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Aszonapyrone A Isolated from *Neosartorya spinosa* IFM 47025 Inhibits the NF-κB Signaling Pathway Activated by Expression of the Ependymoma-Causing Fusion Protein ZFTA-RELA

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Abstract

Ependymoma is a rare and chemotherapy-resistant brain tumor, which has resulted in a delay in the development of drugs to treat it. A subclass of supratentorial ependymomas (ST-EPN), designated ST-EPN-zinc finger-translocation-associated (ZFTA, ST-EPN-ZFTA), exhibits the expression of a fusion protein comprising ZFTA and v-*rel* reticuloendotheliosis viral oncogene homolog A (RELA), an effector transcription factor of the nuclear factor-kappa B (NF-κB) pathway (ZFTA-RELA). The expression of ZFTA-RELA results in the hyperactivation of the oncogenic NF-κB signaling pathway, which ultimately leads to the development of ST-EPN-ZFTA. To identify inhibitors of the NF-κB signaling pathway activated by the expression of ZFTA-RELA, we used a doxycycline-inducible ZFTA-RELA-expressing NF-κB reporter cell line and found that extracts of the fungus *Neosartorya spinosa* IFM 47025 exhibited NF-κB inhibitory activity. We identified eight compounds [aszonapyrone A (**2**), sartorypyrone A (**3**), epiheveadride (**4**), acetylaszonalenin (**5**), (*R*)-benzodiazepinedione (**6**), aszonalenin (**7**), sartorypyrone E (**8**) and (*Z*, *Z*)-*N*,*N*'-(1,2-bis[(4-methoxyphenyl)methylene]-1,2-ethanediyl)bis-formamide (**9**)] from *N. spinosa* IFM 47025 culture extract using a variety of chromatographic techniques. The structures of these compounds were identified through the analysis of various instrumental data (1D, 2D-NMR, MS, and optical rotation). The NF-κB responsive reporter assay indicated that compounds **2**, **3**, **5**, **7**, and **9** exhibited inhibitory activity. We further evaluated the

inhibitory activity of these compounds against the expression of endogenous NF-κB responsive genes (*CCND1*, *L1CAM*, *ICAM1*, and *TNF*) and found that compound **2** showed significant inhibitory activity. Further studies are required to elucidate the mechanism of action of compound **2**, which may serve as a lead compound for the development of a novel therapy for ST-EPN-ZFTA.

Keywords

Aszonapyrone A, *Neosartorya spinosa*, NF-κB Signaling Pathway, Ependymoma, ZFTA-RELA

1. Introduction

Fungi are an important biological resource known to produce numerous useful compounds [1]-[3]. In particular, fungi of the genus *Aspergillus* are widely distributed throughout the world and have been reported to produce a wide variety of secondary metabolites together with their sexual counterparts, *Neosartorya* [4] [5]. Our research group has been engaged in the search for useful compounds for drug development from the secondary metabolites produced by *Aspergillus* fungi [6].

Ependymoma is a rare brain tumor for which the development of effective pharmaceutical treatment has been hindered by the limited number of patients and their resistance to chemotherapy [7]. Consequently, there is a pressing need to identify useful compounds for the development of therapeutics for this disease. A subclass of supratentorial ependymomas (ST-EPN), designated ST-EPN-zinc finger-translocation associated (ZFTA, ST-EPN-ZFTA), exhibits the expression of a fusion protein comprising a nuclear localization protein with zinc finger domains, ZFTA, and v-*rel* reticuloendotheliosis viral oncogene homolog A (RELA), an effector transcription factor of the nuclear factor-kappa B (NF-κB) pathway (hereafter referred to as ZFTA-RELA) [8]-[10]. The NF-κB pathway is an intracellular signaling pathway that transmits extracellular stimuli, such as inflammatory cytokines, into cells [11] [12]. It is engaged in a number of processes, including acute and chronic inflammatory reactions, cell proliferation, apoptosis, and tumorigenesis. In the absence of extracellular stimuli, RELA binds to inhibitor of κB (IκB) and localizes to the cytoplasm. Upon stimulation, IκB is phosphorylated and subsequently degraded, resulting in RELA translocation to the nucleus and regulation of its target gene expression. Even in the absence of stimulation, ZFTA-RELA constitutively localizes to the nucleus and hyperactivates the oncogenic NF-κB signaling pathway through the expression of its target genes, which ultimately leads to the development of ST-EPN-ZFTA [8]. Given the lack of effective chemotherapy for ST-EPN-ZFTA, we and others have conducted screening to isolate compounds that inhibit NF-κB activity caused by the expression of ZFTA-RELA [6] [13]. In a previous report [6], we isolated *epi*-aszonalenin B (**1**) from the extract of

Aspergillus novofumigatus IFM 55215 as an inhibitor of NF-κB activation caused by ZFTA-RELA (**Figure 1**). The present study was carried out to isolate similar NF-κB activation inhibitors from the extract of the culture of *Neosartorya spinosa* IFM 47025.

2. Material and Methods

2.1. General

Ultra pressure liquid chromatography (UPLC)-Electrospray ionization (ESI)-mass spectrometry (MS) analysis was performed using a Xevo G2-XS QTof System (Waters Corporation, Milford, MA, USA). Low pressure liquid chromatography (LPLC) was used with an SSC-3160 pump (Senshu Scientific Co., Ltd., Tokyo, Japan) and ULTRA PACK ODS-SM-50A column (50 µm, 300 × 11 mm; Yamazen Corp., Osaka, Japan) equipped with a YRU-8883 RI-UV detector (Shimamuratach, Tokyo, Japan). High performance liquid chromatography (HPLC) analysis was performed using a Jasco PU-4185 pump, an AS-4150 auto sampler, and an MD-4140 PDA detector (JASCO Co. Ltd., Tokyo, Japan). The substrates were isolated using a Jasco PU-2089 pump (JASCO Co. Ltd.) and an MS-4010 PDA detector (JASCO Co. Ltd.) equipped with a CHF122SC fraction collector (ADVANTEC, Tokyo, Japan) or a Waters 1525 Binary HPLC Pump and a DOCOSIL column (5 µm, 6 mm × 250 mm, Senshu Scientific) maintained at a temperature of 30°C using a CHRATEC column oven and equipped with a Waters 2998 PDA detector. NMR spectra were obtained using a JEOL (Tokyo, Japan) ECZ500R spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C) in $CDCl_3$, MeOD-d_4, or dimethyl sulfoxide (DMSO)-d_6 solvent. ^{1}H and ^{13}C chemical shifts were referenced to solvent signals ($CDCl_3$: δ_H 7.26 and δ_C 77.0, MeOD-d_4: δ_H 3.31 and δ_C 49.0, DMSO-d_6: δ_H 2.50 and δ_C 39.5 ppm). Optical rotations were measured using a P-1000 photopolarimeter (JASCO Co. Ltd.).

2.2. Fungal Material

N. spinosa IFM 47025 [14] was provided by the Medical Mycology Research Center, Chiba University through the National BioResource Project, Japan.

2.3. Fermentation of *N. spinosa* IFM 47025 and Isolation of Compounds 2 - 9

Milled rice (Akitakomachi, 600 g) was soaked in tap water for 60 min and transferred to 50 mL tubes with 0.2 µm filtered lids. After sterilization, *N. spinosa* IFM 47025, which was cultured on a PDA agar medium (Nissui Pharmaceutical Co., Ltd.) for 7 days at 30˚C, was inoculated into the rice medium and incubated at 30˚C for 21 days. The culture was extracted two times with methanol overnight, and the methanol solution was filtered through filter paper. The supernatant was concentrated to obtain the residue (**Figure 2(a)**). This residue was added to an ethyl acetate (AcOEt) and 2.8 mol/L hydrochloric acid solution (3 mL) and extracted. This extract solution was combined with the first extract solution and

concentrated in an evaporator. This AcOEt extract (3.3 g) was dissolved in acetonitrile and defatted twice with *n*-hexane. Acetonitrile was removed by evaporator to obtain acetonitrile extract (2.7 g). The acetonitrile extract was added to methanol and centrifuged to obtain methanol-insoluble (535.3 mg) and methanol-soluble fraction. This solution was fractioned into five fractions (Fractions No. 1 - 5) using silica-gel column chromatography. The column was eluted in the following order: 300 mL of *n*-hexane/AcOEt (8:2), 100 mL of *n*-hexane/AcOEt (6:4), 100 mL of *n*-hexane/AcOEt (4:6), 100 mL of *n*-hexane/AcOEt (2:8), 100 mL of AcOEt/acetone (2:1), 100 mL of AcOEt/acetone (2:5), 100 mL of acetone, and methanol. Epiheveadride (4, 15.4 mg) was isolated from 63.0 mg of Fraction No. 1 (260.9 mg) with the HPLC system under the conditions described in the Supplementary Information. Fraction No. 3 was added to methanol to obtain aszonapyrone A (**2**, 179.9 mg) as methanol-insoluble and methanol-soluble fractions. Sartorypyrone A (**3**, 16.3 mg) was isolated from this methanol soluble fraction with LPLC and the HPLC system. Acetylaszonalenin (**5**, 250.5 mg) was isolated from Fraction No. 4 as methanol-insoluble with the addition of methanol. (*R*)-benzodiazepinedione (**6**, 3.9 mg) was isolated from the methanol-soluble fraction of Fraction No. 5. In the isolation of compounds from the large-scale cultures (**Figure 3**), the incubation and extraction were repeated three times under the same conditions as described above to obtain AcOEt extract (8.9 g). This was extracted with acetonitrile and *n*-hexane to obtain acetonitrile (MeCN) extract (6.4 g) and hexane extract (2.6 g), respectively. The methanol (MeOH) insoluble material generated when MeOH was added to the MeCN extract was washed several times with MeOH to obtain aszonalenin (**7**, 1.3 g). The MeOH-soluble fraction was further fractionated into six fractions using silica-gel column chromatography. The MeOH-insoluble material generated when MeOH was added to fraction No. 4 (1.7 g) was washed with MeOH to obtain acetylaszonalenin (**5**, 729.6 mg). Fraction No. 5 (243.2 mg) was further fractionated into 13 fractions using LPLC to obtain fractions No. 5-10 as sartorypyrone E (**8**, 15.9 mg). Also, (*R*)-benzodiazepine (**6**, 6.3 mg) and (*Z*, *Z*)-*N*, *N'*-[1,2-bis([4-methoxyphenyl]methylene)-1,2-ethanediyl]bis-formamide (**9**, 4.4 mg) were isolated from fractions No. 5 - 5 (17.8 mg) and No. 5 - 6 (11.3 mg) using HPLC. These isolated compounds were identified by the comparison of ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data (**Figure 4-11**), the analysis of 1D and 2D NMR data, high resolution MS analysis and optical rotations for the comparison of the stereochemistry of **6** and **8** with that of references [15]-[17].

Aszonapyrone A (2) HR-ESI-MS obsd 457.2951, calcd for $C_{28}H_{41}O_5$ $[M+H]^+$ 457.2954; ^{1}H NMR: δ ($CDCl_3$) 6.01 (1H, s, H-5'), 4.94 (1H, brs, H-16), 4.75 (1H, brs, H-16), 4.46 (1H, dd, *J* = 11.5, 5.2 Hz, H-3), 2.60 (2H, d, *J* = 6.9 Hz, H-15), 2.39 (1H, dd, *J* = 9.2, 5.7 Hz, H-14), 2.33 (1H, d, *J* = 11.5 Hz, H-12), 2.17 (3H, s, H-7'), 2.05 (3H, s, H-2''), 1.96 (1H, m, H-7), 1.95 (1H, m, H-1), 1.70 (1H, m, H-12), 1.65 (2H, m, H-2), 1.61 (1H, m, H-11), 1.60 (1H, m, H-6), 1.45 (1H, m, H-7), 1.41 (2H, m, H-6), 1.34 (1H, m, H-11), 1.10 (1H, dd, *J* = 12.3, 2.3 Hz, H-9), 1.03 (1H, dt, *J* =

12.6, 4.0 Hz, H-1), 0.92 (1H, d, J = 11.5 Hz, H-5), 0.84 (3H, s, H-18), 0.84 (3H, s, H-19), 0.84 (3H, s, H-20), 0.76 (3H, s, H-17).; ^{13}C NMR: δ ($CDCl_3$) 171.5 (C-1''), 167.3 (C-2'), 159.6 (C-6'), 149.7 (C-13), 106.5 (C-16), 103.1 (C-3'), 81.2 (C-3), 59.8 (C-9), 55.3 (C-5), 54.4 (C-14), 40.3 (C-8) , 40.1 (C-7), 37.8 (C-4), 37.4 (C-10), 38.2 (C-1), 38.2 (C-12), 28.0 (C-19), 23.6 (C-2), 23.4 (C-11), 21.4 (C-2''), 19.7 (C-7'), 18.7 (C-15), 18.7 (C-6), 16.4 (C-18), 16.3 (C-20), 15.0 (C-17).

Sartorypyrone A (3) HR-ESI-MS obsd 457.2952, calcd for $C_{28}H_{41}O_5$ $[M+H]^+$ 457.2954; ^{1}H NMR: δ ($CDCl_3$) 5.89 (1H, brs, H-5'), 5.31 (1H, dd, J = 7.5, 6.3 Hz, H-14), 5.04 (1H, dd, J = 6.9, 5.7 Hz, H-9), 4.88 (1H, brs, H-18), 4.64 (1H, dd, J = 8.6, 4.6 Hz, H-3), 4.61 (1H, brs, H-18), 3.21 (2H, d, J = 7.5 Hz, H-15), 2.30 (1H, dt, J = 13.8, 5.7 Hz, H-1), 2.18 (3H, s, H-7'), 2.09 (2H, m, H-11), 2.07 (3H, s, H-2''), 2.06 (1H, m, H-7), 2.05 (2H, m, H-12), 2.03 (1H, m, H-1), 1.83 (1H, m, H-2), 1.77 (3H, s, H-16), 1.76 (1H, m, H-7), 1.71 (1H, dd, J = 10.3, 2.3 Hz, H-5), 1.57 (2H, m, H-6), 1.57 (3H, s, H-17), 1.55 (1H, m, H-2), 0.93 (3H, s, H-19), 0.77 (3H, s, H-20); ^{13}C NMR: δ ($CDCl_3$) 171.1 (C-1''), 166.3 (C-2'), 165.8 (C-4'), 160.1 (C-6'), 146.6 (C-10), 139.4 (C-13), 135.7 (C-8), 123.9 (C-9), 120.4 (C-14), 109.2 (C-18), 101.2 (C-3'), 100.8 (C-5'), 79.0 (C-3), 51.0 (C-5), 39.6 (C-12), 39.2 (C-4), 38.2 (C-7), 31.8 (C-1), 28.6 (C-2), 26.2 (C-11), 26.0 (C-19), 23.5 (C-6), 22.6 (C-15), 21.3 (C-2''), 19.7 (C-7'), 17.4 (C-20), 16.3 (C-16), 16.0 (C-17).

Epiheveadride (4) HR-ESI-MS obsd 331.1182, calcd for $C_{18}H_{19}O_6$ $[M+H]^+$ 331.1182; ^{1}H NMR: δ ($CDCl_3$) 3.14 (2H, m, H-3), 2.88 (1H, dd, J = 13.2, 2.9 Hz, H-7), 2.45 (1H, m, H-9), 2.23 (2H, m, H-4), 2.16 (1H, m, H-3'), 2.10 (1H, m, H-8), 2.10 (1H, m, H-2''), 1.76 (1H, t, J = 12.6 Hz, H-7), 1.53 (1H, m, H-3'), 1.14 (2H, m, H-2'), 1.08 (3H, dd, J = 7.5, 6.9 Hz, H-1''), 1.01 (1H, m, H-2''), 0.86 (3H, t, J = 7.5 Hz, H-1'); ^{13}C NMR: δ ($CDCl_3$) 165.2 (C-11), 165.2 (C-12), 164.8 (C-13), 164.0 (C-10), 146.1 (C-2), 146.0 (C-1), 145.0 (C-6), 144.1 (C-5), 47.8 (C-8), 40.8 (C-9), 31.5 (C-3'), 27.9 (C-7), 23.4 (C-2''), 22.0 (C-4), 21.7 (C-2'), 21.4 (C-3), 13.9 (C-1'), 12.9 (C-1'').

Acetylaszonalenin (5) HR-ESI-MS obsd 416.1974, calcd for $C_{25}H_{26}N_3O_3$ $[M + H]^+$ 416.1974; ^{1}H NMR: δ ($CDCl_3$) 8.00 (1H, d, J = 8.0 Hz, H-7), 7.68 (1H, dd, J = 7.5, 1.0 Hz, H-21), 7.40 (1H, dt, J = 8.5, 1.0 Hz, H-19), 7.26 (1H, dt, J = 8.5, 1.0 Hz, H-6), 7.23 (1H, d, J = 7.5 Hz, H-4), 7.16 (1H, t, J = 7.5 Hz, H-20), 7.08 (1H, t, J = 7.5 Hz, H-5), 6.91 (1H, d, J = 8.0 Hz, H-18), 5.88 (1H, s, H-2), 5.88 (1H, dd, J = 17.0, 10.5 Hz, H-2'), 5.13 (1H, d, J = 10.5 Hz, H-1'), 5.10 (1H, d, J = 17.0 Hz, H-1'), 3.89 (1H, t, J = 8.5 Hz, H-11), 3.38 (1H, dd, J = 13.5, 8.5 Hz, H-10), 2.59 (3H, s, 1-CO<u>CH$_3$</u>), 2.43 (1H, dd, J = 14.0, 9.0 Hz, H-10), 1.18 (3H, s, H-5'), 0.98 (3H, s, H-4'); ^{13}C NMR: δ ($CDCl_3$) 170.5 (1-<u>CO</u>CH$_3$), 169.3 (C-17), 166.4 (C-13), 143.3 (C-2'), 142.0 (C-8), 133.7 (C-15), 133.4 (C-9), 132.4 (C-19), 131.1 (C-21), 128.9 (C-6), 127.5 (C-14), 125.5 (C-20), 124.2 (C-4), 124.0 (C-5), 120.4 (C-18), 119.2 (C-7), 114.3 (C-1'), 82.1 (C-2), 60.1 (C-3), 56.5 (C-11), 40.7 (C-3'), 30.6 (C-10), 24.1 (1-CO<u>CH$_3$</u>), 22.8 (C-5'), 22.5 (C-4').

(*R*)-benzodiazepinedione (6) $[\alpha]_D^{25}$ −41.3° (c 0.3, MeOH), HR-ESI-MS obsd 304.1084, calcd for $C_{18}H_{14}N_3O_2$ $[M-H]^-$ 304.1086; ^{1}H NMR: δ ($CDCl_3$) 7.78 (1H,

d, $J = 8.0$ Hz, H-21), 7.52 (1H, t, $J = 8.0$ Hz, H-19), 7.42 (1H, d, $J = 7.5$ Hz, H-4), 7.30 (1H, d, $J = 8.0$ Hz, H-7), 7.23 (1H, t, $J = 7.5$ Hz, H-20), 7.13 (1H, s, H-2), 7.12 (1H, d, $J = 8.5$ Hz, H-18), 7.04 (1H, t, $J = 7.5$ Hz, H-6), 6.93 (1H, t, $J = 7.5$ Hz, H-5), 4.09 (1H, dd, $J = 9.0, 4.0$ Hz, H-11), 3.38 (1H, dd, $J = 15.0, 6.0$ Hz, H-10), 3.13 (1H, dd, $J = 15.5, 9.0$ Hz, H-10); ^{13}C NMR: δ ($CDCl_3$) 173.7 (C-17), 170.9 (C-13), 138.1 (C-15), 138.1 (C-8), 134.2 (C-19), 131.7 (C-21), 128.4 (C-14), 127.1 (C-9), 125.8 (C-20), 124.8 (C-2), 122.5 (C-18), 122.3 (C-6), 119.8 (C-5), 118.8 (C-4), 112.4 (C-7), 110.5 (C-3), 54.5 (C-11), 25.0 (C-10).

Aszonalenin (7) HR-ESI-MS obsd 374.1872, calcd for $C_{23}H_{24}N_3O_2$ $[M+H]^+$ 374.1872; ^{1}H NMR: δ ($CDCl_3$) 7.82 (1H, dt, $J = 7.5, 1.2$ Hz, H-21), 7.45 (1H, td, $J = 8.0, 1.4$ Hz, H-19), 7.22 (1H, t, $J = 7.5$ Hz, H-20), 7.15 (1H, d, $J = 6.9$ Hz, H-4), 7.08 (1H, dt, $J = 7.5, 1.2$ Hz, H-6), 6.93 (1H, d, $J = 8.0$ Hz, H-18), 6.72 (1H, dt, $J = 7.5, 1.2$ Hz, H-5), 6.62 (1H, d, $J = 7.9$ Hz, H-7), 6.11 (1H, dd, $J = 17.8, 10.9$ Hz, H-2’), 5.58 (1H, s, H-2), 5.12 (1H, d, $J = 10.9$ Hz, H-1’), 5.11 (1H, d, $J = 17.2$ Hz, H-1’), 4.00 (1H, brt, $J = 8.1$ Hz, H-11), 3.47 (1H, dd, $J = 13.8, 7.5$ Hz, H-10), 2.42 (1H, dd, $J = 14.0, 9.0$ Hz, H-10), 1.14 (3H, s, H-5’), 1.05 (3H, s, H-4’); ^{13}C NMR: δ ($CDCl_3$) 169.9 (C-17), 166.9 (C-13), 149.0 (C-15), 143.7 (C-2’), 134.1 (C-8), 132.7 (C-19), 131.2 (C-9), 131.2 (C-21), 128.6 (C-6), 126.8 (C-14), 125.3 (C-4), 124.9 (C-20), 120.6 (C-18), 118.4 (C-5), 109.2 (C-7), 114.3 (C-1’), 81.7 (C-2), 60.7 (C-3), 57.0 (C-11), 41.4 (C-3’), 33.4 (C-10), 22.7 (C-4’), 22.5 (C-5’).

Sartorypyrone E (8) $[\alpha]_D^{25}$ −8.5˚ (c 0.8, MeOH), HR-ESI-MS obsd 431.2792, calcd for $C_{26}H_{39}O_5$ $[M-H]^-$ 431.2797; ^{1}H NMR: δ ($CDCl_3$) 5.97 (1H, s, H-5), 5.15 (1H, t, $J = 7.5$ Hz, H-8), 5.15 (1H, t, $J = 7.5$ Hz, H-16), 5.07 (1H, t, $J = 7.0$ Hz, H-12), 3.22 (1H, dd, $J = 10.5, 2.0$ Hz, H-20), 3.05 (2H, d, $J = 6.5$ Hz, H-7), 2.23 (1H, m, H-18), 2.18 (3H, m, H-22), 2.04 (1H, m, H-10), 2.04 (2H, m, H-15), 2.04 (1H, m, H-11), 1.97 (1H, m, H-11), 1.95 (1H, m, H-10), 1.95 (2H, m, H-14), 1.95 (1H, m, H-18), 1.72 (3H, s, H-23), 1.68 (1H, m, H-19), 1.60 (3H, s, H-25), 1.56 (3H, s, H-24), 1.34 (1H, m, H-19), 1.14 (3H, s, H-27), 1.11 (3H, s, H-26); ^{13}C NMR: δ ($CDCl_3$) 168.3 (C-2), 167.5 (C-4), 161.7 (C-6), 136.4 (C-9), 136.0 (C-17), 135.9 (C-13), 125.6 (C-16), 125.4 (C-12), 122.6 (C-8), 103.1 (C-3), 101.6 (C-5), 79.1 (C-20), 73.8 (C-21), 40.8 (C-10), 40.8 (C-14), 38.0 (C-18), 30.9 (C-19), 27.7 (C-11), 27.5 (C-15), 25.6 (C-27), 25.0 (C-26), 22.8 (C-7), 19.6 (C-22), 16.3 (C-23), 16.2 (C-25), 16.1 (C-24).

(*Z,Z*)-*N,N’*-[1,2-bis[(4-methoxyphenyl)methylene]-1,2-ethanediyl]bis-foramide (9) HR-ESI-MS obsd 351.1345, calcd for $C_{20}H_{19}N_2O_4$ $[M-H]^-$ 351.1345; ^{1}H NMR: δ (DMSO-d_4) 9.59 (1H, s, NH, *cis*), 9.47 (1H, s, NH, *cis*), 9.38 (1H, d, $J = 11$ Hz, NH, *trans*), 9.31 (1H, d, $J = 11$ Hz, NH, *trans*), 8.20 (1H, s, H-9/9’, *cis*), 8.19 (1H, s, H-9/9’, *cis*), 7.88 (1H, brd, H-9/9’, *trans*), 7.80 (1H, d, $J = 11$ Hz, H-9/9’, *trans*), 7.50-7.42 (m, H-4/4’), 6.96-6.90 (m, H-5/5’), 6.56 (1H, s, H-2/2’), 6.54 (1H, s, H-2/2’), 6.50 (1H, s, H-2/2’), 3.78 (3H, s, 5-OCH_3), 3.78 (3H, s, 5’-OCH_3); ^{13}C NMR: δ (DMSO-d_4) 164.5 (C-9, *trans*), 160.3 (C-9, *cis*), 160.2 (C-9, cis), 158.8 (C-6/6’), 158.6 (C-6/6’), 158.6 (C-6/6’), 158.3 (C-6/6’), 131.8 (C-3/3’), 131.6 (C-3/3’), 130.7 (C-1/1’), 130.6 (C-4/4’), 130.6 (C-8/8’), 130.5 (C-4/4’), 130.5 (C-8/8’),

130.4 (C-4/4’), 130.4 (C-8/8’), 130.3 (C-4/4’), 130.3 (C-8/8’), 130.2 (C-1/1’), 123.2 (C-2/2’), 122.1 (C-2/2’), 121.8 (C-2/2’), 120.3 (C-2/2’), 114.1 (C-5/5’), 114.1 (C-5/5’), 114.1 (C-7/7’), 114.1 (C-7/7’), 113.9 (C-5/5’), 113.9 (C-7/7’), 113.8 (C-5/5’), 113.8 (C-7/7’), 55.1 (5-O$\underline{CH_3}$), 55.1 (5’-O$\underline{CH_3}$).

2.4. UPLC-ESI-MS Analysis

The samples were analyzed using UPLC-ESI-MS under the conditions that were previously described [6].

2.5. Cell Culture

The NF-*κ*B responsive luciferase reporter cell line 6E8 was previously established [6] and was cultured in Dulbecco’s modified Eagle medium (Nacalai Tesque) supplemented with 4.5 g/L glucose, 110 mg/L sodium pyruvate, 10% (v/v) fetal bovine serum (Biowest), and antibiotic-antimycotic solution (Nacalai Tesque), hereafter referred to as culture media, at 37˚C in 5% CO_2 conditions. The expression of ZFTA-RELA in 6E8 cells was induced by the addition of 150–300 ng/mL doxycycline (DOX, Clontech) to the culture medium.

2.6. Luciferase and Relative Viable Cell Count Assays

For the luciferase assay, cells were seeded at 25,000 cells/well in 96-well clear-bottom plates (Thermo Fisher Scientific) and cultured in the culture media supplemented with or without the indicated fungal extracts overnight. To induce the expression of ZFTA-RELA in 6E8, DOX was added to the culture media at the indicated concentration. The luciferase assay was conducted using the TriStar LB 941 instrument (Berthold) and the following reaction buffer: 100 mM Tris-HCl (pH7.8, nacalai tesque), 5 mM $MgCl_2$ (Wako Pure Chemical), 250 μM coenzyme A trilithium salt (Oriental Yeast), 150 μM adenosine triphosphate (nacalai tesque), 150 μg/mL *D*-luciferin potassium salt (Cayman Chemical), 0.5 mM dithiothreitol (Nacalai tesque), 50 μM ethylenediaminetetraacetic acid (pH8.0, Dojindo), and 0.1% Triton X-100 (Sigma-Aldrich).

The relative viable cell number was quantified using the Cell Count Reagent SF (Nacalai Tesque) in accordance with the manufacturer’s instructions using the TriStar LB 941 (Berthold) instrument. IC_{50} was determined using Prism 9 (GraphPad).

2.7. Quantitative Reverse-Transcription PCR (qPCR)

Total RNAs were purified using NucleoSpin RNA (Macherey-Nagel) and reverse-transcribed into cDNAs by ReverTra Ace (Toyobo), following the manufacturers’ instructions. qPCR was performed using TB Green Premix Ex Taq II (TaKaRa) on a StepOne real-time PCR (Thermo Fisher Scientific). The relative mRNA expression level was determined by the $2^{-\Delta\Delta Ct}$ method using *TATA box binding protein* (*TBP*) as an endogenous control to normalize the samples [6] [18]. The primers used in the present study were as follows: (1) *Cyclin D1* (*CCND1*) F, 5’-CAA

TGA CCC CGC ACG ATT TC-3'; R, 5'-CAT GGA GGG CGG ATT GGA A-3′, (2) *intercellular adhesion molecule 1* (*ICAM1*) F, 5'-ATG CCC AGA CAT CTG TGT CC-3'; R, 5'-GGG GTC TCT ATG CCC AAC AA-3', (3) *L1 cell adhesion molecule* (*L1CAM*) F, 5'-TGT CAT CAC GGA ACA GTC TCC-3'; R, 5'-CTG GCA AAG CAG CGG TAG AT-3', (4) *TBP* F, 5'-GAG CCA AGA GTG AAG AAC AGT C-3'; R, 5'-GCT CCC CAC CAT ATT CTG AAT CT-3', and (5) *tumor necrosis factor* (*TNF*) F, 5'-CCT CTC TCT AAT CAG CCC TCT G-3'; R, 5'-GAG GAC CTG GGA GTA GAT GAG-3'.

2.8. Statistics

The means of three or more groups were compared with a one-way analysis of variance (ANOVA) with Tukey's or Dunnett's post-hoc tests using Prism 9 (GraphPad). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Isolation of Compounds from *N. spinosa* IFM 47025

Previously, we established the HEK293-derived cell line 6E8, which exhibits ZFTA-RELA expression and NF-κB responsive luciferase activity in a DOX dose-dependent manner [6]. To identify fungal culture extracts that contain NF-κB activity inhibitors, a screening test was conducted using 13 different fungal culture extracts at 5 μg/mL and 6E8 cells (data not shown). The fungal culture extracts were selected based on their inhibitory activity against NF-κB responsive luciferase activity and their cytotoxic activity in 6E8 cells. The culture extract of *N. spinosa* IFM 47025 inhibited the NF-κB-responsive luciferase activity by 57% compared to the DMSO control cells, in which the expression of ZFTA-RELA was induced with DOX but no fungal extracts were added. This extract also showed a relatively low cytotoxic activity on 6E8 cells, with an 11% reduction in viable cell numbers compared to the DMSO control cells. Therefore, rice cultures of this fungus were extracted with MeOH and fractionated using various organic solvents to examine their NF-κB inhibitory activity (**Figure 2(a) and (b)**). The search for compounds from the five fractions obtained by chromatographic fractionation revealed the isolation of two known compounds, aszonapyrone A (**2**) [19] [20] and sartorypyrone A (**3**) [20], from the fraction No.3 (**Figure 1** and **2**). Additionally, the three known compounds, epi-heveadride (**4**) [21], acetylaszonalenin (**5**) [22] [23] and (*R*)-benzodiazepinedione (**6**) [22], were isolated (**Figure 1**). Although the fraction No. 5 showed NF-κB activation inhibitory activity, an active compound could not be identified due to its low content in a small-scale culture. Therefore, a larger-scale culture was conducted to isolate more active compounds. As a result, three additional compounds {aszonalenin (**7**) [23], sartorypyrone E (**8**) [15], and (*Z*,*Z*)-*N*, *N'*-[1,2-bis([4-methoxyphenyl]methylene)-1, 2-ethanediyl]bis-formamide (**9**) [24]} were isolated along with the aforementioned compounds **5** and **6** (**Figure 3**).

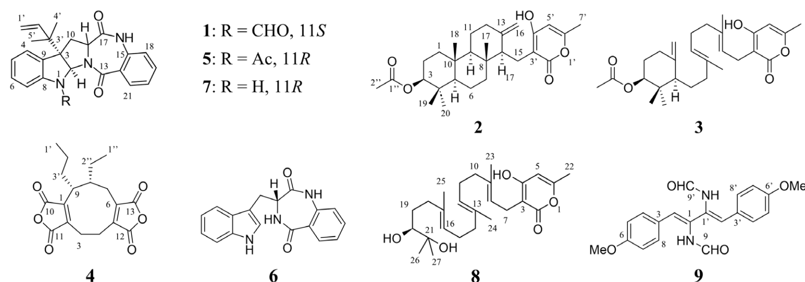

Figure 1. Structures of isolated compounds **2** - **9** from *N. spinosa* IFM 47025 and *epi*-aszonalenin B (**1**).

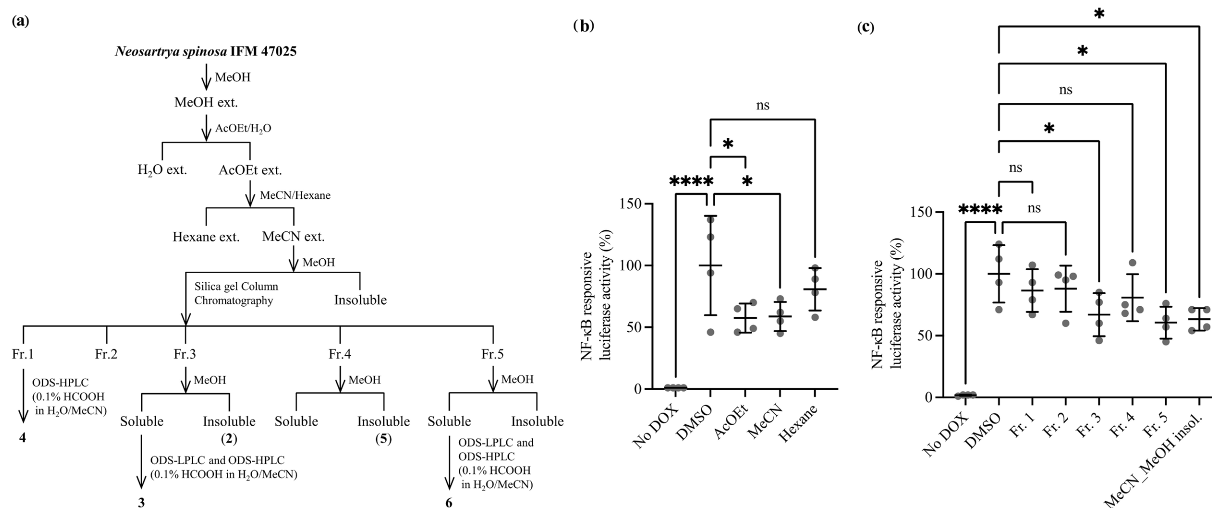

Figure 2. Purification of inhibitors for ZFTA-RELA-induced NF-*κ*B responsive luciferase activity from *N. spinosa* IFM 47025 extracts. (a) Isolation scheme of compounds from *N. spinosa* IFM 47025. (b) and (c) Effect of extracts and fractions from culture of *N. spinosa* IFM 47025 on NF-*κ*B responsive luciferase activity induced by the expression of ZFTA-RELA in 6E8 cells. Each extract and fraction were added at a final concentration of 5 μg/mL in 0.05% DMSO. The mean value of the DMSO control was set to 100%. N = 4 each. Data shown are the mean ± standard deviation (SD). The dots on the graph are the relative luciferase activity of individual samples. ns, not significant; *, $p < 0.05$; ****, $p < 0.0001$.

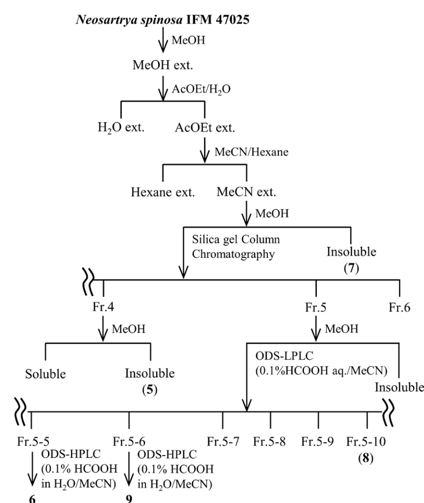

Figure 3. Isolation and purification of compounds **5** - **9** from *N. spinosa* IFM 47025 extracts of large-scale culture.

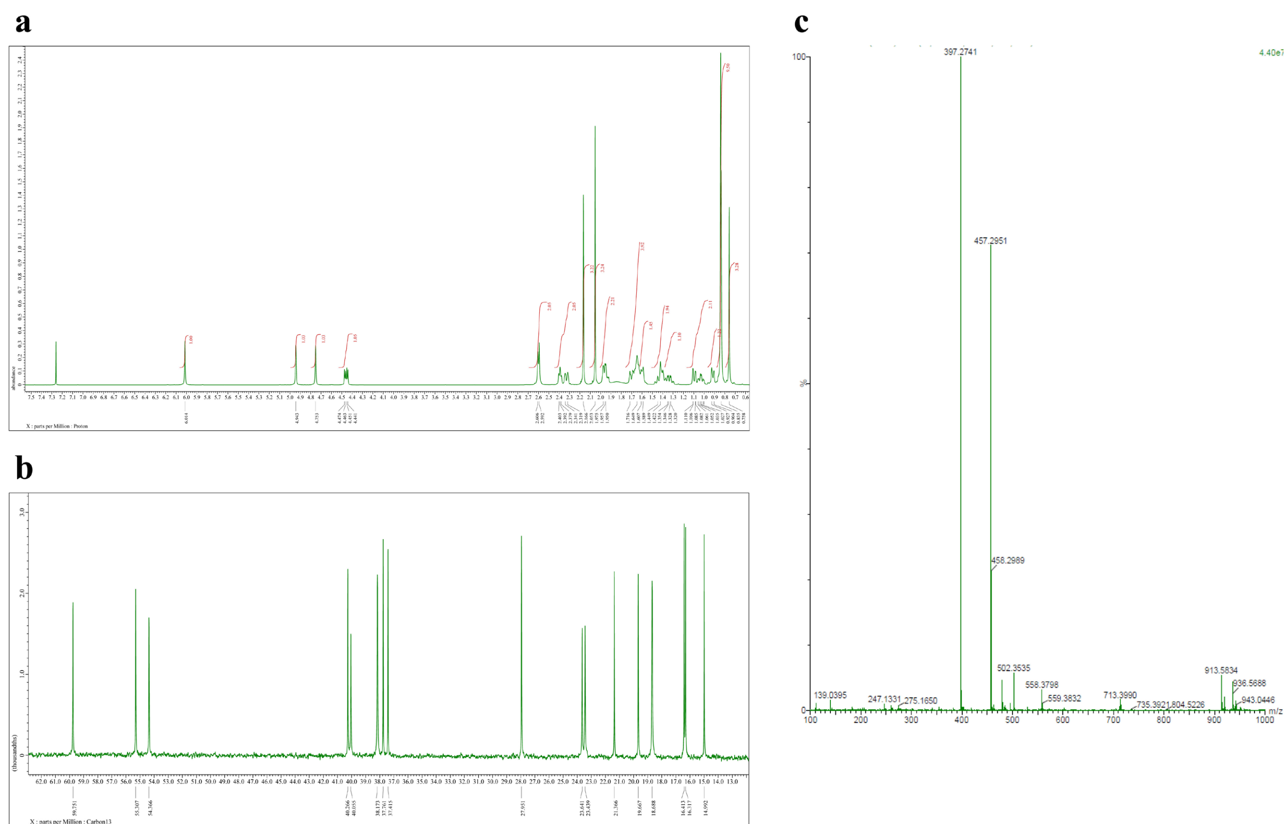

Figure 4. ^{1}H, ^{13}C NMR and MS spectra of aszonapyrone (**2**).

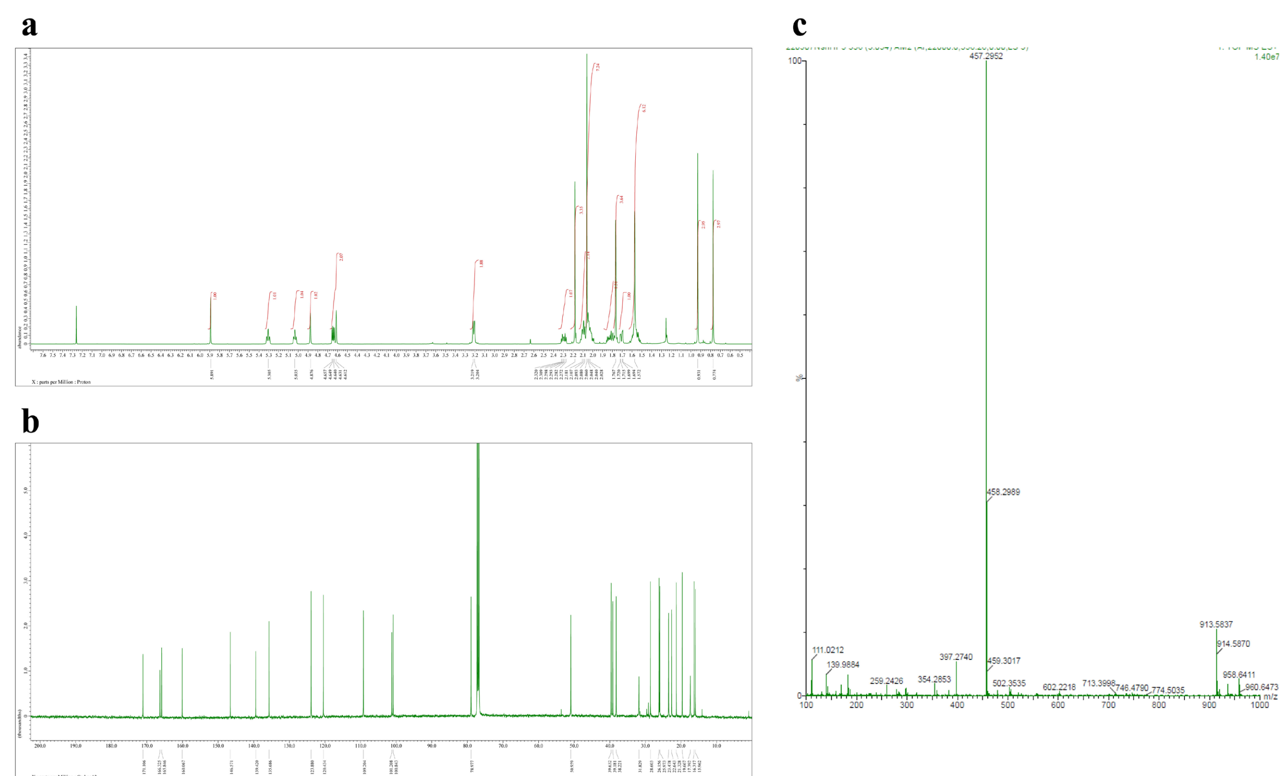

Figure 5. ^{1}H, ^{13}C NMR and MS spectra of sartorypyrone A (**3**).

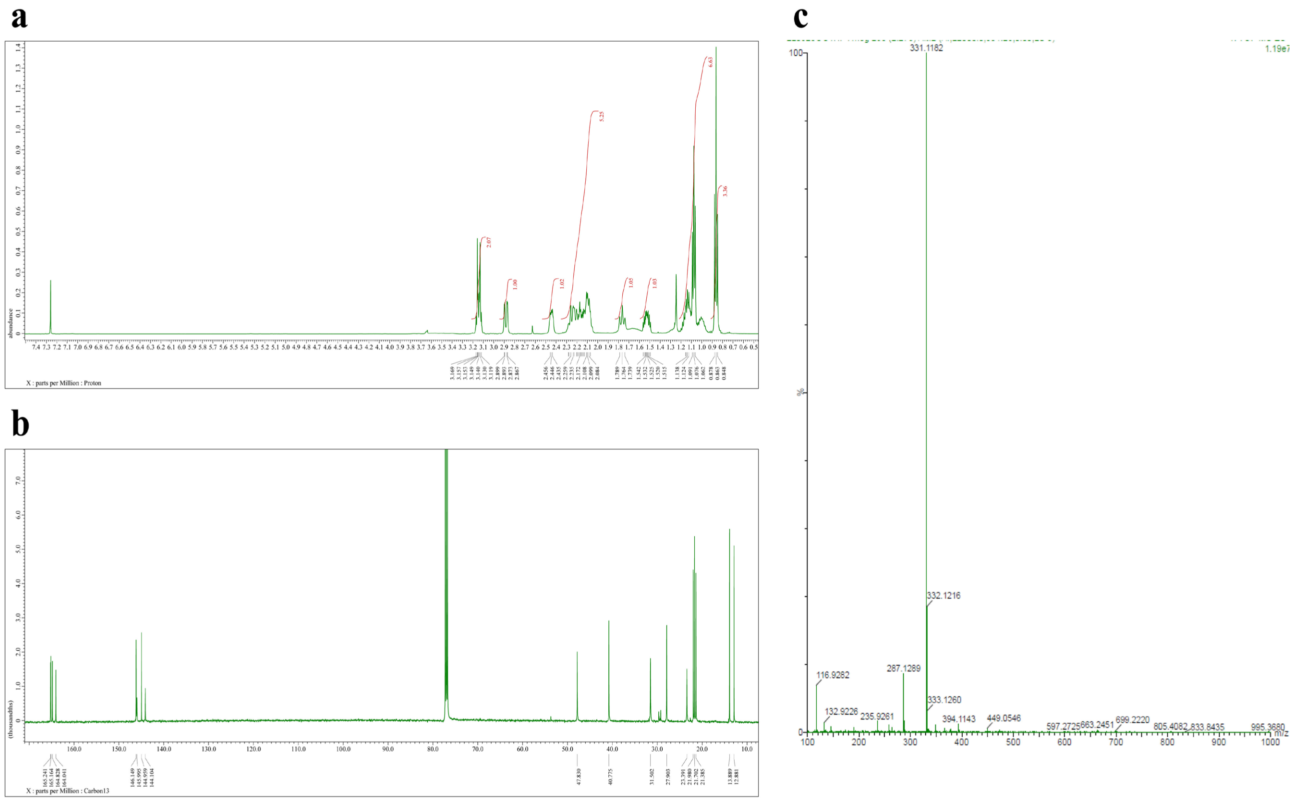

Figure 6. ^{1}H, ^{13}C NMR and MS spectra of epiheveadride (**4**).

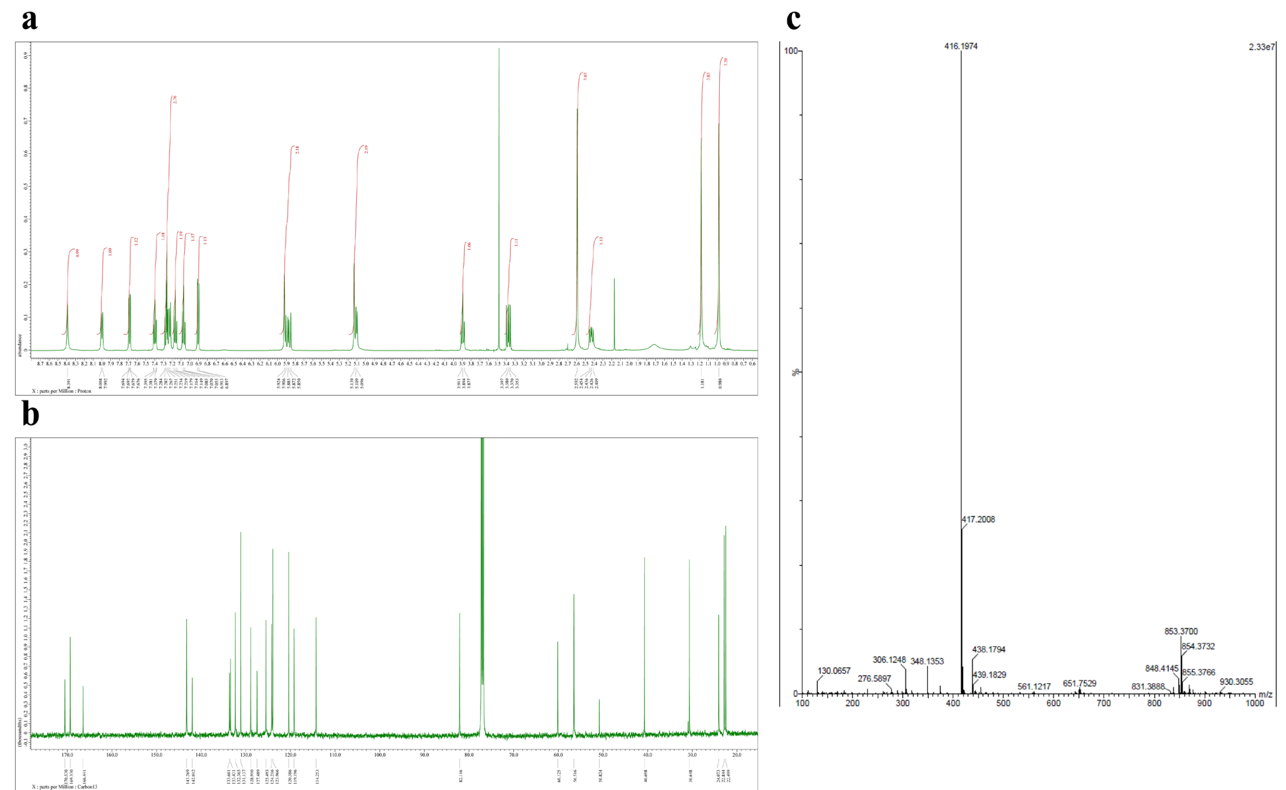

Figure 7. ^{1}H, ^{13}C NMR and MS spectra of acetylaszonalenin (**5**).

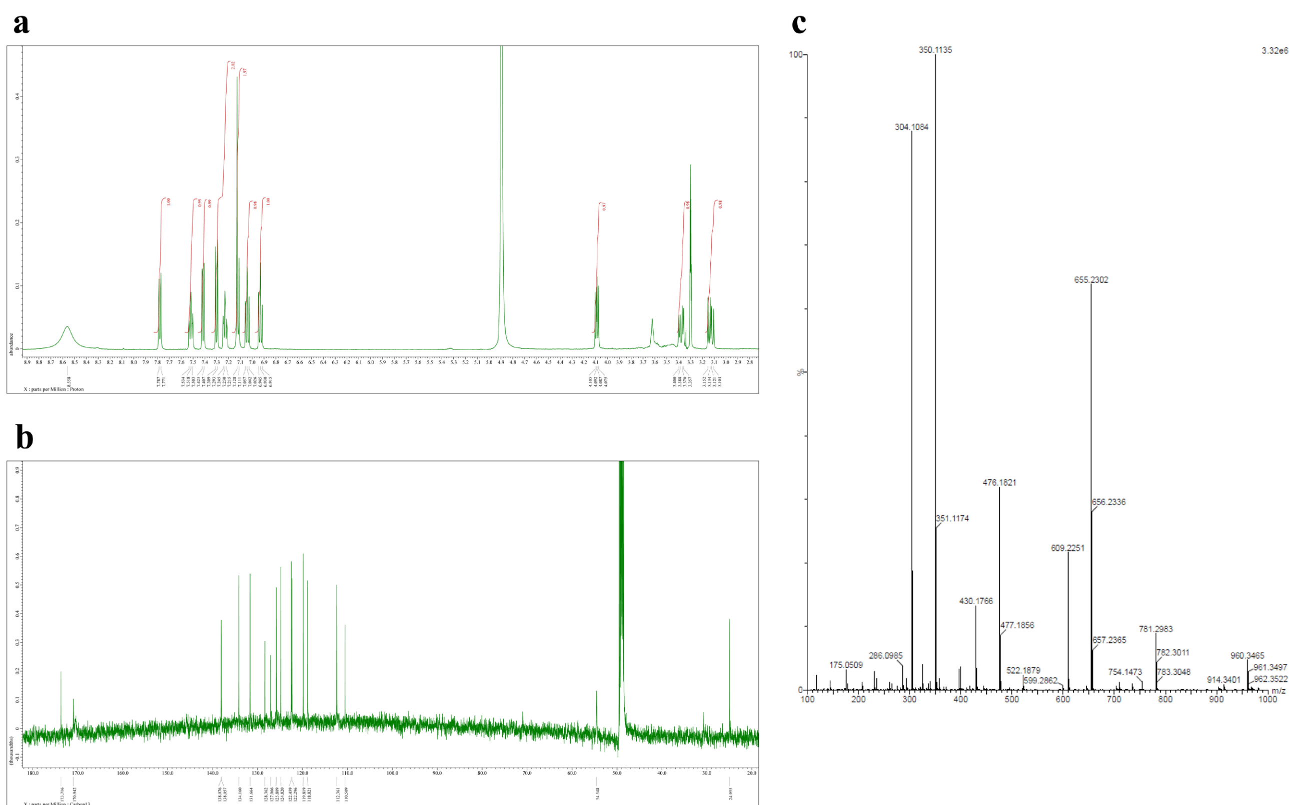

Figure 8. ^{1}H, ^{13}C NMR and MS spectra of (*R*)-benzodiazepinedione (**6**).

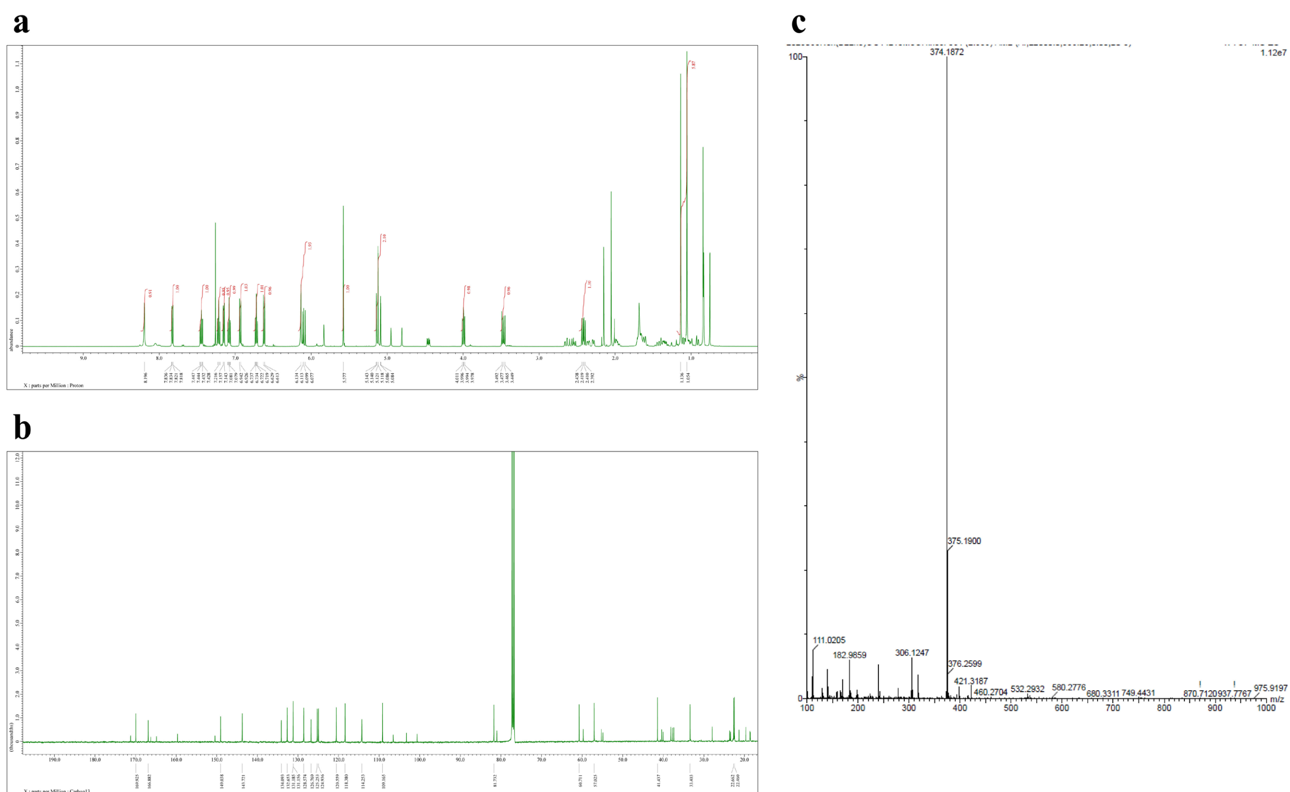

Figure 9. ^{1}H, ^{13}C NMR and MS spectra of aszonalenin (**7**).

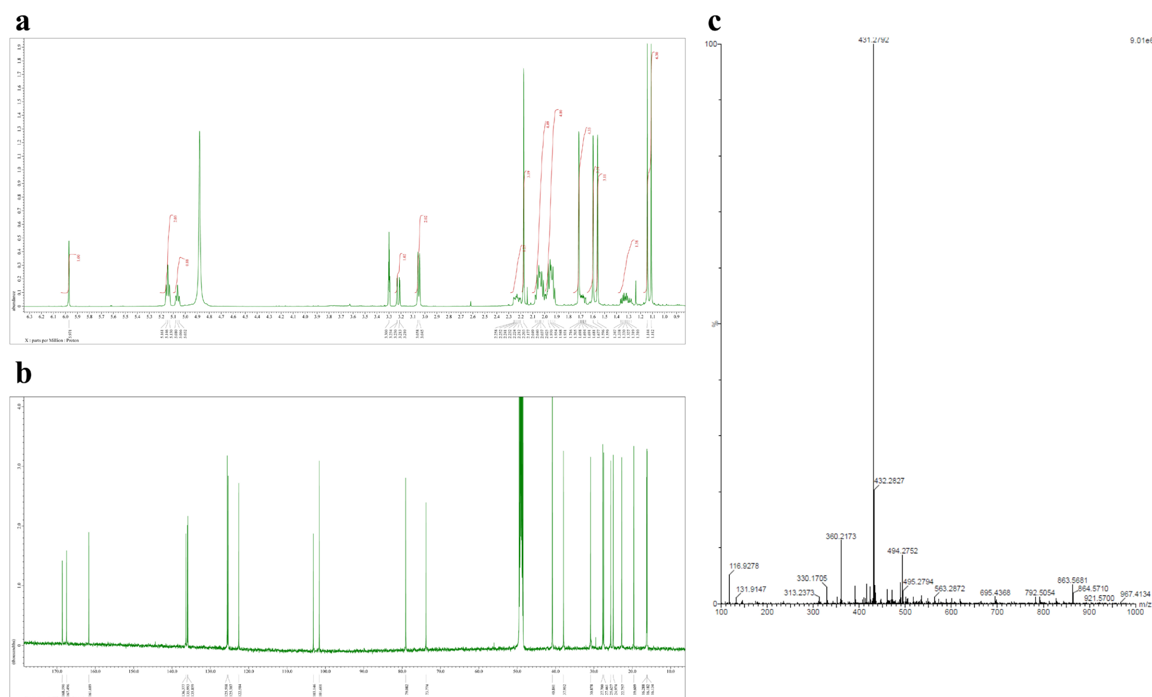

Figure 10. ^{1}H, ^{13}C NMR and MS spectra of sartorypyrone E (**8**).

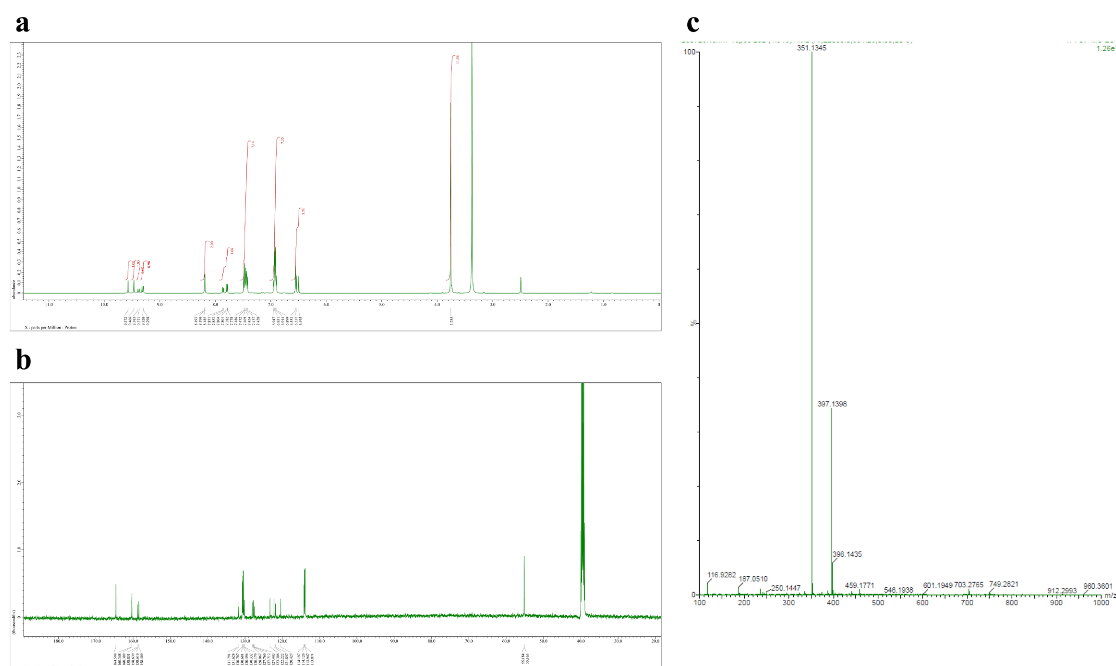

Figure 11. ^{1}H, ^{13}C NMR and MS spectra of (*Z*,*Z*)-*N*,*N'*-[1,2-bis[(4-methoxyphenyl)methylene]-1,2-ethanediyl]bis-formamide (**9**).

3.2. NF-*κ*B Activation Inhibitory Activity of Isolated Compounds

To investigate the inhibitory activity of **2**-**9** from *N. spinosa* IFM 47025 on the NF-*κ*B-responsive luciferase activity, we used **1**, which was previously identified as an inhibitor of NF-*κ*B activity induced by ZFTA-RELA [6], as a positive control. Of these compounds, all but compound **6** exhibited NF-*κ*B activation inhibitory

activity compared to the DMSO control (**Figure 12(a)**). Induction of ZFTA-RELA expression increased relative cell numbers (no DOX vs. DMSO in **Figure 12(b)**), suggesting that activation of the NF-*κ*B pathway increased cell numbers in 6E8 cells. Compounds **2**, **3**, **5**, **7** and **9** at 5 μg/mL reverted the relative cell numbers to levels similar to those without DOX, which is probably due to inhibition of the NF-*κ*B pathway (**Figure 12(b)**). Notably, these compounds did not cause a significant decrease in relative cell numbers compared to the no DOX control (No DOX vs **2**, **3**, **5**, **7** and **9** in **Figure 12(b)**). This indicates that these compounds are not cytotoxic in 6E8 cells at this concentration. As these compounds significantly inhibited NF-*κ*B-responsive luciferase activity induced by the expression of ZFTA-RELA (**Figure 12(a)**) but did not exhibit significant cytotoxicity in 6E8 cells (**Figure 12(b)**), we next tested the effect of these compounds on the expression of endogenous NF-*κ*B-responsive genes, namely *CCND1*, *L1CAM*, *ICAM1*, and *TNF* [6] [18] [25] [26]. In addition to compound **1**, which we previously showed to inhibit NF-*κ*B reporter activity and expression of endogenous NF-*κ*B responsive genes [6], compound **2** showed significant inhibitory activity (**Figure 13**). The inhibitory effect of compounds **1** and **2** on NF-*κ*B-responsive luciferase activity induced by the expression of ZFTA-RELA was observed in a dose-dependent manner (**Figure 14(a)**). The IC_{50} values of **1** and **2** were 2.0 μg/mL (5.0 μM) and 5.3 μg/mL (11.6 μM), respectively. At high concentrations (30 μg/mL), these compounds significantly reduced the relative viable cell numbers (**Figure 13(b)**), suggesting that they may possess cytotoxic properties at higher concentrations.

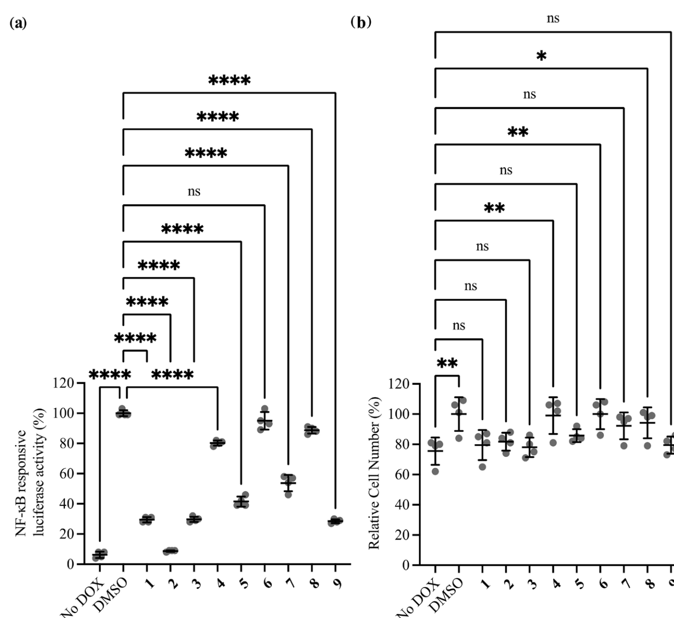

Figure 12. Effect of isolated compounds **2**-**9** from *N. spinosa* IFM 47025 and *epi*-aszonalenin B (**1**) on the NF-*κ*B responsive luciferase activity and relative viable cell number in 6E8 cells. NF-*κ*B responsive luciferase activity induced by the expression of ZFTA-RELA (a) and relative viable cell number (b) in 6E8 cells cultured overnight in the absence (No DOX) or presence (DMSO, **1**-**9**) of 300 ng/mL DOX. Each compound was added at a final concentration of 5 μg/mL in 0.05% DMSO. The mean value of the DMSO control was set to 100%. Data shown are the mean ± SD. Each point on the graph is the luciferase activity (a) and relative cell number (b) of an individual sample. N = 4 each. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

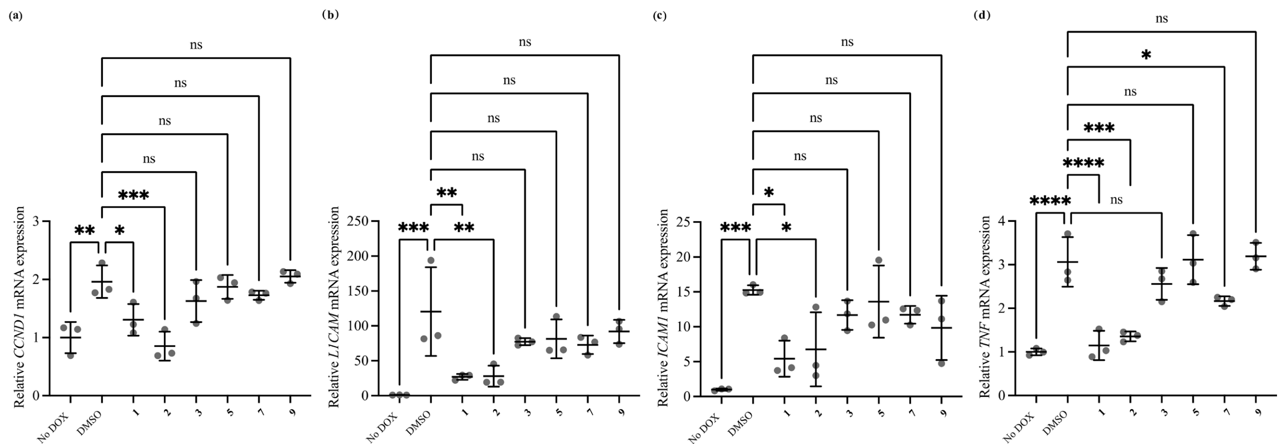

Figure 13. Effect of isolated compounds (2, 3, 5, 7 and 9) from *N. spinosa* IFM 47025 and *epi*-aszonalenin B (1) on the expression of endogenous NF-*κ*B responsive genes. Relative expression levels of *CCND1* (A), *L1CAM* (B), *ICAM1* (C), and *TNF* (D) mRNAs in 6E8 cells cultured overnight in the absence (No DOX) or presence (DMSO, 1, 2, 3, 5, 7, and 9) of 150 ng/mL DOX. Each compound was added at a final concentration of 10 μg/mL in 0.1% DMSO. The mRNA expression levels were normalized to the No DOX controls. Data shown are the mean ± SD. Each point on the graph is the relative mRNA expression level of an individual sample. N = 3 each. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0005$; ****, $p < 0.0001$.

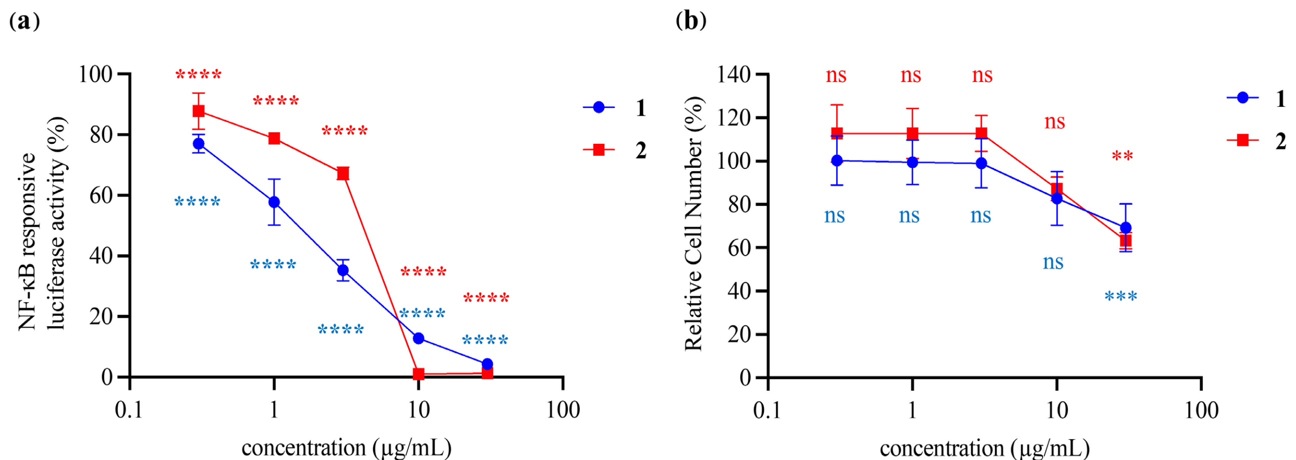

Figure 14. Dose-response curves of compounds 1 (shown in blue) and 2 (shown in red) on the NF-*κ*B responsive luciferase activity (a) and relative viable cell number (b) in 6E8 cells. 6E8 cells were cultured overnight with 300 ng/mL DOX and either 1 or 2 at the indicated concentration in 0.3% DMSO. The mean value of the DMSO control was set to 100%. Data shown are the mean ± SD. N = 4 each. The statistic comparisons were conducted between the test samples and the DMSO controls. ns, not significant; **, $p < 0.004$; ***, $p = 0.0001$; ****, $p < 0.0001$.

4. Discussion

The present study sought to identify compounds that inhibit the oncogenic NF-*κ*B activity from the culture extract of *N. spinosa* IFM 47025 using an assay system constructed in our previous study [6]. As a result, aszonapyrone A (**2**) was identified as an inhibitor of NF-*κ*B responsive luciferase reporter activity from the MeCN extract. Furthermore, compound **2** inhibited the expression of endogenous NF-*κ*B responsive genes. These results suggest that compound **2** functions as an inhibitor of NF-*κ*B activity induced by the expression of ependymoma-causing ZFTA-RELA fusion protein. Compound **2** was first isolated from *Aspergillus*

zonatus IFO8817 [23] and has been demonstrated to exhibit anti-bacterial activity against bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) [27], growth inhibitory effects on human cancer cells [20], and inhibitory activities against NADH-fumarate reductase [28]. Kanokmedhakul *et al.* also converted this compound to chevalones B and C by chemical cyclization and reported the cytotoxic activity of these compounds against cancer cells and their antimicrobial activity against *Mycobacterium tuberculosis* [29]. However, the inhibitory activity of compound **2** against NF-*κ*B signaling and its isolation from *N. spinosa* are reported for the first time in this paper.

While compounds **3**, **5**, **7**, and **9** significantly inhibited NF-*κ*B-responsive luciferase activity induced by ZFTA-RELA expression but not the expression of endogenous NF-*κ*B-responsive genes in the present study. The observed discrepancy may be attributed to differences in sensitivity between these assays. Further investigation of experimental conditions may facilitate the detection of the inhibitory effects of these compounds on the expression of endogenous NF-*κ*B-responsive genes.

Compounds **5** and **7** exhibited inhibitory activity against NF-*κ*B-responsive luciferase activity, whereas their putative biosynthetic intermediate, compound **6**, did not (**Figure 12**). These results suggest that a certain planarity of molecular structure due to multiple consecutive ring structures is important for NF-*κ*B signaling inhibitory activity. While compound **2** demonstrated inhibitory activity against the expression of endogenous NF-*κ*B responsive genes, compounds **3** and **8**, which were presumed biosynthetic intermediates of compound **2** [29], showed no activity despite having similar structures (**Figure 13**). This result suggests that the presence of B and C rings of compound **2** may be responsible for the inhibitory activity. In addition, dysiherbol A, a meroterpene compound with a multiple-ring structure possessing the same decalin ring as **2**, has been reported to exhibit relatively strong NF-*κ*B inhibitory and cytotoxic activities under different activity evaluation conditions [30]. On the other hand, the oxidized form of dysiherbol A at the C-3 position of the A ring exhibited a 10-fold decrease in both activities. The activity of **2** may be reduced due to the presence of an acetoxy group at the C-3 of the A ring. Therefore, deoxygenation of the C-3 of **2** may enhance NF-*κ*B inhibitory activity.

Similar to compound **1**, which was previously identified as an NF-*κ*B signaling inhibitor [6], compound **2** showed NF-*κ*B signaling inhibition (**Figures 12** and **13**). However, their direct application for the treatment of ST-EPN-RELA is precluded by the observation that both compounds have been demonstrated to exert cytotoxic effects on cultured cells at higher concentrations (**Figure 14(b)**). It is of the utmost importance to elucidate the pharmacological mechanism of action of these compounds and modify them to obtain derivatives that inhibit NF-*κ*B signaling at lower concentrations and possess a wider safety range. Given that ZFTA-RELA localizes to the nucleus in a stimulus-independent manner [8], it can be inferred that the inhibitory effect of compound **2** is not on pathways leading to

IκB degradation [11] [12]. Rather, it is likely to be on the nuclear transport, transcriptional activation, and/or post-translational modifications of ZFTA-RELA.

5. Conclusion

Eight known compounds **2**-**9** were isolated from the culture extract of *N. spinosa* IFM 47025 and their structures were identified through a comparison of the data presented in the relevant literatures. The inhibitory activity of compounds **2**, **3**, **5**, **7** and **9** was demonstrated in the NF-κB responsive reporter assay. Moreover, **2** showed significant inhibitory activity in the expression of endogenous NF-κB responsive genes and a dose-dependent inhibition in the NF-κB responsive reporter assay. However, the cytotoxic effects of compound **2** on 6E8 cells at high concentrations indicate the necessity for the development of compounds with higher activity and safety. This study provides useful lead compounds for the development of ST-EPN-RELA therapeutics.

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Authors' Contributions

K. Ishikawa, conceptualization, data curation, funding acquisition, investigation, methodology, writing original draft, writing review & editing. N. Kamiya, investigation, isolation and structure determination. M. Ishii and K. Ichinose, funding acquisition and writing review & editing. T. Yaguchi, resources and writing review & editing. S. Ohata, conceptualization, data curation, funding acquisition, investigation, methodology, writing original draft, writing review & editing.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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