Natural Products

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Development of Highly Cytotoxic and Actin-Depolymerizing Biotin Derivatives of Aplyronine A**

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Aplyronine A (ApA, **1**), a 24-membered macrolide isolated from the sea hare *Aplysia kurodai*, exhibits potent antitumor activities in vivo against P388 murine leukemia (T/C 545%, 0.08 mg kg^{-1}) and several cancers.[1,2] This macrolide has been shown to depolymerize fibrous actin (*F*-actin) and inhibit the polymerization of actin by forming a 1:1 complex with the monomeric globular molecule (*G*-actin, $K_d = 100$ nM).[3] Actin is one of the most abundant proteins in the cytoskeleton and is essential for the regulation of various functions, such as muscle contraction, cell motility, and cell division. Recently, various actin-depolymerizing agents have been found in marine invertebrates,[4] and some compounds, such as ulapualides,[5] mycalolides,[6] kabiramides,[7] sphinxolides/reidispongiolides,[8] swinholides,[9] and bistramides,[10] which are similar to **1**, show extremely strong cytotoxicities. However, when comparing the amount of abundant actin molecules with the amount of **1** incorporated into the cells, the significant antitumor activities of **1** may not be accounted for only by its *F*-actin-severing properties. The modes of action and the target proteins of ApA and related actin-targeting natural products in tumor cells have rarely been clarified, despite their great potential as preclinical candidates for use in cancer chemotherapy.[11,12]

The structure of aplyronine A can be divided into two characteristic parts: the C1–C23 macrolactone and the C24–C34 aliphatic tail (Figure 1a). Structure–activity relationship studies[13] and photoaffinity labeling experiments[14] have established that the tail part of **1** is important for potent actin-depolymerizing activity. In fact, the C21–C34 synthetic analogue of aplyronine A specifically binds to actin at the same position as **1**. Meanwhile, the functional groups of **1** that were important for cytotoxicity were found to be the trimethylserine group on C7, the hydroxy group on C9, and a conjugated diene moiety on the macrolactone ring.

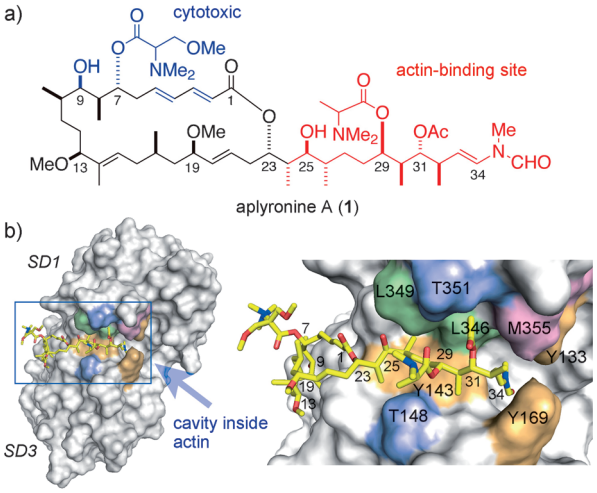

Figure 1. a) Structure of **1** and b) binding of **1** to actin (PDB code: 1wua). Hydrophobic amino acid residues (T, Y, M, L) at the binding site of actin are shown in color. L = leucine, M = methionine, T = threonine, Y = tyrosine.

Recently, an X-ray analysis of the actin/aplyronine A complex was performed at a resolution of 1.45 Å, and showed that the tail of **1** intercalates into the hydrophobic cleft between subdomains (SD) 1 and 3 of actin (Figure 1b).[15] The amino acid residues of actin, such as Y133, Y143, T148, Y169, L346, L349, T351, and M355, participate in the hydrophobic interaction with **1**. In contrast, the terminal *N*-formyl enamide moiety of **1** is located in a very hydrophilic environment and interacts with the water molecules inside the actin. The complex structures of actin with other macrolides, such as kabiramide C,[16] sphinxolide B,[17] reidispongiolides A and C,[17] and swinholide A,[18] as well as a polyketide bistramide A[19] have also been reported. The contacts between actin and each macrolide were similar to those observed in the actin/aplyronine A complex. On the other hand, the trimethylserine moiety of aplyronine A, which is a unique functional group among actin-binding macrolides, and is also important for cytotoxicity, protrudes from the binding position of the macrolactone ring of **1** toward the bulk solvent. This molecular arrangement implies that binding protein(s) (other than actin) would be able to attach to this moiety of **1** in the actin/aplyronine A complex and thus may contribute to the potent antitumor effect of aplyronine A. To clarify this hypothesis, we have performed chemical and biological studies on aplyronine A and related actin-targeting macrolides.[20] Herein we describe the synthesis and biological activities of aplyronine A derivatives that possess a biotin

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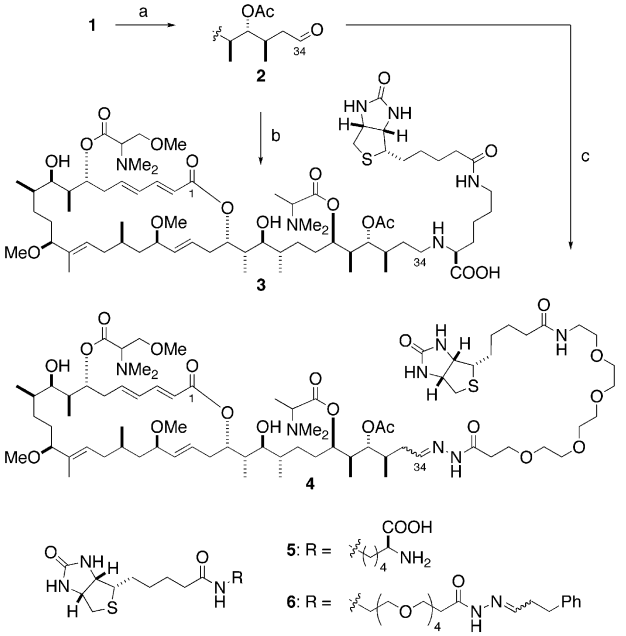
moiety, as well as an investigation of their target proteins in tumor cells.

It has been suggested that the *N*-formyl enamide moiety, which is common to various actin-depolymerizing macrolides, is essential for their potent cytotoxicities.[2,4a,13] However, because there is a cavity structure inside the 1,3-cleft of the actin molecule (Figure 1), we anticipated that ApA could be modified at C34 without a significant loss of activity by elongating a tethered biotin moiety with hydrophilic groups. Acidic hydrolysis of the *N*-formyl enamide moiety of natural aplyronine A (**1**)[11] gave aldehyde **2** (Scheme 1). Reductive aminoalkylation of **2** with biocytin (**5**) gave ApA derivative **3**. Similarly, condensation of **2** with a PEG-linked biotin hydrazide gave another derivative (**4**).[21] For comparison, PEG-linked biotin analogue **6** was also prepared from 3-phenylpropionaldehyde.



Scheme 1. Synthesis of biotinylated ApA derivatives. a) 2 M HCl/1,4-dioxane = 1:3, 50 °C, 80 min; b) **5**, MeOH/H_2O = 1:1, RT, 1 h; $NaBH_3CN$, RT, 36 h, 24% from **1**; c) EZ-Link biotin-PEG_4-hydrazide, MeOH/AcOH = 4:1, RT, 48 h, 38% from **1**.

The cytotoxicities and in vitro actin-depolymerizing activities of ApA analogues and model compounds were compared to those of **1** (Table 1). The biocytin-bearing analogue **3** was found to be 1500 times less cytotoxic than **1**,[22] whereas the PEG-linked analogue **4** was only approximately 10 times less active than **1** (IC_{50} 96 pM). Meanwhile, analogues **3** and **4** showed potent actin-depolymerizing properties, which were comparable to those of natural ApA. In contrast, model compounds **5** and **6**, which lack the whole parts of **1**, did not exhibit either cytotoxicity or actin-depolymerizing properties.

We also examined the ability of biotinylated ApA analogue **4** to depolymerize *F*-actin in living cells (Figure 2). After treatment with **4** (5 μM) for 2 h, actin disassembly and the formation of bubble-like blebs in the cytoplasm were observed, as with **1** (Figure 2b,c). For the detection of

Table 1: Biological activities of biotinylated ApA derivatives.

	Cytotoxicity (HeLa S3) IC_{50} [nM]	Actin-depolymerizing activity[a] EC_{50} [μM]
ApA (**1**)	0.010	1.6
3	15	2.1
4	0.096	1.8
5	>10000	>100
6	>10000	>100

[a] Activity was monitored by measuring the fluorescence intensity of pyrenyl actin. Values indicate the concentrations required to depolymerize *F*-actin (3 μM) to 50% of its control amplitude.

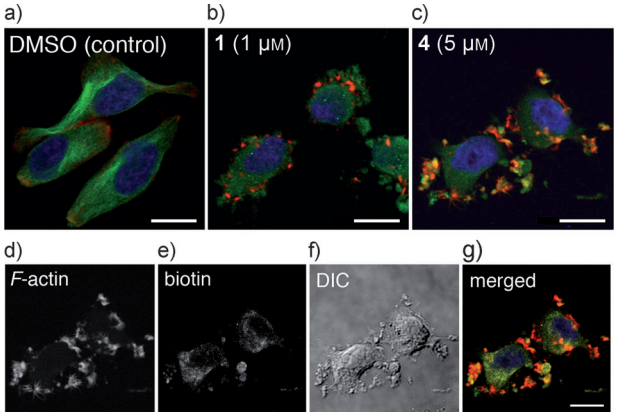


Figure 2. Confocal fluorescence images of HeLa S3 cells treated with **1** and **4** for 2 h. a–c) Cells were immunostained with anti-β-tubulin antibody (green), and co-stained with DAPI (blue) and rhodamine-phalloidin (red). Scale bars = 20 μm. d–g) Monochroic and bright-field images of the cells in (c). Cells were also stained with Alexa Fluor 647 streptavidin (yellow, pseudo color), and four colors (BGRY) were merged in (g). Scale bar = 20 μm. DAPI = 4′,6-diamidino-2-phenylindole, DIC = differential interference contrast, DMSO = dimethylsulfoxide.

compound **4** that had been incorporated into the cells, intracellular biotin was visualized with fluorescently labeled streptavidin. In fact, most of the *F*-actin and biotin in the cytoplasm were differentially localized (Figure 2d–g). These significant morphological changes in HeLa S3 cells strongly indicated that compound **4** may be distributed in the cytoplasm as a 1:1 complex with *G*-actin after the rapid disassembly of multiple filaments. Thus, the actin-severing mechanism and intracellular behaviour of **4** may be identical to that of natural **1**.

The biotinylated ApA derivatives were then examined to determine their specificities for binding to actin. When the binding proteins were competitively eluted by an excess of **1**, only the actins that were attached to biotinylated ApA analogues **3** or **4** were eluted (Figure 3a). In contrast, actins were eluted in all lanes by boiling the resins in SDS buffer (Figure 3b). Thus, considerable amounts of actins were nonspecifically absorbed to the hydrophobic agarose resin because of the presence of the lipophilic parts of the actin molecule (i.e., the 1,3-cleft). Despite this behaviour, resin-bound compounds **3** and **4** were found to exhibit high affinity for actin and to have the same binding properties as **1**.

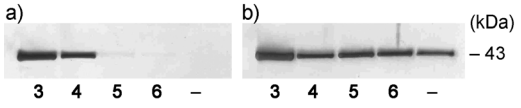


Figure 3. Interaction of biotinylated ApA derivatives with actin. Actin in G buffer was treated with compounds **3**–**6** or DMSO (shown as –) that were preloaded on NeutrAvidin agarose. Binding actin was eluted by a) treatment with **1** (5 μM) or by b) boiling in SDS buffer, and detected with silver stain.

Furthermore, we investigated the interactions of biotinylated ApA analogue **4** with whole cellular proteins. To minimize interference with intrinsic biotin-binding molecules, an excess amount of **4** was pretreated with NeutrAvidin agarose, on which the lysate of HeLa S3 cells was loaded. When the binding proteins were eluted by boiling in SDS buffer, two bands with molecular masses of 43 and 200 kDa were clearly detected along with several nonspecific bands (Figure 4a, lane 4). Through the use of peptide mass fingerprinting (PMF) and immunoblot analyses, the two main bands were identified to be β actin and nonmuscle myosin II (heavy chain), respectively (Figure 4a,d).[23] Myosin II is an *F*-actin binding protein with ATPase activity. However, no significant differences in the staining patterns of myosin II were observed between **4** and other biotinylated compounds **3**, **5**, and **6**.[23] Thus, myosin II may bind not directly to the ApA derivatives, but rather to the abundant actin molecules that were trapped on the resin directly or through biotinylated compounds.

To confirm the interactions of **4** and actin, biotinylated proteins were purified by a pull-down assay and subsequently detected by immunoblotting analysis using a horseradish peroxidase (HRP) conjugated streptavidin (Figure 4b). Upon

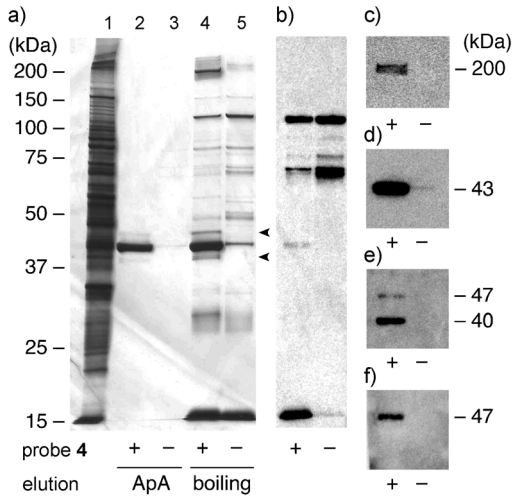


Figure 4. Pull-down assay of ApA-binding proteins in cell lysate. a) HeLa S3 cell lysate (lane 1) was treated with **4** (probe +) or DMSO (probe –), which were preloaded on NeutrAvidin agarose. Binding proteins were eluted by treatment with **1** (5 μM) or by boiling in SDS buffer, subjected to 10% SDS-PAGE, and detected with silver stain. Arrowheads indicate Arp2 and Arp3. b–f) Immunoblotting analysis. Proteins in lanes 4 and 5 in (a) were detected with b) HRP-conjugated streptavidin, c) anti-myosin II, or d) anti-β-actin, respectively. Similarly, proteins in lanes 2 and 3 in (a) were detected with e) anti-Arp2 and anti-Arp3 (1:1 mix) or f) anti-ACTR3.

treatment with **4**, a weak band corresponding to actin was observed, along with nonspecific bands (120 and 74–72 kDa, biotin carboxylase). In the same experiments, biotin-containing compounds, namely **4** and/or compounds derived from **4**, were detected in the region of the lowest molecular weight. Meanwhile, the amounts of bound actins that were eluted by boiling of the resins were mostly the same as those with ApA elution (Figure 4a, lanes 2 and 4). These results suggested that most of the actins bound to **4** were competitively eluted by ApA, and that the ApA analogue **4** was noncovalently but highly specifically bound to actin, as in an actin-binding assay.

In addition, two proteins with molecular masses of 40 and 47 kDa were purified with abundant actin from the lysate of HeLa S3 cells by using a PEG-linked biotinylated ApA analogue **4** (Figure 4a, lane 4), but not when a biocytinylated ApA analogue **3** or model compounds **5** and **6** were used.[23] By a detailed MS/MS analysis of the tryptic peptide fragments obtained by the in-gel digestion, we confirmed that the 40 and 47 kDa proteins were actin-related proteins 2 and 3 (Arp2 and Arp3), respectively. Notably, as with actin, both Arp2 and Arp3 were also competitively eluted from the resin by **1** (Figure 4a, lane 2), a result that was established by immunoblot analysis (Figure 4e,f). These results suggested the presence of specific interactions between ApA and actin-related proteins. Arp2 and Arp3 are the key proteins of the Arp2/3 complex, which binds to the sites of an existing actin filament and initiates growth of a new actin filament to form branched-actin-filament networks.[24,25] The amino acid sequences and three-dimensional structures of Arp2 and Arp3 are highly similar (46% and 36% identity) to those of monomeric actin.[26,27] Moreover, the remaining components of the Arp2/3 complex, ARPC1–5 (16–40 kDa), were not purified by the pull-down assay with **4**. Thus, it was suggested that ApA (**1**) may bind to Arp2 or Arp3 to give 1:1 complexes, as with the actin/aplyronine A complex, and inhibit the ability of the Arp2/3 complex to bind to and branch *F*-actin.[28] These properties may help to enhance the potent actin filament disassembly caused by **1**.

Two small aromatic molecules, CK-636 and CK-548, were recently discovered and were shown to bind to the Arp2/3 complex and inhibit its ability to nucleate actin filaments.[29] Molecules CK-636 and CK-548 bind between Arp2 and Arp3 or insert into the hydrophobic core of Arp3 in the Arp2/3 complex, but do not depolymerize *F*-actin. Meanwhile, to the best of our knowledge, this is the first example in which actin-depolymerizing molecules can also bind to actin-related proteins.

In summary, highly bioactive biotinylated derivatives of aplyronine A (**1**) have been developed. Since the *N*-formyl enamide moiety in **1** is a conserved structure on various actin-depolymerizing marine macrolides, this method of introducing a PEG linker at the end of the aliphatic tail should offer perspectives for developing and using versatile actin-targeting molecular probes.[30] Further structural and functional analyses of ApA-binding proteins as well as their roles in the antitumor activities of **1** are currently underway.

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[1] For the isolation and structures of aplyronines, see: a) K. Yamada, M. Ojika, T. Ishigaki, Y. Yoshida, H. Ekimoto, M. Arakawa, *J. Am. Chem. Soc.* **1993**, *115*, 11020; b) M. Ojika, H. Kigoshi, T. Ishigaki, I. Tsukada, T. Tsuboi, T. Ogawa, K. Yamada, *J. Am. Chem. Soc.* **1994**, *116*, 7441; c) K. Suenaga, T. Ishigaki, A. Sakakura, H. Kigoshi, K. Yamada, *Tetrahedron Lett.* **1995**, *36*, 5053; d) M. Ojika, H. Kigoshi, T. Ishigaki, K. Yamada, *Tetrahedron Lett.* **1993**, *34*, 8501; e) M. Ojika, H. Kigoshi, T. Ishigaki, M. Nishiwaki, I. Tsukada, K. Mizuta, K. Yamada, *Tetrahedron Lett.* **1993**, *34*, 8505; for the synthesis of aplyronines, see: f) H. Kigoshi, M. Ojika, T. Ishigaki, K. Suenaga, T. Mutou, A. Sakakura, T. Ogawa, K. Yamada, *J. Am. Chem. Soc.* **1994**, *116*, 7443; g) H. Kigoshi, M. Ojika, K. Suenaga, T. Mutou, J. Hirano, A. Sakakura, T. Ogawa, M. Nisiwaki, K. Yamada, *Tetrahedron Lett.* **1994**, *35*, 1247; h) H. Kigoshi, K. Suenaga, T. Mutou, T. Ishigaki, T. Atsumi, H. Ishiwata, A. Sakakura, T. Ogawa, M. Ojika, K. Yamada, *J. Org. Chem.* **1996**, *61*, 5326; i) M. Ojika, H. Kigoshi, Y. Yoshida, T. Ishigaki, M. Nishiwaki, I. Tsukada, M. Arakawa, H. Ekimoto, K. Yamada, *Tetrahedron* **2007**, *63*, 3138.

[2] For reviews, see: a) K. Yamada, M. Ojika, H. Kigoshi, K. Suenaga, *Nat. Prod. Rep.* **2009**, *26*, 27; b) K. Yamada, M. Ojika, H. Kigoshi, K. Suenaga, *Proc. Jpn. Acad. Ser. B* **2010**, *86*, 176; c) K. Suenaga, H. Kigoshi, *J. Synth. Org. Chem. Jpn.* **2006**, *64*, 1273 (in Japanese).

[3] S. Saito, S. Watabe, H. Ozaki, H. Kigoshi, K. Yamada, N. Fusetani, H. Karaki, *J. Biochem. (Tokyo)* **1996**, *120*, 552.

[4] For general reviews on actin-depolymerizing natural products, see: a) J. S. Allingham, V. A. Klenchin, I. Rayment, *Cell. Mol. Life Sci.* **2006**, *63*, 2119; b) K. S. Yeung, I. Paterson, *Angew. Chem.* **2002**, *114*, 4826; *Angew. Chem. Int. Ed.* **2002**, *41*, 4632, and references therein.

[5] E. Vincent, J. Saxton, C. Baker-Glenn, I. Moal, J. D. Hirst, G. Pattenden, P. E. Shaw, *Cell. Mol. Life Sci.* **2007**, *64*, 487.

[6] a) M. Hori, S. Saito, Y. Z. Shin, H. Ozaki, N. Fusetani, H. Karaki, *FEBS Lett.* **1993**, *322*, 151; b) S. Saito, S. Watabe, H. Ozaki, N. Fusetani, H. Karaki, *J. Biol. Chem.* **1994**, *269*, 29710; c) S. Wada, S. Matsunaga, S. Saito, N. Fusetani, S. Watabe, *J. Biochem.* **1998**, *123*, 946.

[7] a) J. Tanaka, Y. Yan, J. Choi, J. Bai, V. A. Klenchin, I. Rayment, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 13851; b) C. Petchprayoon, K. Suwanborirux, J. Tanaka, Y. Yan, T. Sakata, G. Marriott, *Bioconjugate Chem.* **2005**, *16*, 1382.

[8] a) X. Zhang, L. Minale, A. Zampella, C. D. Smith, *Cancer Res.* **1997**, *57*, 3751; b) R. D. Perrins, G. Cecere, I. Paterson, G. Marriott, *Chem. Biol.* **2008**, *15*, 287.

[9] a) M. R. Bubb, I. Spector, A. D. Bershadsky, E. D. Korn, *J. Biol. Chem.* **1995**, *270*, 3463; b) S. Saito, S. Watabe, H. Ozaki, M. Jobayashi, T. Suzuki, H. Kobayashi, N. Fusetani, H. Karaki, *J. Biochem. (Tokyo)* **1998**, *123*, 571.

[10] a) A. V. Statsuk, R. Bai, J. L. Baryza, V. A. Verma, E. Hamel, P. A. Wender, S. A. Kozmin, *Nat. Chem. Biol.* **2005**, *1*, 383; b) S. A. Rizvi, D. S. Courson, V. A. Keller, R. S. Rock, S. A. Kozmin, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 4088; c) S. A. Rizvi, S. Liu, Z. L. Chen, C. Skau, M. Pytynia, D. R. Kovar, S. J. Chmura, S. A. Kozmin, *J. Am. Chem. Soc.* **2010**, *132*, 7288.

[11] For reviews on small molecules that affect actin dynamics and the cytoskeleton, see: a) G. Fenteany, S. Zhu, *Curr. Top. Med. Chem.* **2003**, *3*, 593; b) J. R. Peterson, T. J. Mitchison, *Chem. Biol.* **2002**, *9*, 1275.

[12] For reviews on cancer chemotheraphy, see: a) I. Spector, F. Braet, N. R. Shochet, M. R. Bubb, *Microsc. Res. Tech.* **1999**, *47*, 18; b) A. Giganti, E. Friederich, *Prog. Cell Cycle Res.* **2003**, *5*, 511.

[13] a) K. Suenaga, N. Kamei, Y. Okugawa, M. Takagi, A. Akao, H. Kigoshi, K. Yamada, *Bioorg. Med. Chem. Lett.* **1997**, *7*, 269; b) H. Kigoshi, K. Suenaga, M. Takagi, A. Akao, K. Kanematsu, N. Kamei, Y. Okugawa, K. Yamada, *Tetrahedron* **2002**, *58*, 1075.

[14] T. Kuroda, K. Suenaga, A. Sakakura, T. Handa, K. Okamoto, H. Kigoshi, *Bioconjugate Chem.* **2006**, *17*, 524.

[15] K. Hirata, S. Muraoka, K. Suenaga, K. Kuroda, K. Kato, H. Tanaka, M. Yamamoto, M. Takata, K. Yamada, H. Kigoshi, *J. Mol. Biol.* **2006**, *356*, 945.

[16] V. A. Klenchin, J. S. Allingham, R. King, J. Tanaka, G. Marriott, I. Rayment, *Nat. Struct. Biol.* **2003**, *10*, 1058.

[17] J. S. Allingham, A. Zampella, M. V. D'Auria, I. Rayment, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 14527.

[18] V. A. Klenchin, R. King, J. Tanaka, G. Marriott, I. Rayment, *Chem. Biol.* **2005**, *12*, 287.

[19] S. A. Rizvi, V. Tereshko, A. A. Kossiakoff, S. A. Kozmin, *J. Am. Chem. Soc.* **2006**, *128*, 3882.

[20] M. Kita, H. Watanabe, T. Ishitsuka, Y. Mogi, H. Kigoshi, *Tetrahedron Lett.* **2010**, *51*, 4882, and references therein.

[21] Stereoisomers of the imines in **4** and **6** were not separable (E/Z = 2:1), and therefore they were used as mixtures in biochemical studies.

[22] Biocytinylated compound **3** bears a carboxyl group, which may influence its ability to permeate into living cells or give it lower affinity for targeted molecules, thus causing a moderate decrease in cytotoxicity.

[23] See the Supporting Information for details.

[24] a) R. D. Mullins, J. A. Heuser, T. D. Pollard, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6181; b) L. Blanchoin, K. J. Amann, H. N. Higgs, J. B. Marchand, D. A. Kaiser, T. D. Pollard, *Nature* **2000**, *404*, 1007.

[25] For a review on the Arp2/3 complex, see: E. D. Goley, M. D. Welch, *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 713.

[26] R. C. Robinson, K. Turbedsky, D. A. Kaiser, J. B. Marchand, H. N. Higgs, S. Choe, T. D. Pollard, *Science* **2001**, *294*, 1679.

[27] The hydrophobic cleft between SD1 and SD3 of actin, to which **1** binds, includes the helixes αD and αK and the sheets β10–β12. These secondary structures are highly conserved among actin, Arp2, and Arp3. See the Supporting Information for details.

[28] The interactions of ApA with Arp2/Arp3 were shown only by pull-down experiments at this stage, and we could not eliminate the possibility that ApA targets both actin and the Arp2/3 complex to form the ternary complex. We hope to verify this behaviour in future studies.

[29] B. J. Nolen, N. Tomasevic, A. Russell, D. W. Pierce, Z. Jia, C. D. McCormick, J. Hartman, R. Sakowicz, T. D. Pollard, *Nature* **2009**, *460*, 1031.

[30] There is one report on the biocytinylated mycalolide and kabiramide derivatives (see Ref. [6c]). However, the cytotoxicities and actin-depolymerizing activities of these compounds have not been described. Biocytinylated mycalolide B was shown to bind to some cellular proteins including actin, but its binding properties were nonspecific because of the formation of covalent bonds at the unsaturated ketone moiety.

