition curves in the presence of normal alcohols (top), unsaturated alcohols (bottom) and diols (right). (B) Array according to the estimated IC_{50} values of solute molecules tested in this study. Structural formulae stand for 1-propanol (**10**), 1-butanol (**11**), 1-pentanol (**12**), 1-hexanol (**13**), crotyl alcohol (**14**), 4-pentene-1-ol (**15**), *trans*-2-pentene-1-ol (**16**), *cis*-2-pentene-1-ol (**17**), *cis*-2-hexene-1-ol (**18**), ethylene glycol (**19**), 1,2-propanediol (**20**), 2-methyl-2,4-pentanediol (**21**) and glycerol (**22**).

Figure S3 (A) Comparison of structurally relative helix-grip fold proteins. Primary substrate or ligand of each protein is displayed at the bottom. Structural formulae stand for abscisic acid (**23**) and octaketide–acyl carrier protein conjugate (**24**). (B) Superposition around the entrance. Cartoon representations of *AcLFS* (green), MLP15 (orange), PYL10 (cyan) and ZhuI (magenta). Superposed view from Floor to Gate of the pocket. (C) Cluster of hydrophobic residues on Gate layer. Spheres are drawn as respective van der Waals radii of carbon (yellow) and sulfur (orange).

Figure S4 Structure-based amino acid sequence alignment of *AcLFS* and structural homologs. Conserved residues represent blue characters. Residues are highlighted for Wall and Floor (green), Gate (yellow), direct association with ABA (orange in PYL10) and CAPS (orange in ZhuI). The key residues of *AcLFS* are marked with magenta dots and secondary structure elements drawn upside are for *AcLFS*. Arrowheads indicate residues pointing inward.