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Communication

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Interactions of the Antitumor Macrolide Aplyronine A with Actin and Actin-Related Proteins Established by Its Versatile Photoaffinity Derivatives

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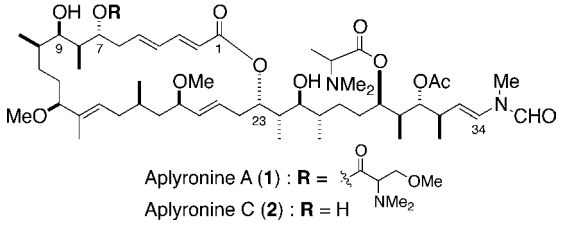
Supporting Information

ABSTRACT: The antitumor and apoptogenic macrolide aplyronine A (ApA) is a potent actin-depolymerizing agent. We developed an ApA acetylene analog that bears the aryldiazirine group at the C34 terminus, which formed a covalent bond with actin. With the use of the photoaffinity biotin derivatives of aplyronines A and C, Arp2 and Arp3 (actin-related proteins) were specifically purified as binding proteins along with actin from tumor cell lysate. However, Arp2 and Arp3 did not covalently bind to aplyronine photoaffinity derivatives. Thus, actin-related proteins might indirectly bind to ApA as the ternary adducts of the actin/ApA complex or through the oligomeric actin.

Aplyronine A (ApA, **1**), a 24-membered macrolide isolated from the sea hare *Aplysia kurodai*, exhibits remarkable antitumor activities *in vivo* against P388 murine leukemia cells (T/C 545%, 0.08 mg/kg) and several cancers.[1] The primary target of **1** was found to be actin, a cytoskeleton protein that is essential for various biological functions, such as muscle contraction, cell motility, and cell division.[2] In fact, ApA potently depolymerizes fibrous actin (F-actin) and inhibits the polymerization of actin by forming a 1:1 complex with the monomeric globular molecule (G-actin, K_d = 100 nM).[3] Studies on structure−activity relationships,[4] an X-ray analysis of the actin−ApA complex,[5] and photoaffinity labeling experiments[6] have established that the tail part (C24/C34) of **1** is important for its actin-binding property. Meanwhile, aplyronine C (ApC, **2**), an extremely minor congener of **1** in *A. kurodai* that lacks the C7 trimethylserine moiety, shows significant actin-depolymerizing activity and is highly accumulated in the cytoplasm, similar to **1** but is much less cytotoxic than **1** (~1/1000).[1a,d,7] Interestingly, this C7 ester moiety of **1** protrudes toward the bulk solvent region in the actin−ApA complex.[5] Thus, the significant antitumor activities of **1** may not be due solely to its F-actin-severing properties and instead may involve its interactions with protein(s) other than actin.



Aplyronine A (**1**) : **R** =
Aplyronine C (**2**) : **R** = H

Recent progress in chemical biology research has accelerated identification of the targets of bioactive natural products as well as observations of their dynamic behaviors in living systems.[8] In addition, photoaffinity labeling combined with mass spectrometric approaches has been developed as a useful proteomics tool.[9] To develop novel actin-targeting antitumor agents, we performed chemical and biological studies on ApA and related actin-targeting macrolides. With the use of fluorescent derivatives of aplyronines, we have shown that **1** causes rapid disassembly of the actin cytoskeleton and the dephosphorylation of focal adhesion kinase in tumor cells with caspase-dependent apoptosis.[7] Furthermore, with the use of biotinylated ApA analogs, we recently identified actin-related proteins 2/3 (Arp2 and Arp3) as presumed targets of **1**.[10] Arp2 and Arp3 constitute the Arp2/3 complex, which binds to the sides of an existing actin filament and initiates growth of a new actin filament to form branched actin filament networks.[11,12] Due to the high structural similarity between G-actin and actin-related proteins, especially around the hydrophobic clefts between subdomains 1 and 3 (ApA-binding site of actin), we expected that **1** might bind to Arp2 or Arp3 to give 1:1 complexes and inhibit the ability of the Arp2/3 complex. These properties might enhance the potent disassembly of actin filaments caused by **1**. However, the detailed molecular mechanism and binding modes of **1** with actin and actin-related proteins are still unclear due to their polymerization properties and instability as a complex *in vitro*. We prepared aplyronine photoaffinity derivatives that can covalently bind to targets and investigated the binding modes of **1** to these proteins as well as its mechanisms of action.

Based on the finding that the C34 *N*-formyl enamide moiety of **1** can be replaced with oximes without a significant loss of activity,[7a] we introduce here an aryltrifluoromethyldiazirine[13] (photoreacting) group via L-lysine (L-Lys) linkers (Figure 1). To detect photolabeled proteins, we use fluorescent labeling with azide/acetylene 1,3-dipolar cyclization (Hüisgen reaction) after the photoreactions as well as affinity purification and the immunoblotting detection of biotin groups using resin-bound or enzyme-conjugated avidins. Thus, we designed an ApA photoaffinity acetylene analog (ApA/PA, **4**) and an ApA photoaffinity PEG-linked biotin analog (ApA/PB, **5**). To compare, we also synthesized a tolyl analog **3**, which lacks

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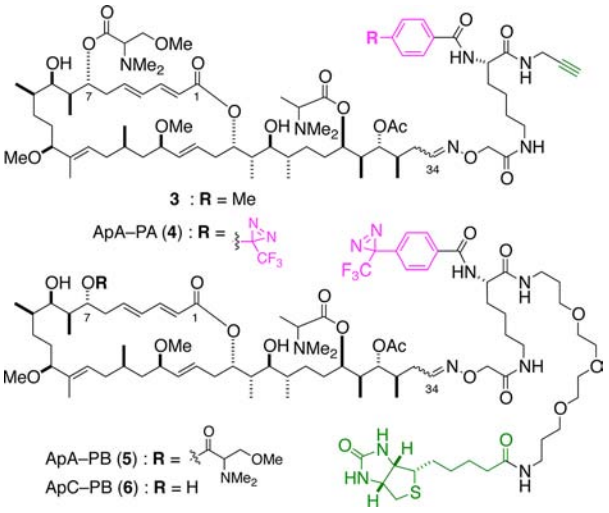


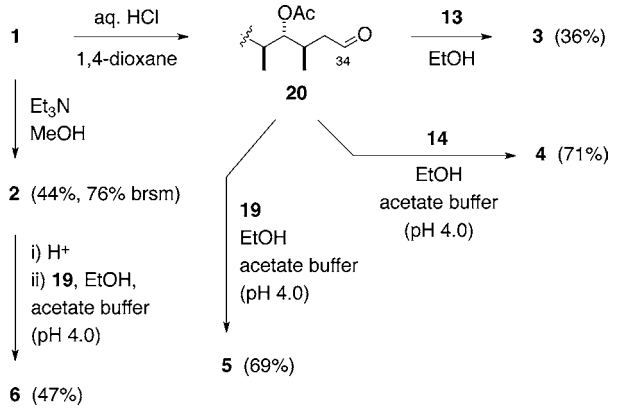
Figure 1. Structures of PA and PB derivatives of aplyronines. Photoreacting (aryl diazirine) and detecting (acetylene or biotin) groups are highlighted in pink and green, respectively.

the diazirine moiety of **4**, and an ApC photoaffinity biotin analog (ApC/PB, **6**).

Scheme 1 summarizes the synthesis of the two diazirine-containing alkoxyamines **14** and **19**. Condensation of the carboxylic acid **7**[14] derived from L-Lys with propargylamine using EDC and HOBt gave amide **8** (71%). Acidic cleavage of the Boc group in **8** with TFA followed by coupling with the succinyl ester **9**[15] (R = Me) in the presence of NMM and DMAP yielded tolyl amide **11** in a moderate yield (49%). Similarly, aryldiazirine amide **12** was prepared from **8** and succinyl ester **10**[16] (R = C(N═N)CF_3) (48%). Fmoc deprotection in **11** and **12** with piperidine in DMF afforded alkoxyamines **13** and **14** in quantitative yield, respectively, while phthalimide (Phth)-protected carboxylic acid **15**[17] was condensed with the primary amine **16**[18] bearing a PEG-linked biotin by using EDC and HOAt to give amide **17** (74%). Subsequent acidic cleavage of the Boc group in **17** with TFA followed by coupling with compound **10** in the presence of DIPEA yielded aryldiazirine amide **18** (82%). Finally, deprotection of the Phth group in **18** with hydrazine monohydrate quantitatively yielded alkoxyamine **19**. Since all three of the neutral alkoxyamines **13**, **14**, and **19** were unstable, they were immediately used for the next coupling reactions with aldehydes.

With diazirine reagents **14** and **19** in hand, we synthesized photoaffinity derivatives of aplyronines (Scheme 2). Acidic

Scheme 2. Preparation of Aplyronine Photoaffinity Derivatives



hydrolysis of **1** afforded the C34 aldehyde **20**,[10] which was condensed with alkoxyamine **13** in EtOH to give the tolyl analog **3** (36%). With the addition of acetate buffer (5 mM), however, the coupling reactions of aldehyde **20** with alkoxyamines **14** or **19** were accelerated to provide **4** (71%) and **5** (69%), respectively. Due to the scarcity of **2** from natural sources (e.g., 3×10^{-7}% yield based on wet wt),[1a] we examined the conversion from ApA (**1**). After several attempts, we found that methanolysis of **1** with 0.1% triethylamine preferentially cleaved the trimethylserine ester to give **2** in 44% yield (with 42% of recovered **1**) (Figure S1). Subsequent acidic hydrolysis of **2** followed by condensation with alkoxyamine **19** gave **6** (47%).

Biological activities of aplyronine analogs were compared with those of natural compounds (Table 1). The ApA analogs **3**–**5** all showed potent cytotoxicities against HeLa S3 cells (IC_{50} 0.16–1.2 nM), whereas the ApC analog **6** was ~260 times less cytotoxic than **5**. By using pyrene-labeled (pyrenyl) actin, we established that ApA tolyl analog **3** significantly depolymerized F-actin *in vitro* (EC_{50} 2.0 μM against 3 μM actin), as with **1** (EC_{50} 1.3 μM) (Figure S2). Also, **5** potently

Scheme 1. Synthesis of the Diazirine-Containing Alkoxyamines 14 and 19

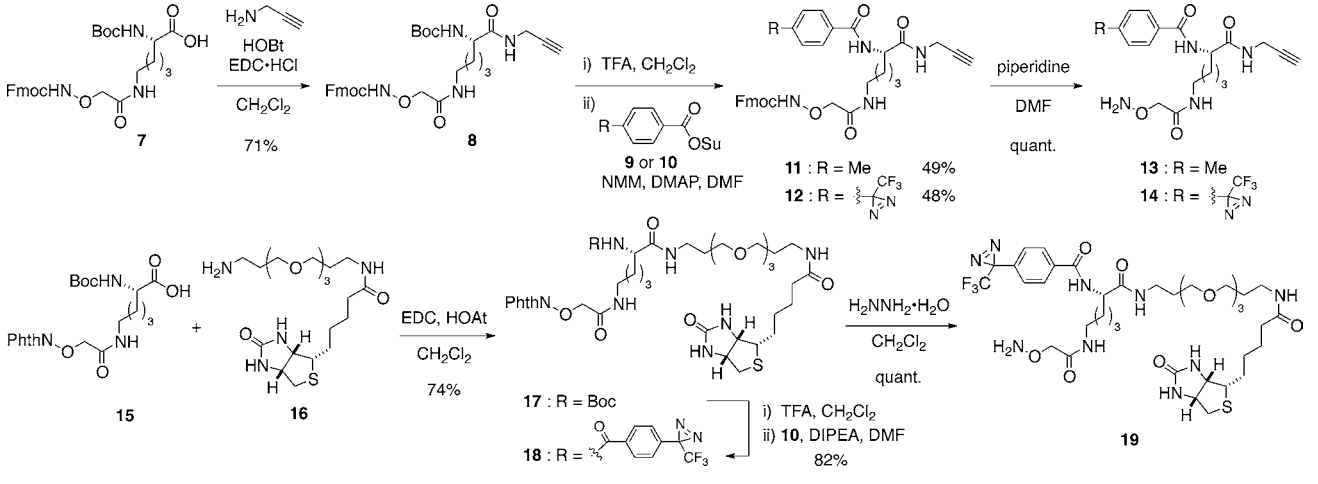


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Table 1. Biological Activities of ApA and Its Derivatives

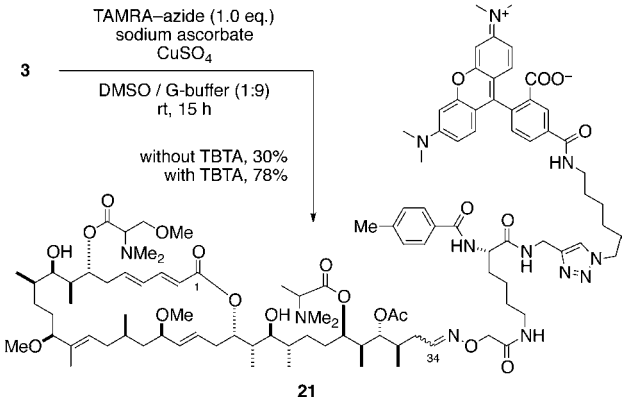
	cytotoxicity (HeLa S3) IC_{50} (nM)	actin-depolymerizing activity EC_{50} (μM)[a]
1	0.010	1.3
2	17	1.4
3	0.16	2.0
4	0.76	–[b]
5	1.2	–[b,c]
6	310	–[b]

[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. [b]Not examined. [c]Confirmed by ultracentrifugation.

inhibited F-actin sedimentation, which was confirmed by ultracentrifugation (Figure S3).[7a]

To develop effective actin labeling and detection methods, we next examined the model Hüisgen reactions using ApA/tolyl analog **3** (Scheme 3). While 1,3-dipolar cycloaddition

Scheme 3. Fluorescent Labeling Experiments for Alkyne 3 in an Aqueous Phase



between the alkyne in **3** and tertamethylrhodamine (TAMRA)/azide using sodium ascorbate and copper sulfate gave an ApA/TAMRA conjugate **21**, the reaction was very slow in G-buffer-rich [2 mM Tris·HCl (pH 8.0), 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol] solution (30%). However, the addition of tris[(1-benzyl-*1H*-1,2,3-triazol-4-yl)methyl]amine (TBTA)[19] to stabilize the Cu(I) active species considerably improved the yield of tetrazole **21** (78%, Figure S5).

Photolabeling experiments of actin using **4** were then carried out (Figure 2). After covalent bond formation by irradiation with UV (365 nm) at 0 °C for 15 min, the probe-bound actin was further labeled with TAMRA by the Hüisgen reaction. Notably, the most highly fluorescent actin bands were observed when the Hüisgen reactions were applied for 30 min (lane 3). Meanwhile, prolonged azide treatment over 1 h resulted in the significant attenuation of actin fluorescence, due to the degradation of actin protein itself (lanes 5–8). While the amount of TAMRA–bound actin was slightly increased without **4** in association with the Hüisgen reactions (lanes 2, 4, and 6), we concluded that ApA analog **4**, bearing an aryldiazirine group at the C34 terminus, formed a covalent bond with actin, in which the protein/probe complex was efficiently fluorescence-labeled for 30 min.

Furthermore, we sought to identify the target proteins of aplyronines in tumor cell lysate using biotinylated photoaffinity

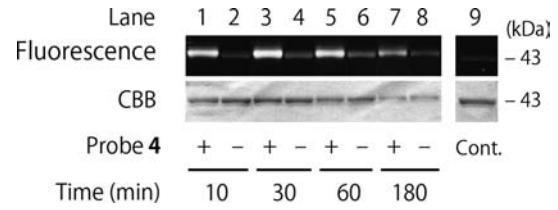


Figure 2. Photolabeling experiments for actin with **4**. After actin (10 μM) was incubated with or without **4** (20 μM) in DMSO/G-buffer (1:9), samples were irradiated with UV_{365}, TAMRA azide, sodium ascorbate, $CuSO_4$, and TBTA were added, and the mixture was incubated for the times shown at the bottom. Labeled actin was detected by TAMRA fluorescence and CBB (Coomassie brilliant blue) stain. The control (lane 9) consisted of actin without UV irradiation or fluorescence labeling.

derivatives. Upon photoreaction with **5** followed by affinity purification using NeutrAvidin agarose, three strong bands corresponding to β-actin, Arp2, and Arp3 (43, 40, and 47 kDa, respectively) were detected with silver stain as well as with anti-Arp2 and Arp3 (Figure 3a,b, lane 1). Binding of these three

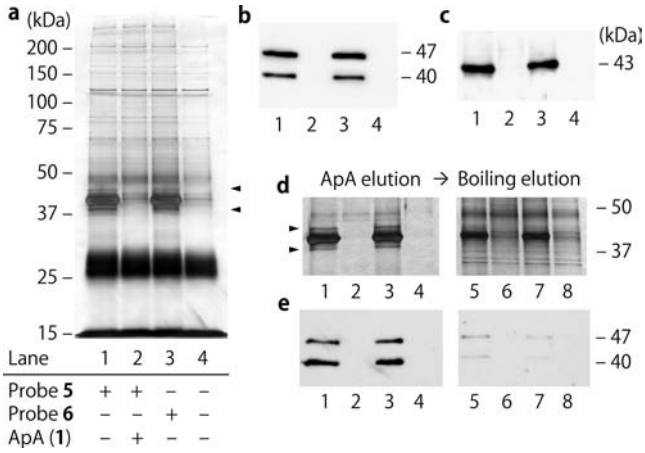


Figure 3. Purification of ApA-binding proteins in tumor cells. Target proteins in HeLa S3 cell lysate were photolabeled with the **5** or **6** preloaded on the NeutrAvidin agarose in the absence or presence of excess **1**. (a) Resulting biotin-bound proteins were eluted by boiling in SDS buffer and detected with silver stain. (b,c) Immunoblotting analysis. Proteins in (a) were detected with (b) anti-Arp2/Arp3 (1:1 mix) or (c) streptavidin-HRP conjugate. (d,e) Stepwise elution. Binding proteins on the resin were eluted by treatment with **1** (10 μM, lanes 1–4) followed by boiling in SDS buffer (lanes 5–8) and detected with (d) silver stain or (e) anti-Arp2/Arp3. Arrowheads in (a,d) indicate Arp2 and Arp3.

proteins with compound **5** was competitively inhibited by excess **1** (lane 2), and the results were similar to those with the biotinylated ApA analogs.[10] These results established that there are specific interactions between the ApA analogs and actin or actin-related proteins, rather than their simple absorptions on the agarose resin.

To confirm the properties of binding between ApA and its target proteins, photoreacted products were detected by streptavidin-HRP conjugate. As expected, a strong band corresponding to actin was observed upon treatment with **5** (Figure 3c, lane 1), and the band disappeared with the addition of excess **1** (lane 2). In contrast, neither Arp2 nor Arp3 was detected as a biotinylated protein. Thus, actin surely formed a covalent bond with **5**, while actin-related proteins did not.

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When the photolabeled products were competitively eluted with **1**, both Arp2 and Arp3 were liberated from the resin with a quantity of actin (Figure 3d,e, lane 1), while the actin covalently bound to ApA derivatives was eluted only after the boiling process (lane 5). These results indicated that Arp2 and Arp3 might indirectly bind to ApA as the ternary adducts of the actin/ApA complex or through the oligomeric actin that interacts with ApA derivatives. Furthermore, through affinity purification with **6**, both Arp2 and Arp3 were obtained with actin from cell lysate, as with **5**, in which only the actin was photolabeled (Figure 3a–e, lane 3). Since both **5** and **6** with different cytotoxicity bound to Arp2 and Arp3 in same extent, we concluded that actin-related proteins were not critical target proteins of **1** that are essential for its potent antitumor activity.

In summary, through the use of photoaffinity derivatives of aplyronines, we have established their interactions with actin and actin-related proteins. We concluded that actin-related proteins might interact with ApA via the actin/ApA complex. Further investigations on the target proteins of ApA in living cell systems as well as its mechanisms of action are currently underway. Since the alkoxyamine reagents **14** and **19** can be easily coupled with various substances that contain aldehyde or hemiacetal groups and the conjugated oximes were considerably stable under physiological conditions (pH 6–8),[20,21] they may serve as quite useful photolabeling tools for analyzing the binding sites and properties of various biomacromolecules and active substances.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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