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SYNTHESIS, CYCLIZATION, AND EVALUATION OF THE ANTICANCER ACTIVITY AGAINST HeLa S-3 CELLS OF ETHYL 2-ACETYLAMINO-3-ETHYNYLAZULENE-1-CARBOXYLATES †

Marie Hyoudou, Hajime Nakagawa, Takahiro Gunji, Yoshino Ito, Yu Kawai, Reiko Ikeda, Takeo Konakahara, and Noritaka Abe*

Department of Pure and Applied Chemistry, Faculty of Science and Technology, Tokyo University of Science, Noda, Chiba 278-8510, Japan
E-mail noriabe@rs.noda.tus.ac.jp

Abstract – Reaction of ethyl 2-aminoazulene-1-carboxylate with NIS in $CHCl_3$ gave 2,2'-diamino-3,3'-diethoxycarbonyl-1,1'-biazulene (**3**) and a terazulene derivative. The coupling of azulenes was catalyzed by acid. Operation of the reaction in the presence of Et_3N in CH_2Cl_2 for 7 min at −7 °C retarded the coupling of the azulene nuclei to give ethyl 2-amino-3-iodoazulene-1-carboxylate (**1a**) in 95% yield. Sonogashira cross-coupling of ethyl 2-acetylamino-3-iodoazulene-1-carboxylate (**1b**) gave ethyl 2-acetylamino-3-ethynylazulene-1-carboxylates (**5a** and **5b**) in good yields. Cyclization of ethyl 2-acetylamino-3-phenylethynylazulene-1-carboxylate with Pd-catalyst gave azuleno[2,1-*b*]pyrrole derivatives. Compounds (**3** and **5b**) showed potent cytotoxic activity against HeLa S-3 cells (IC_{50} [μM] : **3**: 2.9 ± 0.2, **5b**: 13.4 ± 1.1).

INTRODUCTION

The chemistry of azulenes began as natural product chemistry at first, and is attracted attention from peculiarities of their structures and reactions, as well as their numerical pharmaceutical utilities.[1] As the potential application of azulenes, very recently, antigastric ulcer activity of guaiazulene derivatives was reported.[2] Heterocycle-fused azulenes have also been described from the early studies of azulene chemistry,[1e,1f] and recently reviewed.[3] Some heterocycle-fused azulenes also attracted from their

† Dedicated to Prof. Ei-ichi Negishi on the occasion of his 77th birthday.

pharmaceutical utilities; especially, the reports about linderazulene and its derivatives, such as gorgiallylazulene, were often seen.[4] Pharmaceutical utilities of heterocycles-fused azulenes other than furan ring were also reported; *e.g.*, azuleno[5,6-*b*]indole displays antineoplastic activity,[5] a 3,4-dihydroazuleno[4,3a,3-*bc*]pyridin-5(5*H*)-one derivative inhibits protein kinases (serine/threonine kinases),[6] azuleno[4,3-*f*]pyrazoles show antiphlogistic, sedative, and analgestic effects,[7] and azuleno[2,1-*b*]pyridines are anti-inflammatory.[8]

As a synthetic method for heterocycles, the electrophilic cyclization and the transition-metal-catalyzed annulation of alkynes with preferentially situated substituents were efficient methods; *e.g.*, Sonogashira cross-coupling of 2-haloanilines and successive base-mediated (or electrophilic) cyclization; Pd-mediated cyclization of 2-ethynylanilines; the reaction of 2-haloanilines with Cu-acetylide known as Cacci reaction; 2-iodoaniline with internal alkynes being referred as the Larock heteroannulation, *etc*.[9]

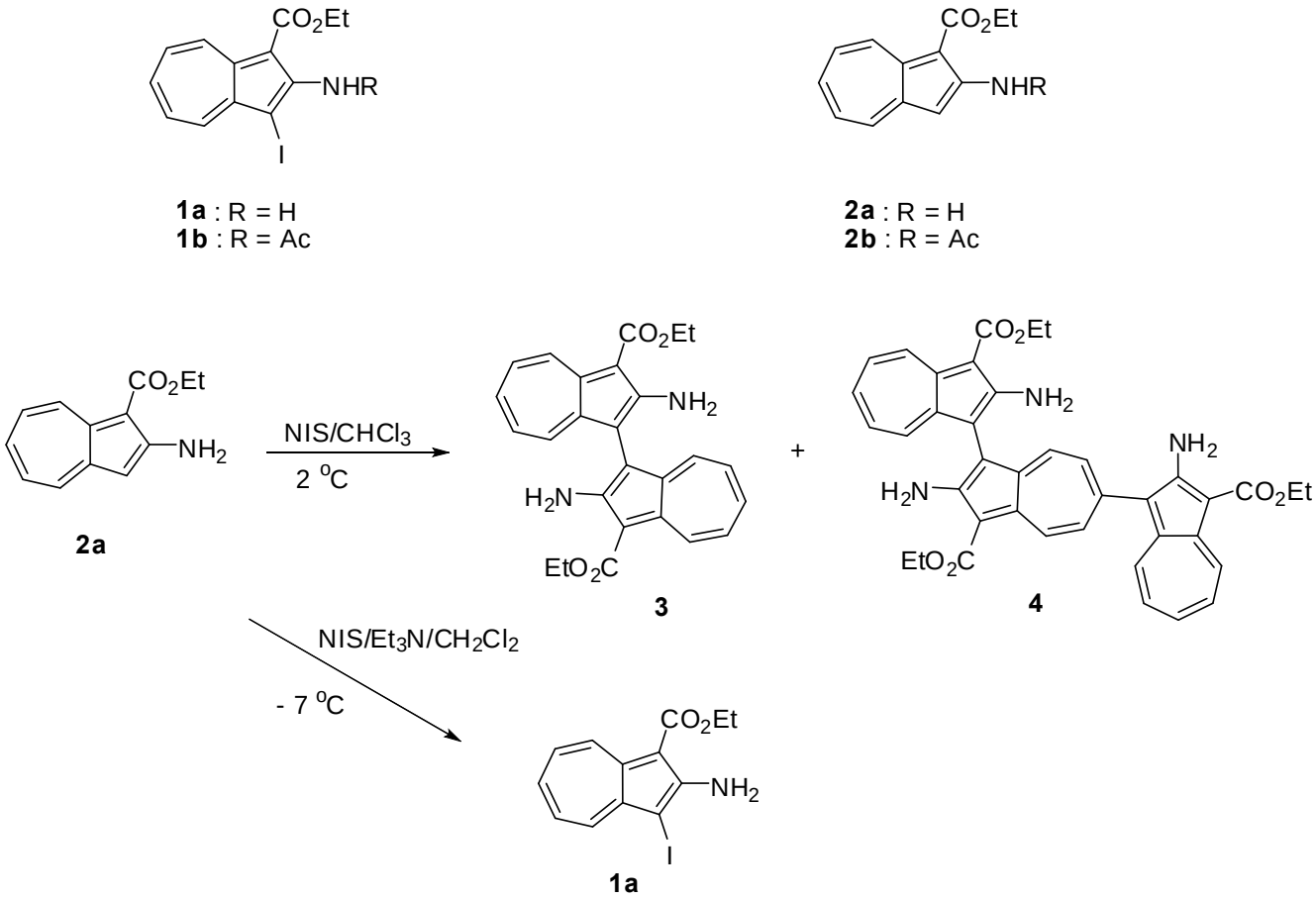
Ethynylazulenes mainly have been investigated from the respect to the construction of advanced materials for electronic and photonic applications.[1f,10,11] In spite of the synthetically and pharmaceutical potentiality of (amino substituted)-ethynylazulenes, attempts of synthesis and reactions of the ethynylazulene were little; only related report was Cacci reaction of ethyl 2-acetylamino-3-iodoazulene-1-carboxylate[12] with Cu-phenylacetylide; where azuleno[2,1-*b*]pyrrole derivative was obtained, but ethyl 2-acetylamino-3-ethynylazulene-1-carboxylates were not isolated.[13] For extension of the synthesis of heterocycle-fused azulenes and the inquiry of bioactivity of azulenes, we investigated the synthesis and reactions of ethyl 2-acetylamino-3-ethynylazulene-1-carboxylate.

RESULTS AND DISCUSSION

For the synthesis of ethyl 2-amino-3-ethynylazulene-1-carboxylate, we examined the synthesis of ethyl 2-amino-3-iodoazulene-1-carboxylate (**1a**), at first. According to Morita's methods,[12] we treated ethyl 2-aminoazulene-1-carboxylate[12,14] (**2a**) with *N*-iodosuccinimide (NIS) in CH_2Cl_2 for 15 min at 5 °C. From the reaction mixture, **1a** was not obtained, so we reinvestigated about the synthesis of **1a**. When **2a** was treated with NIS in $CHCl_3$ for 15 min at 2 °C, 2,2'-diamino-3,3'-diethoxycarbonyl-1,1'-biazulene (**3**) and terazulene derivative (**4**) were isolated in 46% and 6% yields, respectively (Scheme 1). The structure of **3** was deduced from its ^{1}H NMR, ^{13}C NMR, IR, and HRMS spectra. The molecular formula of **3** was $C_{26}H_{24}N_2O_4$ from its HRMS spectrum. The ^{1}H NMR spectrum of **3** showed seven-membered protons at δ 7.19 (dd, *J* 10.1 and 9.6, H-5,5'), 7.29 (t, *J* 10.1, H-7,7'), 7.41 (t, *J* 10.1, H-6,6'), 7.42 (d, *J* 10.1, H-4,4'), and 9.03 (d, *J* 9.6, H-8,8'), together with NH protons at δ 5.62 (4H, br s) and ethyl protons. The molecular formula of **4** was deduced to $C_{39}H_{35}N_3O_6$ from its HRMS spectrum. In the ^{1}H NMR spectrum of **4**, six doublet signals for eight protons, at δ 7.30 (2H, d, *J* 10.2), 7.56 (2H, d, *J* 10.2), 7.94 (1H, d, *J* 10.2), 8.99

(1H, d, *J* 9.6), 9.04 (1H, d, *J* 9.6), and 9.07 (1H, d, *J* 9.6), were seen together with other six aromatic protons, three ethyl ester protons, and amino protons. Existence of couples of doublets at δ 7.30 (2H, d, *J* 10.2) and 7.56 (2H, d, *J* 10.2) suggested that the centered azulene nucleus was substituted at the 6-position by another azulene.

It was described that the bromination of azulene derivatives with NBS affords dimer, trimers and oligomers, and the reaction was suggested to proceed by radical mechanism.[15] Shoji *et al*. reported that



Scheme 1

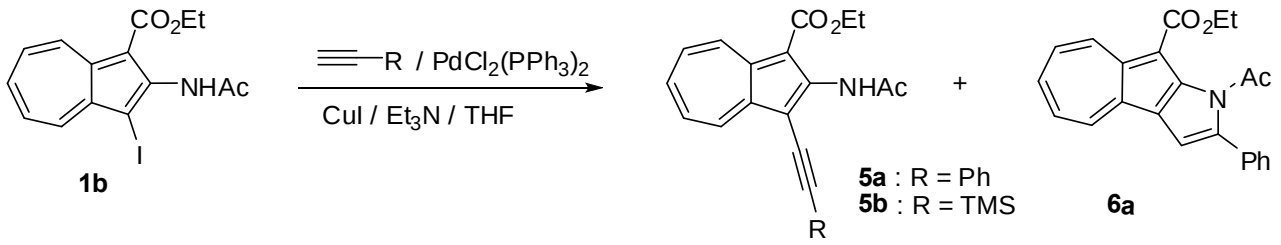
1-azulenyl sulfoxides gave 3,3'-bis(methylthio)-1,1'-biazulene derivatives in the presence of acid.[16] They proposed a radical mechanism with reference to reports of Razus.[17] Apart from this, forming of 2,2'-diamino-3,3'-diethoxycarbonyl-8,8'-diphenyl-1,1'-biazulene by radical coupling of ethyl 2-amino-4-phenylazulene-1-carboxylate in the presence of $FeCl_3$ was reported.[18] It seems that our results resemble to Shoji's report. So we examined the behavior of **1a** in the presence of acid. The solution of **1a** in CH_2Cl_2, which was prepared from **2a** with NIS, did not convert at rt for 5 h under tracing the reaction

with TLC. Addition of a small amount of TsOH to the solution, and stirring the mixture for 24 h led to consume **1a**. From the reaction mixture, **3** was obtained in 61% yield together with a small amount of **1a**. For considering the instability of haloazulenes with acid,[15] we achieved the synthesis of **1a** in 95% yield by treating of **2a** with NIS in CH_2Cl_2 for 7 min at −7 °C in the presence of Et_3N.

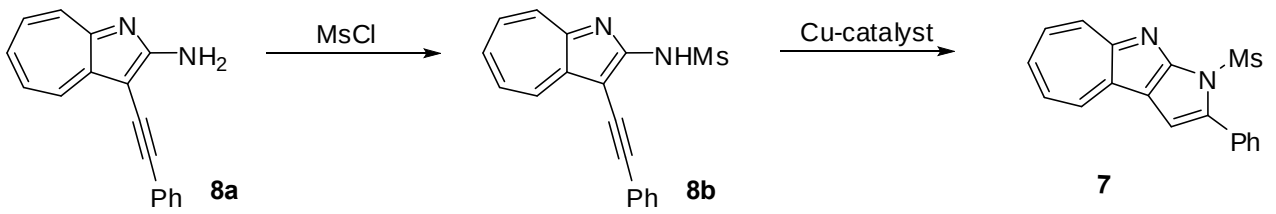
Because **1a** was rather labile, we decided to use 2-acetylamino-3-iodoazulene-1-carboxylate[12] (**1b**) as a raw material for Sonogashira cross-coupling. Thus **1b** was synthesized in 96% yield by the treatment of **2b** with NIS in CH_2Cl_2 for 15 min at 5 °C.

The reaction of **1b** with phenylacetylene was carried out in the presence of $PdCl_2(PPh_3)_2$, CuI, and Et_3N in THF for 24 h at rt, and ethyl 2-acetylamino-3-phenylethynylazulene-1-carboxylate (**5a**) was obtained in 76% yield. When the reaction operated in the presence of a large excess of Et_3N, ethyl 1-acetylamino-2-phenylazuleno[2,1-*b*]pyrrole-9-carboxylate (**6a**) was obtained in 10% yield together with **5a** (63%) (Scheme 2). The molecular formula of **5a** was $C_{23}H_{19}NO_3$ from the HRMS spectrum. In its IR spectrum, peaks at 3300 (NH), 2200 and 2140 cm^{-1} (C≡C) were seen. The molecular formula of **6a** was $C_{23}H_{19}NO_3$ from its HRMS spectrum. In its ^1H NMR spectrum, 1H-singlet was seen at δ 6.99, and methyl signal was appeared at δ 2.36 but NH signal was not observed. It is known that 1H-singlet in the ^1H NMR spectrum of 2a-aza-2*H*-cyclopent[*cd*]azulene (**7**) was seen at δ 6.87,[19] therefore the signal at δ 6.99 of **6b** is considered to be reasonable.

In a similar manner, treatment of **1b** with trimethylsilylacetylene gave ethyl 2-acetylamino-3-trimethylsilylethynylazulene-1-carboxylate (**5b**) in 83% yield.

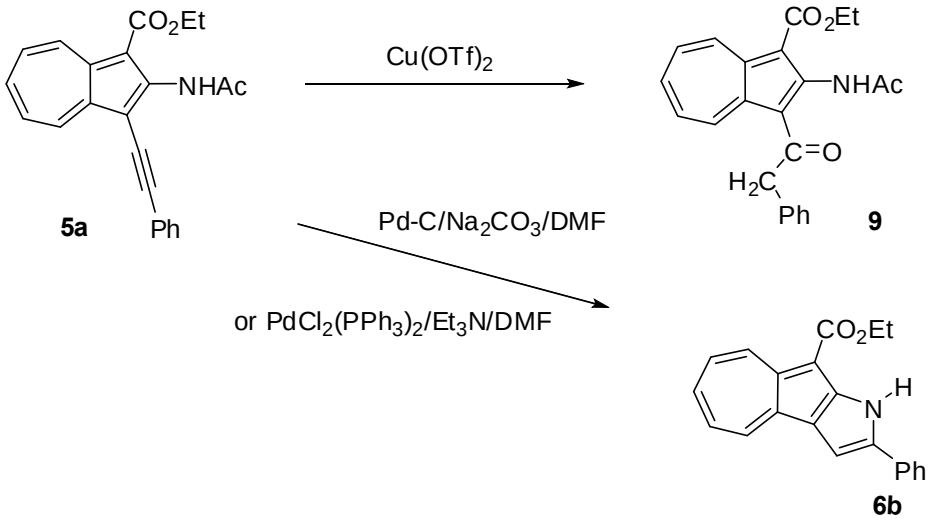


Scheme 2



Scheme 3

It was reported that cyclization of 2-mesylamino-3-phenylethynyl-1-azaazulene (**8b**), prepared from **8a**, underwent in the presence of Cu-catalyst and gave 2a-aza-2*H*-cyclopent[*cd*]azulene (**7**) (Scheme 3).[19] As for cyclization of **5a**, we adopted similar condition at first. Thus we treated **5a** with an equivalent of $Cu(OTf)_2$ in toluene for 3 h at 110 °C in a sealed tube. The reaction showed complex feature, and no identified product was isolated. When the reaction was performed in CH_2Cl_2 for 3 h at 40 °C, the reaction showed complex feature again, but ethyl 2-acetylamino-3-phenacylazulene-1-carboxylate (**9**) was isolated in 9% yield from the mixture. The molecular formula of **9** was $C_{23}H_{21}NO_4$ from the HRMS spectrum. In its ^{1}H NMR spectrum, phenyl protons were seen at δ 7.10−7.16 (5H, m), and *o*-situated hydrogens of phenyl group did not shift to low field. From the results, we assigned the structure. Then we treated **5a** with an equivalent of CuI in toluene for 4 h at 110 °C in a sealed tube, but the reaction did not undergo and **5a** was recovered.



Scheme 4

Next, we examined the cyclization of **5a** by Pd-catalyst. Treatment of **5a** with 2 mol% of 5% Pd-C and Na_2CO_3 in DMF for 24 h at 120 °C gave ethyl 2-phenyl-3*H*-azuleno[2,1-*b*]pyrrole-4-carboxylate (**6b**)[13] in 33% yield. The reaction of **5a** with $PdCl_2(PPh_3)_2$ and Et_3N in DMF for 24 h at 100 °C gave **6b** in 72% yield. Thus cyclization of **5a** was achieved by the treatment of Pd-catalyst, but in the reaction deacetylation occurred.

On the contrary of the case of **5a**, treatment of **5b** with $Cu(OTf)_2$ in CH_2Cl_2 for 3 h at 40 °C gave a complex mixture and no distinct product was obtained. The reaction of **5b** with CuI did not undergo at all. Reaction of **5b** with Pd-C gave a complex mixture, and recovered **5b** (24%) was only identified compound.

Evaluation of cytotoxic activity

Compounds (**1b**, **3**, **5a**, **5b**, and **8a**) were evaluated for their cytotoxic activity against HeLa S-3 cells. The IC_{50} values [μM] are summarized in Table 1. The compounds (**3** and **5b**) showed potent cycotoxic activity. On the other hand the compound (**1b** and **5a**) showed scarcely cytotoxic activity. From the facts as above and that 2-amino-3-phenylethynyl-1-azaazulene (**8a**) has scarcely cytotoxic activity against HeLa S-3 cells, it seems that the phenyl group obstructed the activity.

Table 1. Cytotoxic evaluation of compounds (**1b**, **3**, **5a**, **5b** and **8a**); IC_{50} were expressed in μM.

	1b	**3**	**5a**	**5b**	**8a**
IC_{50}	> 52.2	2.9 ± 0.2	> 55.8	13.4 ± 1.1	99.0 ± 1.1

CONCLUSION

Reaction of ethyl 2-aminoazulene-1-carboxylate (**2a**) with NIS was reinvestigated. Treatment of **2a** with NIS in $CHCl_3$ at 5 °C gave 2,2'-diamino-3,3'-diethoxycarbonyl-1,1'-biazulene (**3**: 46%) and terazulene derivative (**4**: 6%). The reaction was catalyzed by acid and the reaction of **1a** with TsOH gave **3** in 61%. The synthesis of ethyl 2-amino-3-iodoazulene-1-carboxylate (**1a**) was achieved in the presence of Et_3N in CH_2Cl_2 for 7 min at −7 °C and **1a** was obtained in 95% yield. Ethyl 2-acetylamino-3-iodoazulene-1-carboxylate (**1b**) was obtained in 96% yield by the reaction of 2-acetylaminoazulene-1-carboxylate (**2b**) with NIS. Sonogashira cross-coupling of **1b** with terminal acetylenes gave corresponding ethyl 2-acetylamino-3-ethynylazulene-1-carboxylates (**5a** and **5b**) in good yields. Cyclization of ethyl 2-acetylamino-3-phenylethynylazulene-1-carboxylate (**5a**) with Pd-catalyst gave azuleno[2,1-*b*]pyrrole derivatives. Compounds (**3** and **5b**) showed potent cytotoxic activity against HeLa S3 cells (IC_{50} [μM] : **3**: 2.9 ± 0.2, **5b**: 13.4 ± 1.1).

EXPERIMENTAL

Melting points were determined with a Bibby Sterilin melting point SMP-3 apparatus and were uncorrected. ^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a JEOL JNM ECP-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) using $CDCl_3$ as a solvent with tetramethylsilane as an internal standard unless otherwise stated; *J* values are recorded in Hz. IR spectra were recorded for KBr pellets on a JASCO FT/IR-6100 unless otherwise stated. Mass specra were taken with JEOL JMS-T100LC. ESI-MS was measured using dried methanol as a solvent. High-resolution mass spectra (HRMS) were found to be with in ± 5% of the theoretical values. Merck Kieselgel 60 was used for silica gel column

chromatography, and Wako activated Alumina (300 mesh) was used for alumina column chromatography.

Reaction of ethyl 2-aminoazulene-1-carboxylate (2a) with NIS

a) A mixture of **2a**[12,14] (0.215 g, 1.00 mmol) and NIS (0.225 g, 1.00 mmol) in $CHCl_3$ (10 mL) was stirred at 2 °C for 15 min, then evaporated. Chromatography of the residue on silica gel with CH_2Cl_2 gave **3** (0.0984 g, 46%) and **4** (0.0155 g, 6%), successively.

3: Red needles (from hexane-CH_2Cl_2), mp 176−179 °C; ^{1}H NMR δ 1.40 (6H, t, *J* 7.1, CH_3), 4.50 (4H, q, *J* 7.1, OCH_2), 5.62 (4H, br s, NH_2), 7.19 (2H, dd, *J* 10.1 and 9.6, H-5, 5'), 7.29 (2H, t, *J* 10.1, H-7, 7'), 7.41 (2H, t, *J* 10.1, H-6, 6'), 7.42 (2H, d, *J* 10.1, H-8, 8'), 9.03 (2H, d, *J* 9.6, H-4, 4'); ^{13}C NMR δ 14.7, 59.6, 98.40, 107.08, 128.41, 129.08, 129.55, 129.62, 131.32, 142.58, 143.42, 158.93, and 166.86; ν_{max}/cm^{-1} 3485, 3335 (NH_2), 1669 (C=O). MS (ESI$^+$) *m*/*z* 451 ([M + Na]$^+$). Calcd. for $C_{26}H_{24}N_2O_4$: M = 428; HRMS (ESI$^+$): Calcd. for $^{12}C_{26}{}^{1}H_{24}{}^{14}N_2{}^{23}Na_1{}^{16}O_4$: 451.1634. Found: *m*/*z* 451.1627. *Anal.* Calcd. for $C_{26}H_{24}N_2O_4$: C, 72.88; H, 5.65; N, 6.64. Found: C, 72.65; H, 5.41; N, 6.47.

4: Dark red powders (from hexane-CH_2Cl_2), mp 206−208 °C; ^{1}H NMR δ 1.49 (3H, t, *J* 6.9), 1.51 (3H, t, *J* 7.2), 1.52 (3H, t, *J* 6.9), 4.49 (2H, q, *J* 6.9), 4.50 (2H, q, *J* 7.2), 4.53 (2H, q, *J* 6.9), 5.5-7.0 (6H, br), 7.17 (1H, like t, *J* 9.4), 7.19 (1H, like t, *J* 9.6), 7.20 (1H, like t, *J* 9.6), 7.30 (2H, d, *J* 10.2), 7.31 (1H, like t, *J* 9.6), 7.39 (1H, like t, *J* 9.6), 7.43 (1H, like t, *J* 10.5), 7.56 (2H, d, *J* 10.2), 7.94 (1H, d, *J* 10.2), 8.99 (1H, d, *J* 9.6), 9.04 (1H, d, *J* 9.6), and 9.07 (1H, d, *J* 9.6); ν_{max}/cm^{-1} 3490, 3484, 3450, 3352, 3336, and 3308 (NH_2), 1657 (C=O), 1593 (C=N). MS (ESI$^+$) *m*/*z* 664 ([M + Na]$^+$). Calcd. for $C_{39}H_{35}N_3O_6$: M = 641; HRMS (ESI$^+$): Calcd. for $^{12}C_{39}{}^{1}H_{35}{}^{14}N_3{}^{23}Na_1{}^{16}O_6$: 664.2424. Found: *m*/*z* 664.2415.

b) A mixture of **2a** (0.304 g, 1.41 mmol) and NIS (0.320 g, 1.42 mmol) in CH_2Cl_2 (10 mL) was stirred at −10 °C for 10 min, then the temperature was elevated to rt and stirred for 5 h. Tracing the reaction with TLC showed that only **1a** was forming. To the mixture TsOH (0.01 g) was added, and the mixture was stirred for 24 h at rt, then Et_3N (0.1 mL) was added. The mixture was evaporated, and silica gel chromatography of the residue with CH_2Cl_2 gave **3** (0.182 g, 61%) and **1a** (0.003 g, 0.5%).

Synthesis of ethyl 2-amino-3-iodoazulene-1-carboxylate (1a)

A mixture of **2a** (0.641 g, 2.98 mmol) and NIS (0.671 g, 2.97 mmol) in the presence of Et_3N (3 drops) in CH_2Cl_2 (10 mL) was stirred at −7 °C for 7 min, then evaporated. Chromatography of the residue on silica gel with CH_2Cl_2 gave **1a** (0.968 g, 95%).

1a: Dark red needles (from EtOH), mp 119.6 °C (decomp.) (lit.,[12] mp 121−122 °C); ^{1}H NMR δ 1.48 (3H, t, *J* 7.1, CH_3), 4.71 (4H, q, *J* 7.1, OCH_2), 6.34 (2H, br s, NH_2), 7.37 (2H, tdd, *J* 9.6, 9.1, and 1.4, H-7),

7.38 (1H, td, *J* 9.1 and 1.7, H-5), 7.41 (1H, ddd, *J* 9.1, 8.7, and 1.4, H-6), 7.92 (1H, ddd, *J* 8.7, 1.7, and 1.4, H-4), 8.93 (1H, d, *J* 9.6, H-8); ^{13}C NMR ($CDCl_3$) δ 14.7, 59.6, 98.40, 107.08, 128.41, 129.08, 129.55, 129.62, 131.32, 142.58, 143.42, 158.93, and 166.86; ν_{max}/cm^{-1} 3473, 3313 (NH_2), 1669 (C=O). MS (ESI$^+$) *m*/*z* 363 ([M + Na]$^+$). Calcd. for $C_{13}H_{12}NO_2$: M = 340; HRMS (ESI$^+$): Calcd. for $^{12}C_{13}{}^{1}H_{12}{}^{14}N_1{}^{23}Na_1{}^{127}I_1{}^{16}O_2$: 363.9810. Found: *m*/*z* 363.9795.

Synthesis of ethyl 2-acetylaminoazulene-1-carboxylate (2b)

A mixture of **2a**[12,14] (0.511 g, 2.40 mmol), Ac_2O (5.0 mL), and 2 drops of pyridine was stirred for 3 h at rt, then the precipitate was collected by filtration and recrystallization from benzene gave **2b** (0.408 g, 66%).

2b: Red needles (from benzene), mp 143.6−144 °C (lit.,[14] mp 140−141.5 °C); ^{1}H NMR (DMSO-d_6) δ 1.43 (3H, t, *J* 7.0, CH_3), 2.26 (3H, s, $COCH_3$), 4.44 (2H, q, *J* 7.0, OCH_2), 7.60 (1H, t, *J* 9.9, H-7), 7.65 (1H, t, *J* 9.9, H-5), 7.77 (1H, t, *J* 9.9, H-6), 7.99 (1H, s, H-3), 8.46 (1H, d, *J* 9.9, H-4), 9.20 (1H, d, *J* 9.9, H-8), and 10.65 (1H, br s, NH); ν_{max}/cm^{-1} 3260 (NH), 1693 and 1646 (C=O). MS (ESI$^+$) *m*/*z* 280 ([M + Na]$^+$). Calcd. for $C_{15}H_{15}NO_3$: M = 257; HRMS (ESI$^+$): Calcd. for $^{12}C_{15}{}^{1}H_{15}{}^{14}N_1{}^{23}Na_1{}^{16}O_3$: 280.0950. Found: 280.0933.

Synthesis of ethyl 2-acetylamino-3-iodoazulene-1-carboxylate (1b)

A mixture of **2b** (0.200 g, 0.77 mmol) and NIS (0.170 g, 0.77 mmol) in CH_2Cl_2 (10 mL) was stirred 15 min at 5 °C, then evaporated. Chromatography of the residue on alumina with CH_2Cl_2 (added small amount of Et_3N) gave **1b** (0.29 g, 96%).

1b: Violet needles (from EtOH-acetone), mp 187−189 °C (lit.,[12] mp 188−189 °C); ^{1}H NMR (DMSO-d_6) δ 1.29 (3H, t, *J* 7.0, CH_3), 2.13 (3H, s, $COCH_3$), 4.24 (2H, q, *J* 7.0, OCH_2), 7.68 (1H, t, *J* 9.9, H-7), 7.71 (1H, t, *J* 9.9, H-5), 7.92 (1H, t, *J* 9.9, H-6), 8.42 (1H, d, *J* 9.9, H-4), 9.10 (1H, d, *J* 9.9, H-8), and 10.14 (1H, br s, NH); ^{13}C NMR δ 14.51, 24.51, 60.54, 73.99, 109.03, 128.63, 129.15, 135.76, 137.90, 138.80, 140.24, 142.71, 150.09, 165.28, and 167.61; ν_{max}/cm^{-1} 3273 (NH), 1689 and 1671 (C=O), 595 (C-I). MS (ESI$^+$) *m*/*z* 406 ([M + Na]$^+$). Calcd. for $C_{15}H_{14}NIO_3$: M = 383; HRMS (ESI$^+$): Calcd. for $^{12}C_{15}{}^{1}H_{14}{}^{14}N_1{}^{127}I_1{}^{23}Na_1{}^{16}O_3$: 405.9916. Found: 405.9888.

Synthesis of ethyl 2-acetylamino-3-phenylethynylazulene-1-carboxylate (5a)

a) Under argon atmosphere, a mixture of **1b** (0.200 g, 0.52 mmol), phenylacetylene (0.09 mL, 0.53 mmol), $PdCl_2(PPh_3)_2$ (0.01 g, 0.016 mmol), CuI (0.01 g, 0.05 mmol), and Et_3N (2 mL) in THF (3 mL) was stirred for 24 h at rt, then water was added. The mixture was extracted with $CHCl_3$. The extract was dried over Na_2SO_4 and evaporated. Chromatography of the residue on alumina with hexane-AcOEt (5 : 1) gave **5a** (0.139 g, 76%).

b) Under argon atmosphere, a mixture of **1b** (0.200 g, 0.52 mmol), phenylacetylene (0.09 mL, 0.53 mmol), $PdCl_2(PPh_3)_2$ (0.010 g, 0.016 mmol), CuI (0.010 g, 0.05 mmol), and Et_3N (7 mL) in THF (6 mL) was stirred for 24 h at rt, then water was added. The mixture was extracted with $CHCl_3$. The extract was dried over Na_2SO_4 and evaporated. Chromatography of the residue on alumina with hexane-AcOEt (5 : 1) gave **5a** (0.120 g, 63%) and **6a** (0.019 g, 10%), successively.

5a: Green needles (from hexane-$CHCl_3$), mp 138−139.5 °C; ^{1}H NMR δ 1.50 (3H, t, *J* 7.0, CH_3), 2.36 (3H, s, $COCH_3$), 4.49 (2H, q, *J* 7.0, OCH_2), 7.30-7.35 (3H, m, H-*m*, *p*-Ph), 7.55 (1H, t, *J* 9.9, H-7), 7.58 (1H, dd, *J* 9.9 and 9.5, H-5), 7.64 (2H, dd, *J* 7.3 and 1.5, H-*o*-Ph), 7.71 (1H, t, *J* 9.9, H-6), 8.75 (1H, d, *J* 9.5, H-4), and 9.24 (1H, d, *J* 9.9, H-8), and 10.20 (1H, br s, NH); ^{1}H NMR (DMSO-d_6) δ 1.32 (3H, t, *J* 7.0, CH_3), 2.20 (3H, s, $COCH_3$), 4.28 (2H, q, *J* 7.0, OCH_2), 7.43 (2H, dd, *J* 7.3 and 6.6, H-*m*-Ph), 7.45 (1H, td, *J* 6.6 and 1.1, H-*p*-Ph), 7.65 (2H, t, *J* 9.9, H-5, 7), 7.69 (2H, t, *J* 7.3, H-*o*-Ph), 7.91 (1H, t, *J* 9.9, H-6), 8.68 (1H, d, *J* 9.9, H-4), 9.10 (1H, d, *J* 9.9, H-8), and 10.44 (1H, br s, NH); ^{13}C NMR δ 14.48, 24.55, 60.50, 83.68, 98.72, 103.93, 104.55, 124.14, 127.71, 128.21, 129.05, 129.90, 131.32, 135.63, 135.69, 137.50, 139.92, 144.43, 149.43, 166.27, and 167.35; ν_{max}/cm^{-1} 3300 (NH), 2200 and 2140 (C≡C), 1692, 1678, and 1652 (C=O). MS (ESI$^+$) *m*/*z* 380 ([M + Na]$^+$). Calcd. for $C_{23}H_{19}NO_3$: M = 357; HRMS (ESI$^+$): Calcd. for $^{12}C_{23}{}^{1}H_{19}{}^{14}N_1{}^{23}Na_1{}^{16}O_3$: 380.1249. Found: *m*/*z* 380.1267.

6a: Dark green needles (from hexane-$CHCl_3$), mp 142.0−143.1 °C; ^{1}H NMR δ 1.53 (3H, t, *J* 7.0, CH_3), 2.36 (3H, s, COCH3), 4.49 (2H, q, *J* 7.0, OCH_2), 6.99 (1H, s, H-1), 7.31 (2H, dd, *J* 7.7 and 7.4, H-*m*-Ph), 7.56 (2H, t, *J* 9.9, H-6, 8), 7.60 (1H, dd, *J* 7.7 and 1.2, H-*p*-Ph), 7.61 (2H, dd, *J* 7.4 and 1.2, H-*o*-PH), 7.72 (1H, t, *J* 9.9, H-7), 8.72 (1H, d, *J* 9.9, H-9), and 9.26 (1H, d, *J* 9.9, H-5); ν_{max}/cm^{-1} 1693, 1678, and 1659. MS (ESI$^+$) *m*/*z* 380 ([M + Na]$^+$): Calcd. for $C_{23}H_{19}NO_3$: M = 357; HRMS (ESI$^+$): Calcd. for $^{12}C_{23}{}^{1}H_{19}{}^{14}N_1{}^{23}Na_1{}^{16}O_3$: 380.1263. Found: *m*/*z* 380.1249.

Synthesis of ethyl 2-acetylamino-3-trimethylsilylethynylazulene-1-carboxylate (5b)

Under argon atmosphere, a mixture of **1b** (0.200 g, 0.52 mmol), trimethylsilyllacetylene (0.07 mL, 0.62 mmol), $PdCl_2(PPh_3)_2$ (0.010 g, 0.016 mmol), CuI (0.010 g, 0.05 mmol), and Et_3N (3 mL) in THF (3 mL) was stirred for 24 h at rt, then water was added. The mixture was extracted with $CHCl_3$. The extract was dried over Na_2SO_4 and evaporated. Chromatography of the residue on alumina with hexane-AcOEt (5 : 1) gave **5b** (0.091 g, 53%).

5b: Green needles (from hexane-$CHCl_3$), mp 123−124.5 °C; ^{1}H NMR (DMSO-d_6) δ 0.28 (9H, s, $SiCH_3$), 1.31 (3H, t, *J* 7.0, CH_3), 2.14 (3H, s, $COCH_3$), 4.27 (2H, q, *J* 7.0, OCH_2), 7.69 (2H, t, *J* 9.9, H-5, 7), 7.90 (1H, t, *J* 9.9, H-6), 8.50 (1H, d, *J* 9.9, H-4), 9.11 (1H, d, *J* 9.9, H-8), and 10.27 (1H, br s, NH); ^{13}C NMR δ 0.26, 14.57, 24.37, 60.50, 85.91, 98.56, 1004.01, 104.68, 128.49, 129.18, 135.74, 135.80, 137.55,

139.93, 144.97, 166.15, and 167.21; ν_{max}/cm^{-1} 3250 (NH), 2140 (C≡C), 1693 and 1650 (C=O). MS (ESI$^+$) *m*/*z* 376 ([M + Na]$^+$). Calcd. for $C_{20}H_{23}NO_3Si$: M = 353; HRMS (ESI$^+$): Calcd. for $^{12}C_{20}{}^{1}H_{23}{}^{14}N_1{}^{23}Na_1{}^{16}O_3{}^{28}Si_1$: 376.1345. Found: *m*/*z* 376.1330.

Reaction of ethyl 2-acetylamino-3-phenylethynylazulene-1-carboxylate with Cu(OTf)$_2$

A mixture of **5a** (0.100 g, 0.28 mmol), Cu(OTf)$_2$ (0.100 g, 0.28 mmol) in CH_2Cl_2 (6 mL) was heated at 40 °C for 3 h, then water was added. The mixture was extracted with CH_2Cl_2. The extract was dried over Na_2SO_4 and evaporated. Chromatography of the residue on silica gel with $CHCl_3$ gave **9** (0.009 g, 9%).

9: Red powder (from hexane-$CHCl_3$), mp 126-127.5 °C; ^{1}H NMR δ 1.52 (3H, t, *J* 7.0, CH_3), 2.36 (3H, s, $COCH_3$), 4.00 (2H, s, $COCH_2Ph$), 4.53 (2H, q, *J* 7.0, OCH_2), 7.10-7.16 (5H, m, Ph), 7.47 (1H, t, *J* 9.9, H-7), 7.57 (1H, t, *J* 9.9, H-5), 7.70 (1H, t, *J* 9.9, H-6), 8.62 (1H, d, *J* 9.9, H-4), 9.35 (1H, d, *J* 9.9, H-8), and 11.14 (1H, br s, NH); ν_{max}/cm^{-1} 1703, 1686, and 1649 (C=O). MS (ESI$^+$) *m*/*z* 398 ([M + Na]$^+$). Calcd. for $C_{23}H_{21}NO_4$: M = 375; HRMS (ESI$^+$): Calcd. for $^{12}C_{23}{}^{1}H_{21}{}^{14}N_1{}^{23}Na_1{}^{16}O_4$: 398.1368. Found: *m*/*z* 398.1353.

Reaction of ethyl 2-acetylamino-3-phenylethynylazulene-1-carboxylate with Pd/C

A mixture of **5a** (0.100 g, 0.28 mmol), 5% Pd/C (0.007 g, 0.0056 mmol), Na_2CO_3 (0.089 g, 0.84 mmol) in DMF (2 mL) was heated at 120 °C for 24 h, then water was added. The mixture was extracted with AcOEt. The extract was dried over Na_2SO_4 and evaporated. Chromatography of the residue on silica gel with benzene gave **6b** (0.030 g, 33%).

6b: Dark green needles (from hexane-$CHCl_3$), mp 123.7−124.6 °C (lit.,[13] mp 124 °C); ^{1}H NMR δ 1.53 (3H, t, *J* 6.8, CH_3), 4.53 (2H, q, *J* 7.0, OCH_2), 7.17 (1H, s, H-1), 7.31 (1H, t, *J* 9.9, H-8), 7.45-7.49 (3H, m, H-*m*, *p*-Ph), 7.51 (1H, t, *J* 9.9, H-7), 7.65 (1H, dd, *J* 9.9 and 9.6, H-6), 7.69 (2H, d, *J* 7.0 and 1.5, H-*o*-Ph), 8.68 (1H, d, *J* 9.9, H-9), 9.09 (1H, br s, NH), and 9.48 (1H, d, *J* 9.6, H-5); ^{13}C NMR δ 14.11, 59.75, 97.45, 98.21, 124.35, 124.61, 126.51, 127.24, 127.36, 129.09, 131.99, 132.67, 133.46, 134.55, 134.96, 139.76, 144.59, 148.81, and 165.24; ν_{max}/cm^{-1} 3486 (NH) and 1687 (C=O). MS (ESI$^+$) *m*/*z* 380 ([M + Na]$^+$). Calcd. for $C_{23}H_{21}NO_3$: M = 357; HRMS (ESI$^+$): Calcd. for $^{12}C_{23}{}^{1}H_{21}{}^{14}N_1{}^{23}Na_1{}^{16}O_3$: 380.1263. Found: *m*/*z* 380.1240.

Reaction of ethyl 2-acetylamino-3-phenylethynylazulene-1-carboxylate with PdCl$_2$(PPh$_3$)$_2$

A mixture of **5a** (0.0728 g, 0.207 mmol), $PdCl_2(PPh_3)_2$ (0.0098 g, 0.0014 mmol), and Et_3N (0.02 g, 0.28 mmol) in DMF (2 mL) was heated at 100 °C for 24 h, then water was added. The mixture was extracted with AcOEt. The extract was dried over Na_2SO_4 and evaporated. Chromatography of the residue silica gel

with benzene gave **6b** (0.0526 g, 72%).

Biological assay

HeLa S3 cells were obtained from AIST and used after cultivation. The cultivated HeLa S3 cells were cellcounted and the culture fluid was prepared to the cell consistency of 2 x 10^4 cell/mL. The compounds were added to the medium in DMSO solutions. To the aliquot of the culture fluid, which was incubated for 3 h at 37 °C, the test sample was added and then the culture fluid was incubated for 72 h. To the culture fluid, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution was added, and incubated for 4 h. Then the sample was centrifuged at 3000 rpm for 10 min at 4 °C, and the solvent was evaporated. Then DMSO was added to obtained mixture. The MTT-formazan was dissolved by plate-mixing and OD540 was measured. The rate of outlive determined to refer with un-dosed control. Dose-response curve was drawn up and IC_{50} was pursued. Every experiment in the cytotoxic assay was replicated four times in order to define the IC values.

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