

Identification of Binding Proteins of Fusarielin A as Actin and Tubulin

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Fusarielin A (FSA), isolated from culture of *Fusarium* sp. K432, has anti-fungal/anti-angiogenic activity. Based on our previous structure–activity relationship studies, we prepared FSA-immobilized affinity gel. The results of affinity chromatography with this gel indicated that tubulin and actin are FSA-binding proteins. The binding affinities of tubulin and actin for FSA were estimated by the use of surface plasmon resonance spectroscopy.

Key words fusarielin A; affinity gel; HL-60; binding protein; tubulin; actin

Fusarielin A (**1**; FSA, Fig. 1) is an anti-fungal/anti-angiogenic/anti-proliferative natural product isolated from culture of *Fusarium* sp. K432. For the isolation of FSA, we applied the *Pyricularia oryzae* (*P. oryzae*) P-2b assay method based on the germination of conidia.^{1–3} The germination process is sensitive to various kinds of bioactive compounds (details will be published elsewhere).³ In particular, tubulin polymerization inhibitors, including rhizoxin,⁴ griseofulvin,⁵ noco-dazole⁶ and phomopsidin,⁷ generally induce clear morphological effects, such as curling, in mycelia resulting from germination of the conidia. FSA induced similar morphological changes to the above tubulin polymerization inhibitors, suggesting that it may exert its biological activities through binding to tubulin and affecting tubulin function.^{1,2} However, FSA was found not to inhibit the polymerization/depolymerization of tubulin in typical assays using porcine tubulin protein fractions.^{1–3} Thus, the target molecule(s) and mechanism of action of FSA both remain to be clarified.³

Our previous structure–activity relationship (SAR) studies suggested that the 1-hydroxyl group, the 11,12-epoxy group and the 15,16-epoxy group of FSA are mandatory for the anti-fungal, anti-angiogenic and antiproliferative activities. In contrast, benzylation of the 3-hydroxyl group of FSA (**1**) does not affect these activities. Based on these results, we designed and prepared a photoaffinity labeling agent, CAFSA (coumarin- and azidophenyl-substituted FSA derivative, **2**, Fig. 1), which showed the same biological activities as FSA.³ By using CAFSA and tritium-labeled FSA (prepared biosynthetically), cellular uptake, cytosolic distribution, and the likely presence of specific binding protein(s) of FSA (**1**) have been demonstrated.³ Here, we planned to identify the FSA-

specific binding protein(s) by using FSA-immobilized affinity gel.

In this paper, we describe (i) the design and preparation of the affinity gel, (ii) the identification of actin and tubulin as FSA-specific binding proteins by the use of the prepared affinity gel, and (iii) direct investigation of the binding affinity of actin and tubulin for FSA (**1**) using surface plasmon resonance (SPR) spectroscopy.

Results and Discussion

Preparation of Affinity Gels for the Isolation of FSA-Binding Proteins Previously, we showed that CAFSA (**2**) possesses anti-angiogenic activity [inhibition of tube formation of human umbilical vein endothelial cells (HUVEC)] and anti-proliferative activity (growth inhibition of human leukemia HL-60 cells) with similar potency to FSA (**1**).³ This led us to design and prepare ZAFSA-Gel (**7**; Chart 1), *i.e.*, FSA (**1**) coupled with Affi-Gel 10 (Bio-Rad, Ltd.), a commercially available agarose having surface *N*-hydroxy-succinimide ester groups for the immobilization of primary-amine-containing compounds.

As shown in Chart 1, a spacer moiety containing primary amine was introduced at the 3-hydroxyl group of FSA, and the resulting ligand, named ZAFSA (**6**; Chart 1), was coupled with Affi-Gel 10 by use of the method previously reported.^{7,8} Briefly, the primary alcoholic group (the 1-hydroxyl group) of FSA (**1**) was protected with a *tert*-butyldimethylsilyl (TBS) group, followed by introduction of a *tert*-butoxycarbonylmethine group at the 3-hydroxyl group to afford **4**. Then, the *tert*-butyl ester moiety of **4** was hydrolyzed, followed by coupling with ethylenediamine in the presence of carbonyldiimidazole (CDI) to give **5**. Deprotection of **5** using tetra-*n*-butylammonium fluoride (TBAF) gave ZAFSA (**6**), and then ZAFSA (**6**) was coupled with Affi-Gel 10. Unreacted activated ester groups on the gel were quenched by addition of an excess amount of ethanolamine to give ZAFSA-Gel (**7**). The efficacy of the coupling reaction was estimated by measuring the amount of free ZAFSA (**6**) remaining in the reaction mixture. Under typical reaction conditions, ZAFSA-Gel (**7**) bears 5–10 μmol ZAFSA/ml gel bed volume. In the experiments described in this paper, ZAFSA-Gel (**7**) with 8.7 μmol ZAFSA/ml gel bed volume was used. To confirm the effectiveness of the designed ligand

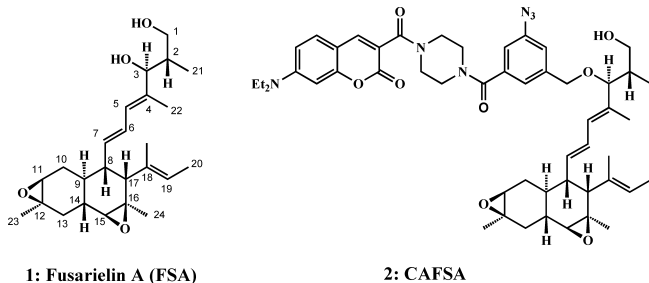
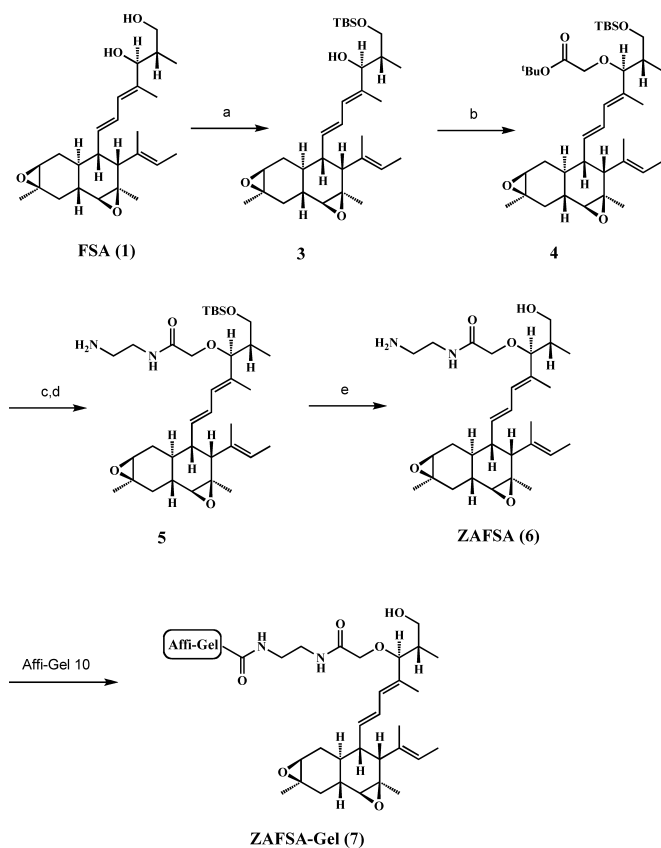


Fig. 1. Structures of FSA (**1**) and CAFSA (**2**)

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Reagents and conditions: (a) TBSCl, Et₃N, DMAP, CH₂Cl₂, RT, 23 h (86%); (b) *tert*-butyl bromoacetate, 12 N NaOH aq., TBAB, toluene, RT, 16 h (47%); (c) 2 N NaOH aq., THF, MeOH, RT, 18 h; (d) ethylenediamine, CDI, DMF, RT, 17 h (54%, 2 steps); (e) TBAF, THF, RT, 17 h (65%).

Chart 1

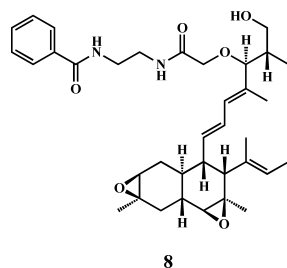


Fig. 2. Structure of 8

moiety, compound 8 (Fig. 2), the *N*-benzoyl derivative of ZAFSA, was prepared and tested for biological activities. It was confirmed to show anti-angiogenic and anti-proliferative activities (measured by means of previously reported methods³) with similar potency to FSA (Table 1), indicating that ZAFSA-Gel should be suitable to purify FSA-binding proteins.

In contrast, the 11,12-epoxy group of FSA (1) is essential for potent biological activities; hydrolysis of this group resulted in a dramatic decrease of the biological activities of FSA (1), especially anti-proliferative activity, though moderate anti-angiogenic activity remained.^{1–3} Therefore, we also designed and prepared DAFSA-Gel (10) as a negative control gel by the method shown in Chart 2. Briefly, nucleophilic attack of ethylenediamine in methanol toward FSA (1) occurred selectively at the 11-position to give DAFSA (9), which was used as a substrate for coupling with Affi-Gel 10

Table 1. Anti-angiogenic and Anti-proliferative Activities of FSA (1), 8 and DAFSA (9)

	FSA (1)	8	DAFSA (9)
Cell growth inhibitory activity HL-60 (IC ₅₀ : μM)	24	25	>100
HUVEC tube formation inhibitory activity (%-inhibition at 50 μM)	48	42	28

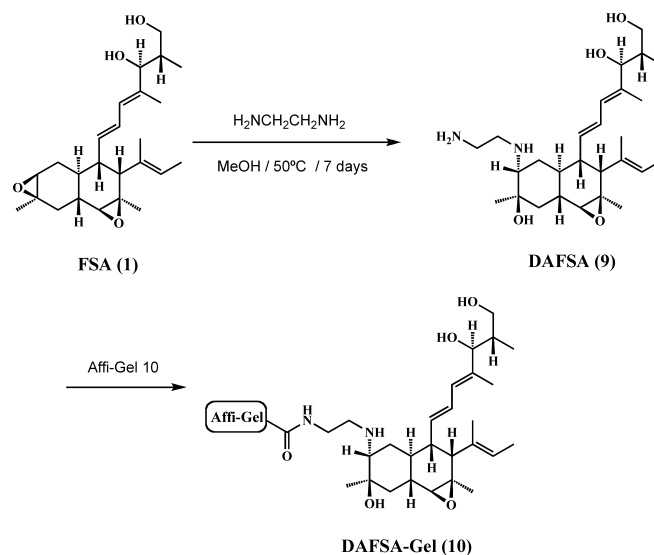


Chart 2. Preparation of DAFSA (9) and DAFSA-Gel (10)

(Chart 2). As expected, DAFSA (9) showed only very weak anti-proliferative activity toward HL-60 cells (IC₅₀ value of >100 μM; growth was inhibited by only 24% at 100 μM concentration) and moderate anti-angiogenic activity measured by assay of HUVEC tube formation-inhibitory activity (Table 1), indicating the suitability of DAFSA-Gel as a negative control gel.

Finally, to identify nonspecifically binding proteins, Affi-Gel 10 was reacted with an excess of ethylenediamine, and the resulting spacer-bearing gel was used as a control gel.

Purification and Identification of FSA-Binding Proteins Our previous studies using HL-60 cells and tritium-labeled FSA showed that FSA (1) taken up in HL-60 cells is distributed into the cytosolic fraction.³ Therefore, the cytosolic fraction prepared from HL-60 cells was used as a source to search for FSA-binding proteins.

Briefly, cytosolic fraction prepared from HL-60 cells was incubated with control gel, ZAFSA-Gel (7), or DAFSA-Gel (10), then washed with phosphate-buffered saline (PBS). The washed gels were treated with hot sodium dodecylsulfate (SDS) solution to elute proteins absorbed on the gels, if any. The supernatant was separated and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and separated proteins were stained with Coomassie Brilliant Blue (CBB).

Two bands at around 50 kDa (one major and one minor, indicated by arrows in Fig. 3, lane B) were detected only in the eluate from ZAFSA-Gel (7). No such bands were observed in the eluate from the control gel (Fig. 3, lane A). In the case of the eluate from DAFSA-Gel (10), the bands were not detected under the same staining conditions, but prolonged staining of the gel resulted in visualization of a weak band

Table 2. Protein Identification of ZAFSA-Gel-Binding Proteins

Band	Protein (ID number)	Matched peptide	Score
1	Tubulin, beta (gi 18088719)	1-MREIVHIQAGQCGNQIGAK-19 63-AILDVLEPGTMDSVR-77 104-GHYTEGAELVDSVLDVVR(K)-122 242-FPGQLNADLR-251 252-(K)LAVNMVPPFR-262 263-LHFFMPGFAPLTSR-276 283-ALTVPELTQQVFDK-297 283-ALTVPELTQQVFDKDMAACDPR-306 309-YLTVAAVFR-318 362-MAVTFIGNSTAIQELFKR-380 381-ISEQFTAMFR(R)-391	85
2	ACTB protein (gi 15277503)	4-AGFAGDDAPR-13 14-AVFPSIVGRPR-24 70-IWHHTFYNELR-80 81-VAPEEHPVLLTEAPLNPK-98 133-TTGIVMDSGDGVTHTVPIYEGYALPHAILR-162 182-GYSFTTAER-191 224-SYELPDGQVITIGNER-239 276-(R)KDLYANTVLSGGTTMYPGIADR-297	74

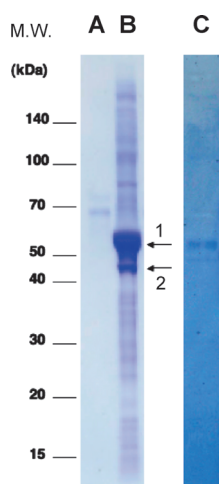


Fig. 3. SDS-PAGE of Affinity Gel (7)-Binding Proteins
A: Control gel, B: ZAFSA-Gel (7), and C: DAFSA-Gel (10).

(Fig. 3, lane C) which seems to correspond to the major band in lane B. Moreover, when the ZAFSA-Gel (7) incubated with HL-60 cytosolic fraction was eluted with an excess amount of FSA (1)-containing solution, these two bands became weaker, suggesting that they represent FSA-selective/specific binding proteins.

Although we used DAFSA-Gel (10) as a negative control gel, DAFSA (10) shows weak biological activity (*i.e.*, it is not completely inactive), as shown in Table 1, so the detection of a weak band in lane C corresponding to the major band in lane B is not problematic.

Next, the two bands detected in lane B (Fig. 3) were excised, subjected to in-gel digestion with trypsin, and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MASS). By using the Mascot online database searching algorithm, these two bands were identified as tubulin beta and ACTB protein (beta actin), respectively (Table 2).

Detection of Direct Binding of FSA (1) with Actin/Tubulin and Determination of Quantitative Binding Para-

meters by Surface Plasmon Resonance (SPR) Spectroscopy

As described above, tubulin and actin were identified as candidate FSA (1)-binding proteins. To verify that FSA (1) really binds to tubulin and/or actin, we directly examined the interaction between FSA (1) and tubulin/actin using SPR spectroscopy. For this purpose, tubulin (isolated/purified from porcine brain) and actin (from rabbit skeletal muscle, Cytoskeleton Inc., Denver, CO, U.S.A.) were immobilized on the carboxymethylated dextran surface of a CM5 sensor chip for SPR analysis using a Biacore T100 (GE Healthcare). We have previously established that the binding of small molecules, including colchicine and 5HPP-33 (both of which are potent tubulin polymerization inhibitors), to tubulin can be detected by this method.⁹⁾

SPR analysis provided clear evidence of direct interactions between FSA and actin/tubulin. The association constants (K_a values) of FSA/actin and FSA/tubulin interaction were calculated to $2.1\text{--}4.7 \times 10^3 \text{ M}^{-1}$ and $1.7\text{--}6.6 \times 10^3 \text{ M}^{-1}$, respectively. These values are consistent with the concentrations at which FSA elicits its biological activities, considering that both actin and tubulin are polymeric macromolecules. These results indicate that actin and/or tubulin are likely to be target protein(s) of FSA. However, it has been reported that FSA does not affect the polymerization/depolymerization of tubulin, as far as investigated for porcine tubulin with a typical turbidity assay.¹⁻³⁾

Determination of the Effects of FSA (1) on Cellular Morphology and Actin Filaments by Using Cytochemical Analysis

To further examine the effects of FSA on actin, we investigated the FSA-induced morphological changes of human embryonic kidney cell line HEK-293. Morphological changes were observed in both optical microscopic (cell shape) and cytochemical fluorescence (actin filament morphology) analyses using actin-staining phalloidin-fluorescein isothiocyanate (FITC) (Fig. 4). Optical microscopy (upper panels, Fig. 4) showed that cells treated with FSA (1) became round, as is observed in cells treated with a typical actin-polymerization inhibitor, cytochalasin B. However, actin filaments showed different morphological changes

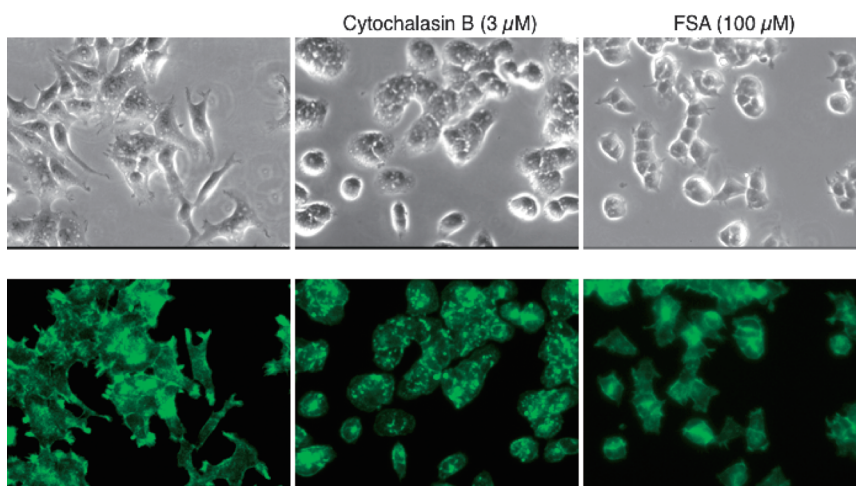


Fig. 4. Morphological Change of HEK-293 Cells

HEK-293 cells were treated with cytochalasin B ($3 \mu\text{M}$) or FSA ($100 \mu\text{M}$), then stained with phalloidin-FITC. Upper panels: observed with an optical microscope. Lower panels: observed with a fluorescence microscope.

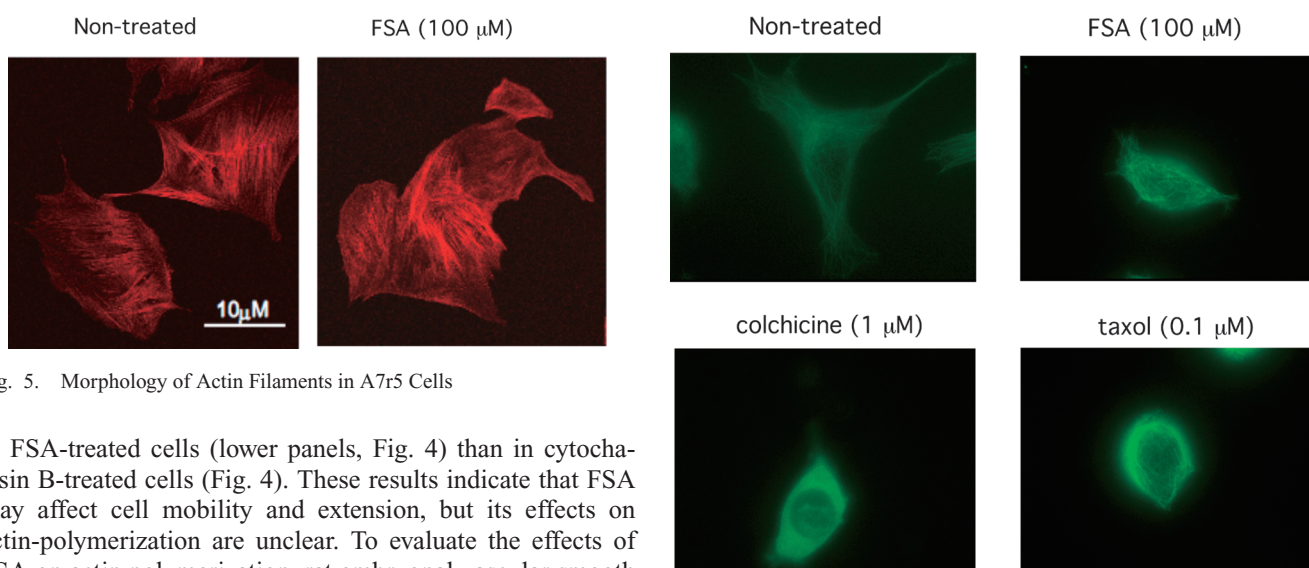


Fig. 5. Morphology of Actin Filaments in A7r5 Cells

in FSA-treated cells (lower panels, Fig. 4) than in cytochalasin B-treated cells (Fig. 4). These results indicate that FSA may affect cell mobility and extension, but its effects on actin-polymerization are unclear. To evaluate the effects of FSA on actin polymerization, rat embryonal vascular smooth muscle cell line A7r5 was employed.^{10,11} The filamentous actin (F-actin) of A7r5 cells could be clearly visualized by means of phalloidin-FITC staining. As shown in Fig. 5, no disruption of actin filaments was observed in FSA (1)-treated cells, suggesting that FSA (1) does not inhibit actin polymerization.

Cytohistochemical fluorescence analysis using anti- α -tubulin/FITC conjugate was also performed using HeLa cells (Fig. 6). Although FSA (1) does not inhibit the polymerization/depolymerization of tubulin (as far as investigated with typical tubulin polymerization/depolymerization assay methods using porcine tubulin protein fractions),¹⁻³ the morphology in FSA (1)-treated cells seemed to be different from that in non-treated cells, and closer to those in cells treated with typical tubulin disruptors, including colchicine and taxol (Fig. 6).

One possible interpretation is that actin and/or tubulin is a target molecule(s) of FSA (1), but the effect(s) of FSA (1) on the function(s) of actin and/or tubulin is different from that of typical known actin- or tubulin-polymerization/depolymerization inhibitors. Cytohistochemical staining revealed disruption of pseudopodium and a rounded cell shape, indi-

Fig. 6. Morphology of Tubulin Microtubules in HeLa Cells

cating that FSA (1) may influence cell mobility, which is controlled by the cytoskeleton. Some proteins, such as spectrin¹² or raft-associated proteins,¹³ were reported to control the cell morphology and mobility by modulating the interaction between cell membrane and cytoskeleton, and FSA (1) might disrupt these interactions.

The anti-angiogenic activity of FSA (1) could be caused by such effects. Another possibility is that the effects of FSA (1) binding to actin and/or tubulin are cell type-specific. Of course, it is also possible that the bindings of FSA (1) to actin and tubulin are unrelated to the biological activities elicited by FSA (1), but this seems implausible, considering that the K_a values for the FSA (1)/actin and FSA (1)/tubulin interactions are consistent with the biologically effective concentration range of FSA (1).

Conclusion

Actin and tubulin were identified as FSA (1)-binding proteins. Although the biological meanings of these interactions remain to be established, the binding affinities of actin and

tubulin for FSA (1) are consistent with the biologically effective concentration range of FSA (1).

Experimental

Preparation of FSA (1) FSA from *Fusarium* sp. K432 was purified using the reported method.^{1–3} Briefly, *Fusarium* sp. K432 strain was cultured in dishes each containing 30 ml of potato dextrose medium (potato 200 g, dextrose 20 g per liter of water) at 20 °C for 21 d. The acetone–benzene extract of a total of 2.41 of culture was separated by silica gel column chromatography. The column was eluted with ethyl acetate–hexane (3:2). The eluate was concentrated, and the residue (2.8 g) was subjected to Sephadex LH-20 column chromatography, eluted with MeOH. The eluate was concentrated, and the residue was applied to a column of activated charcoal. This column was eluted with MeOH, and the eluate was concentrated to give FSA (1) as a white powder (550 mg).

1-(tert-Butyldimethylsilyloxy)-FSA (3) To a solution of FSA (1) (58.6 mg, 0.146 mmol) in CH₂Cl₂ (0.4 ml) were added *tert*-butyldimethylchlorosilane (24.0 mg, 0.159 mmol), triethylamine (25.0 μl, 0.179 mmol), and *N,N*-dimethylaminopyridine (1.0 mg, 0.008 mmol). The mixture was stirred at ambient temperature for 23 h, then applied directly to silica-gel and purified by silica-gel column chromatography (hexane/ethyl acetate=7/1, then 4/1) to give 1-(*tert*-butyldimethylsilyloxy)-FSA (3) (64.3 mg, 0.124 mmol, 86%) as a pale-yellow oil; ¹H-NMR (DMSO-*d*₆) δ: 0.00 (3H, s), 0.01 (3H, s), 0.69 (3H, d, *J*=7.0 Hz), 0.85 (9H, s), 0.92–1.00 (1H, m), 1.11 (3H, s), 1.19–1.28 (1H, m), 1.27 (3H, s), 1.47 (1H, ddd, *J*=3.0, 10.5, 14.0 Hz), 1.54–1.75 (6H, m), 1.59 (3H, s), 1.61 (3H, s), 2.02–2.14 (1H, m), 2.06 (1H, dd, *J*=3.0, 14.0 Hz), 2.43–2.52 (1H, m), 2.70 (1H, s), 2.90 (1H, d, *J*=5.5 Hz), 3.43 (1H, dd, *J*=7.0, 9.5 Hz), 3.61 (1H, dd, *J*=4.0, 8.5 Hz), 3.70 (1H, dd, *J*=4.0, 9.5 Hz), 4.62 (1H, d, *J*=4.0 Hz), 5.13 (1H, dd, *J*=10.5, 15.0 Hz), 5.13–5.20 (1H, m), 5.79 (1H, d, *J*=10.5 Hz), 6.13–6.24 (1H, m). MS (FAB) *m/z*: 539 (M+Na)⁺.

1-(tert-Butyldimethylsilyloxy)-3-(tert-butoxycarbonylmethyl)-FSA (4) To a solution of 3 (4.0 mg, 7.7 μmol) in toluene (45 μl) were added tetra-*n*-butylammonium bromide (0.8 mg, 2.5 μmol), *tert*-butyl bromoacetate (4.0 μl, 24.8 μmol), 12 N NaOH (30 μl, 360 μmol), and the mixture was stirred for 40 h at room temperature. The reaction was quenched by addition of brine and the mixture was extracted with Et₂O, and concentrated under reduced pressure. The product was purified by silica-gel column chromatography (hexane/ethyl acetate=19/1, 9/1 to 6/1) to give 4 (2.3 mg, 3.6 μmol, 47%) as a colorless oil; ¹H-NMR (acetone-*d*₆) δ: 0.056 (3H, s), 0.061 (3H, s), 0.77 (3H, d, *J*=6.8 Hz), 0.91 (9H, s), 1.07 (1H, dd, *J*=12.2, 14.7 Hz), 1.15 (3H, s), 1.27 (3H, s), 1.43 (9H, s), 1.59 (1H, td, *J*=3.1, 11.0 Hz), 1.65 (3H, s), 1.66 (3H, s), 1.71 (1H, t, *J*=13.4 Hz), 1.76–1.87 (2H, m), 2.08 (1H, dd, *J*=3.1, 13.4 Hz), 2.49 (1H, d, *J*=4.9 Hz), 2.68 (1H, s), 2.81 (1H, s), 2.84 (2H, s), 2.88 (1H, d, *J*=5.5 Hz), 3.52 (1H, d, *J*=9.2 Hz), 3.62 (1H, dd, *J*=6.8, 9.2 Hz), 3.71 (1H, d, *J*=15.9 Hz), 3.82 (1H, d, *J*=15.9 Hz), 3.90 (1H, dd, *J*=3.1, 9.2 Hz), 5.25 (1H, t, *J*=4.9 Hz), 5.28 (1H, dd, *J*=10.4, 11.0 Hz), 5.92 (1H, d, *J*=10.4 Hz), 6.31 (1H, t, *J*=12.2 Hz). MS (FAB) *m/z*: 630 (M)⁺.

1-(tert-Butyldimethylsilyloxy)-3-(aminoethylaminocarbonylmethyl)-FSA (5) To a solution of 4 (25.1 mg, 39.8 μmol) in a 1:5 mixture of THF and methanol (0.6 ml) was added 2 N NaOH (80 μl, 160 μmol), and the mixture was stirred for 42 h at room temperature. The reaction was quenched by adding 1 N HCl to make pH 7 and the mixture was extracted with Et₂O. The organic solution was concentrated under reduced pressure. To the residue was added a solution of 1,1'-carbonyldiimidazole (9.4 mg, 58.0 μmol) in DMF (0.2 ml) and the mixture was stirred at room temperature. After 30 min, ethylenediamine (13 μl, 194 μmol) was added, and stirring was continued for 65 h at the same temperature. The reaction was quenched by addition of brine and the mixture was extracted with Et₂O. The organic solution was concentrated under reduced pressure. The product was purified by silica-gel column chromatography (CH₂Cl₂/methanol=19/1) to give 5 (13.2 mg, 21.4 μmol, 54% (2 steps)) as a colorless oil; ¹H-NMR (CD₃OD) δ: 0.055 (3H, s), 0.060 (3H, s), 0.78 (3H, d, *J*=7.3 Hz), 0.91 (9H, s), 1.10 (1H, dd, *J*=12.2, 14.7 Hz), 1.20 (3H, s), 1.27 (1H, s), 1.33 (3H, s), 1.61 (1H, ddd, *J*=3.1, 10.4, 13.4 Hz), 1.658 (3H, s), 1.665 (3H, s), 1.69 (1H, t, *J*=13.4 Hz), 1.78–1.96 (2H, m), 2.13 (1H, dd, *J*=3.1, 13.4 Hz), 2.14–2.21 (1H, m), 2.51 (1H, s), 2.75 (2H, t, *J*=6.1 Hz), 2.77 (1H, s), 2.99 (1H, d, *J*=6.1 Hz), 3.31 (1H, t, *J*=6.1 Hz), 3.54 (1H, d, *J*=9.8 Hz), 3.68 (1H, d, *J*=14.7 Hz), 3.71 (1H, dd, *J*=3.1, 9.8 Hz), 3.80 (1H, dd, *J*=4.9, 5.5 Hz), 3.82 (1H, d, *J*=15.3 Hz), 5.24 (1H, t, *J*=6.1 Hz), 5.28 (1H, dd, *J*=10.4, 15.3 Hz), 5.92 (1H, d, *J*=10.4 Hz), 6.26 (1H, t, *J*=12.2 Hz), 7.04 (1H, s), 7.67 (1H, s). MS (FAB) *m/z*: 617 (M+H)⁺.

ZAFSA: 3-(Aminoethylaminocarbonylmethyl)-FSA (6) To a solution of 5 (13.2 mg, 21.4 μmol) in THF (0.1 ml) was added 1 M tetra-*n*-butylam-

monium fluoride in THF (100 μl, 100 μmol) and the mixture was stirred for 17 h at room temperature. The reaction was quenched by addition of brine and the mixture was extracted with Et₂O. The organic solution was concentrated under reduced pressure, and the residue was purified by preparative TLC (CH₂Cl₂/methanol=4/1) to give 6 (7.0 mg, 13.9 μmol, 65%) as a pale yellow oil; ¹H-NMR (CD₃OD) δ: 0.79 (3H, d, *J*=6.8 Hz), 1.10 (1H, t, *J*=13.4 Hz), 1.20 (3H, s), 1.28 (1H, s), 1.33 (3H, s), 1.61 (1H, td, *J*=3.1, 10.4 Hz), 1.65 (3H, s), 1.66 (3H, s), 1.79–1.92 (2H, m), 2.13 (1H, dd, *J*=3.1, 13.4 Hz), 2.14–2.22 (1H, m), 2.51 (1H, t, *J*=3.1 Hz), 2.78 (1H, s), 2.83 (2H, t, *J*=6.1 Hz), 2.98 (1H, d, *J*=5.5 Hz), 3.35 (1H, sextet, *J*=6.1 Hz), 3.54 (1H, d, *J*=9.8 Hz), 3.61 (1H, dd, *J*=3.1, 10.4 Hz), 3.74 (1H, d, *J*=15.9 Hz), 3.86 (1H, dd, *J*=4.9, 5.5 Hz), 3.87 (1H, d, *J*=15.9 Hz), 5.24 (1H, t, *J*=6.1 Hz), 5.29 (1H, dd, *J*=10.4, 15.3 Hz), 5.94 (1H, d, *J*=10.4 Hz), 6.27 (1H, t, *J*=12.2 Hz). HR-MS [FAB, (M+H)⁺] Calcd for C₂₉H₄₇N₂O₅: 503.3485, Found: 503.3503.

Preparation of Affinity Gels: Control Gel, ZAFSA-Gel (7) and DAFSA-Gel (10) Commercially available Affi-Gel 10 (Bio-Rad, Ltd.) was washed with isopropanol using a filtration apparatus under reduced pressure. Then, the gel (2 ml bed volume) was suspended in isopropanol (2 ml) and to this was added 0.3 mmol of ethanolamine (for control gel), 0.03 mmol of ZAFSA (6) [for ZAFSA-gel (7)] or 0.03 mmol of DAFSA (9) [for DAFSA-gel (10)] in 2 ml of isopropanol. The mixture was incubated at room temperature for 6 h under gentle stirring. For ZAFSA- and DAFSA-gel, 0.3 mmol of ethanolamine was added and incubation was continued at room temperature for an additional 6 h. Then the gel was collected by filtration under reduced pressure, washed with isopropanol 6 times using a filtration apparatus, and resuspended in 2 ml of isopropanol. This stock suspension was stored in a refrigerator (–20 °C).

3-(Benzoylaminoethylaminocarbonylmethyl)-FSA (8) ¹H-NMR (CD₃OD) δ: 0.76 (3H, d, *J*=7.4 Hz), 1.03 (1H, dd, *J*=12.2, 14.7 Hz), 1.15 (3H, s), 1.24–1.29 (m, 1H), 1.28 (3H, s), 1.57 (1H, ddd, *J*=3.1, 10.4, 13.5 Hz), 1.63 (3H, s), 1.65 (6H, s), 1.71 (1H, t, *J*=13.5 Hz), 1.76–1.84 (1H, m), 2.06 (1H, dd, *J*=3.1, 10.4 Hz), 2.10–2.20 (2H, m), 2.46 (1H, d, *J*=4.9 Hz), 2.68 (1H, s), 2.77 (1H, s), 2.83 (2H, s), 2.89 (1H, d, *J*=5.5 Hz), 2.93 (1H, s), 3.35–3.41 (1H, m), 3.45–3.60 (4H, m), 3.67 (1H, d, *J*=15.9 Hz), 3.83 (1H, d, *J*=15.9 Hz), 3.93 (1H, dd, *J*=3.1, 10.4 Hz), 5.20 (1H, dd, *J*=10.4, 15.3 Hz), 5.25 (1H, q, *J*=5.5 Hz), 5.83 (1H, d, *J*=10.4 Hz), 6.26 (1H, t, *J*=12.2 Hz), 7.42–7.47 (2H, m), 7.48–7.53 (1H, m), 7.86–7.90 (2H, m). HR-MS [FAB, (M+H)⁺] Calcd for C₃₆H₅₁N₂O₆: 607.3747, Found: 607.3719.

DAFSA: 11-(Aminoethylaminocarbonylmethyl)-FSA (9) To a solution of FSA (1) (20 mg, 0.050 mmol) in MeOH (0.1 ml) was added ethylenediamine anhydrous (1.25 ml in 1 ml MeOH, 0.187 mmol) and the mixture was stirred at 50 °C for 7 d. The reaction was quenched by adding H₂O and the mixture was extracted with ethyl acetate. The organic solution was dried (MgSO₄) and concentrated under reduced pressure to give DAFSA (9) (10.8 mg, 0.023 mmol, 47%) as a white powder; ¹H-NMR (DMSO-*d*₆, 70 °C) δ: 0.68 (3H, d, *J*=6.5 Hz), 1.12 (3H, s), 1.14 (3H, s), 1.14 (1H, m), 1.35 (1H, m), 1.44 (1H, dd, *J*=3.0, 12.8 Hz), 1.58 (3H, d, *J*=6.0 Hz), 1.58 (1H, m), 1.62 (3H, s), 1.62 (3H, s), 1.62 (1H, m), 1.64 (1H, m), 1.81 (1H, m), 2.14 (1H, ddd, *J*=5.0, 10.3, 10.8 Hz), 2.36 (1H, m), 2.49 (1H, s), 2.56 (4H, m), 2.61 (1H, s), 3.33 (1H, ddd, *J*=4.0, 6.0, 10.2 Hz), 3.53 (1H, ddd, *J*=5.0, 5.0, 10.2 Hz), 3.65 (1H, d, *J*=8.5 Hz), 5.16 (1H, dd, *J*=10.5, 14.5 Hz), 5.21 (1H, q, *J*=7.4 Hz), 5.83 (1H, d, *J*=10.8 Hz), 6.14 (1H, dd, *J*=10.5, 14.5 Hz). HR-MS (FAB, [M+H]⁺) Calcd for C₂₇H₄₇N₂O₄: 463.3536, Found: 463.3536.

Purification and Identification of FSA (1)-Binding Proteins by the Use of ZAFSA-Gel (7) HL-60 cells, maintained in RPMI1640 medium supplemented with 10% v/v fetal bovine serum (FBS), were collected by centrifugation at 2000 rpm for 10 min at 4 °C. The cell pellet was treated with 20 mM Tris–HCl (pH 7.6) for 45 min on ice. Then, the mixture was centrifuged at 105000 g for 1 h at 4 °C, and the supernatant was collected and used as cytosolic fraction. The protein concentration was adjusted to 1.0 mg/ml in PBS, and the solution (1.3 ml) was incubated with ZAFSA-gel (7, bed volume of 40 μl) at 4 °C for 1 h. The mixture was centrifuged at 15000 rpm for 1 min, and the gel pellet was collected and washed. Then, SDS-PAGE sample loading buffer 10 μl was added to the gel pellet, and boiled at 100 °C for 5 min. The resulting mixture was analyzed by SDS-PAGE. Bands were cut from the ZAFSA-gel and subjected to in-gel digestion with trypsin according to the general protocol. Then, the mixture of peptides was extracted from each band and analyzed by MALDI-TOF Mass (Bruker, Microflex), using Mascot online database search (Bruker, Biotoool software).

Preparation of Tubulin Microtubule protein was prepared from porcine brain as described.^{14–16} Pure tubulin dimer was obtained by ion-ex-

change column chromatography. Briefly, prepared microtubule protein was ultracentrifuged at 36000 rpm for 45 min at room temperature. The pellet was taken up in 5 ml 100 mM 2-morpholinoethanesulfonic acid (MES) buffer, 50 ml 100 mM guanosine 5'-triphosphate (GTP) and 5 μ l 1 M 2-mercaptoethanol, and incubated for 25 min on ice. The mixture was ultracentrifuged at 36000 rpm for 45 min at 4 °C, and GTP was added to the supernatant. Tubulin dimer was obtained by means of ion-exchange column chromatography.

Surface Plasmon Resonance (SPR) Analysis A Biacore T100 (GE Healthcare) was used to analyze molecular interactions by means of SPR spectroscopy. Actin (Cytoskeleton Inc., Denver, CO, U.S.A.) from rabbit skeletal muscle was purchased from Wako Pure Chemical Industries, Ltd. Microtubule protein was prepared from porcine brain and tubulin dimer was isolated by ion-exchange column chromatography as previously mentioned. Actin and tubulin dimer were each covalently linked to the carboxymethylated dextran surface of a CM5 sensor chip by using amine-coupling chemistry.¹⁷⁾ A surface density of 167 resonance units (RU) was generated for actin by using 60 μ l of 0.05 μ g/ μ l protein in Tris-HCl buffer (5 mM Tris-HCl, 0.2 mM CaCl₂, pH 4.7). A surface density of 4362 resonance units (RU) was generated for tubulin by using 60 μ l of 0.05 μ g/ μ l protein in MES-KCl buffer (100 mM MES, 1 mM EGTA, 0.5 mM MgCl₂·6H₂O, 0.15 M KCl). A 0.5% DMSO solution of the analyte FSA was injected over the surface at 25 °C, with a flow rate of 15 μ l/min in running buffer (Tri-HCl buffer for actin and MES-KCl buffer for tubulin). After injection, analyte solutions were replaced by the running buffer at a continuous flow rate of 15 μ l/min. Surface regeneration was accomplished by replacing the running buffer for 10 min (150 μ l injections at a flow rate of 15 μ l/min). Each sensorgram (time course of the SPR signal) was corrected for the response observed in the control flow cell and normalized to a baseline of 0 RU. Different concentrations of the analyte were passed for 4 min over the sensor chip bearing immobilized actin and tubulin (60 μ l injections at a flow rate of 15 μ l/min). The interaction rate constants were calculated by using the Biacore T100 Evaluation software, version 2.0.1 (Biacore).

Tubulin-Staining A glass base dish (Iwaki) was treated with PBS buffer containing 20 μ g/ml poly-D-lysine for 1 h at 37 °C, then washed with DMEM medium, and 7.5×10⁴ cells/ml HeLa cells were added. Next day, test compound or no compound was added. The cells were incubated for 16 h, rinsed with PBS buffer, and then treated with PBS buffer containing 3.6% formaldehyde for 30 min at room temperature. Formaldehyde was removed by washing with PBS buffer. The cell membrane was permeabilized by treating the cells with 0.1% Triton X-100 in PBS buffer for 15 min. Then, 0.1% Triton X-100 was removed by washing with PBS buffer and the cells were blocked with PBS buffer containing 1% BSA and 0.05% Tween 20 for 1 h. Then, to the treated cells in PBS buffer, monoclonal anti- α -tubulin clone DM1A FITC conjugate (Sigma) was added and incubation was continued for 1 h under light shielding. Stained α -tubulin was detected using a confocal fluorescence microscope. Each cell was photographed using a ×1000 objective to analyze tubulin.

Actin-Staining Cells were rinsed with PHEM buffer [1,4 piperazine diethylsulfonic acid (PIPES)¹⁸⁾ 60 mM, N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid (HEPES) 25 mM, EGTA 10 mM, and MgCl₂ 2 mM, pH

7.3], and then treated with PHEM buffer containing 3.7% formaldehyde for 15 min at 37 °C. Formaldehyde was then removed by washing with PHEM buffer at room temperature once every 5 min for 15 min. The cell membrane was permeabilized by treating the cells with 0.2% Triton X-100 in PHEM buffer for 90 s. Then, to the treated cells in PHEM buffer, phalloidin-FITC (4 U/ml) was added and the cells were incubated for 1 h. Stained F-actin was detected using a confocal microscope.

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