Synthesis and Structure–Activity Relationship Study of NBRI16716B, an Antitumor Natural Product

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Received March 5, 2015; accepted March 14, 2015

The total synthesis of NBRI16716B (2), a naturally occurring modulator of tumor–stroma interactions, was successfully achieved. Using this synthetic route, a dehydroxy analogue (21) and a derivative lacking the 5-hydroxy-3-methylpentenoyl side chain (22) became accessible. A preliminary structure–activity relationship study to unveil the structural requirements for selective inhibition of tumor cells cocultured with stromal cells revealed that both of the hydroxamate structures of 2 are indispensable, whereas the 5-hydroxy-3-methylpentenoyl side chain is not essential.

Key words antitumor agent; natural product; diketopiperazine; tumor–stroma interaction; structure–activity relationship

Tumor tissues comprise not only of tumor cells, but also the surrounding stroma3) made up of normal cells, including endothelial cells, fibroblast-like cells (termed stromal cells), and extracellular matrix.4) Stromal cells regulate the growth of adjacent tumor cells either positively or negatively via direct or indirect communication influenced by cell adhesion or secreted factors (tumor–stroma interactions).3–6) Signals transmitted from stromal cells are particularly interesting as the machineries come from normal cells in which mutations of participating molecules occur less frequently than those from tumor cells. Hence, the signal molecules from stromal cells responsible for controlling tumor growth could be novel molecular targets of antitumor agents with a lower tendency to develop resistance, which prompted us to screen for modulators of tumor–stroma interactions.7)

Toward this end, we constructed an assay system to select molecules that inhibit the growth of tumor cells cocultured with stromal cells more potently than that of monocultured tumor cells.8,9) Since tumor-stroma interactions are a relatively new research topic in the field of oncology, key signaling molecules involved in these interactions are currently under active investigation. A phenotypic assay system of this type would require identifying the molecular targets of hit compounds, which would provide insight into the communication between tumor and stroma cells. Natural products were used as the major source for our screening platform; the structural variety of natural products is expected to bait a diverse array of counterpart proteins. In fact, several natural products exhibit the desired activity and selectivity, including phthoxazolin A,10) NBR123477 A and B,11) leucinostatins,12) and intervenolin.13,14) Two of NBR116716s (15) (Fig. 1), having a diketopiperazine moiety as the core structure, are also among this class.

The structures of NBR116716A (1), B (2), and C (3) share an identical molecular framework, but differ in OH functionality at the amide nitrogen. These natural products were discovered from the fermentation broth of fungal strain Peri-sporiopsis melioides Mer-f16716 displaying more potent anti-proliferative activity against DU-145 prostate cancer cells cocultured with PrSCs human prostate stromal cells than toward monocultured DU-145 cells. Indeed, evidence supports a close relationship between the prostate stroma and the growth and metastasis of surrounding tumor cells.16,17) Of the three compounds, NBR116716B had the most potent and selective in vitro activity under the cocultured conditions described above, whereas NBR116716C exhibited weak activity, indicating that the OH group at the R2 position (Fig. 1) is crucial. Notably, NBR116716A and B displayed in vivo antitumor activity toward tumor model mice inoculated with DU-145 and PrSCs cells, which renders these compounds potential candidates of lead for anticancer drugs. Although insulin-like growth factor-I secreted by human prostate stromal cells are known to promote the growth of human prostate cancer cells,8,18) no evidence showing that it is the direct molecular target of NBR116716s has been obtained. Herein we disclose a synthetic route to NBR116716B (2), with which structurally related derivatives are accessible, thereby paving the way to preliminary structure–activity relationship (SAR) studies.

Results and Discussion

Total Synthesis of NBRI16716B (2) NBRI16716B (2) has been reported as a degradation product of isotriornitin, a

![Fig. 1. Structure of NBRI16716s](image)
natural product produced by *Epicoccus purpurascens*. It was also found as a natural product by us, however, no synthetic study has been reported to date. The synthetic strategy in this study is straightforward (Chart 1): the diketopiperazine skeleton was constructed by dimerization of suitably protected N-hydroxyornithine derivatives and subsequent cyclization. The two hydroxylamine moieties were then differentially acylated to form the whole framework of NBRI16716B (2).

Toward this end, the hydroxyl group of commercially available N-Cbz-norvaline (4) was converted to the N-Boc-O-Ac-hydroxyamino functionality by the Mitsunobu reaction to give 5. The Cbz group was then removed by conventional methods to afford a free amino acid 6, which was condensed with the norvaline derivative 7 using WSC·HCl (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) as a coupling reagent. The coupling product was obtained with many unidentified byproducts to result in moderate yield. The dipeptide obtained as described above was subjected to standard hydrogenolysis conditions, resulting in the diketopiperazine precursor 9 without any difficulty. Subsequent treatment of 9 with N,N-diisopropylethylamine (DIPEA) led to the formation of a diketopiperazine framework with concomitant removal of Ac protecting groups on the N-hydroxyl moieties, affording 10. The uncyclized deacetyl compounds were found as byproducts. Following the removal of Boc using trifluoroacetic acid (TFA), introduction of the 5-hydroxy-3-methylpentenoyl side chain was achieved using COMU® (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate) as a coupling reagent, while at the same time the tert-butyl diphenylsilyl (TBDPS) group was uninstalled to accomplish the total synthesis of NBRI16716B (2). The high water solubility caused difficulty in work up to ended up with the moderate isolated yield. The physicochemical data and biological activity (*vide infra*) of the synthetic sample were identical to those of the natural product.

**Synthesis of NBRI16716B Derivatives** To assess the importance of the OH groups on the nitrogen of the amide functionalities, and the 5-hydroxy-3-methylpentenoyl side chain, we performed additional SAR studies taking advantage of the synthetic method to access the mother compound NBRI16716B. At first, the dehydroxy derivative 21 was prepared as illustrated in Chart 2. Initially, the hydroxyl group of commercially available N-Cbz-norvaline methyl ester 13 was substituted with the N-Cbz-O-tert-butyldimethylsilyl (TBS)-hydroxyamino group by a Mitsunobu reaction to give 14, from which the Fmoc protecting group was removed by treatment with piperidine to afford an intermediate with an unmasked amino group (15). Next, the amide linkage between the free primary amino group of 15 and the carboxyl group of a known ornithine derivative 16 was formed with WSC·HCl. The resulting fully protected dipeptide 17 was subjected to detachment of the Fmoc group using EtNH₂ with subsequent cyclization to yield the diketopiperazine compound 18. Hydrogenolysis removed the Cbz group to give 19, to which side chain precursor 20 was attached with concomitant removal of the TBS groups to afford the desired dehydroxy analog 21. Regarding length and chemical yield of each step, the synthetic route in Chart 2 more efficient than the one in Chart 1. However, the same set of protecting groups in Chart 2 could not be employed upon synthesis of 2 because of extremely low conversion of the first amide bond formation.

To evaluate the importance of the side chain moiety, the diacetyl analog 22 was also synthesized by simple acetylation of 11 (Chart 3).
compounds against cancer cells cocultured with stromal cells was examined. Originally, NBRI16716B (2) was discovered by the assay system using human prostate cancer and stromal cells, DU-145 and PrSC cells, respectively. Coculture-selective growth inhibition of NBRI16716s was recently reported to be more distinct for the combination of lung cancer and stromal cells, A549 and normal human lung fibroblast (NHLF). In the present study, the in vitro biological activity of the two analogs (21, 22) was evaluated using the A549-NHLF system, and compared with that of NBRI16716B alone (Fig. 2).

The synthetic sample of NBRI16716B (2) displayed identical cocultured conditions (with NHLF) selective growth inhibition toward A-549 (IC\textsubscript{50} synthetic: 23.6 µg/mL for co-cultured, 46.5 µg/mL for mono-cultured; natural: 22.7 µg/mL for co-cultured, 41.8 µg/mL for mono-cultured). Diacetyl analog 22 retained the growth-inhibitory activity and also exhibited substantially lower IC\textsubscript{50} values in the presence of stromal cells (51.7 µg/mL) than in their absence (79.2 µg/mL), suggesting that the 5-hydroxy-3-methylpentenoyl side chain moiety is a target for structural modification in further SAR studies. In contrast, dehydroxy derivative 21 completely lost the activity. This finding, together with the observation that NBRI16716C lacking the OH group on the nitrogen in the 5-hydroxy-3-methylpentenoyl side chain did not inhibit the growth of the cells examined, indicates that the hydroxamate structures in both of the side chains are indispensable.

### Conclusion

The total synthesis of NBRI16716B (2), a naturally occurring modulator of tumor–stroma interactions, was successfully developed. This synthetic route rendered analogs 21 and 22 accessible, allowing for preliminary SAR studies to unveil the structural requirements for selective inhibition of tumor cells cocultured with stromal cells. Further SAR studies using the synthetic method described here as a key technique to develop anticancer leads and molecular probes to identify the primary target of NBRI16716B are ongoing.

### Experimental

#### General Remarks

The reactions were performed in an oven-dried test tube or round bottom flask with a Teflon-coated magnetic stirring bar unless otherwise noted. All work-up and purification procedures were carried out with reagent-grade solvents under ambient atmosphere. Infrared
(140 mg, 0.319 mmol) in 72% yield; α in vacuo. The reaction mixture was concentrated in vacuo. The resulting precipitates were collected, and washed with MeOH thoroughly. Optical rotation was measured using a 2 mL cell with a 1.0 dm path length on a JASCO polarimeter P-1030. High-resolution mass spectra (HR-MS) (electrospray ionization (ESI)-Orbitrap) were measured on ThermoFisher Scientific LTQ Orbitrap XL.

Centrifugal liquid–liquid partition chromatography (CPC) was performed with a CPC240 system (Senshu Scientific Co., Ltd.). Unless otherwise noted, materials were purchased from commercial suppliers and were used without purification. For reaction, tetrahydrofuran (THF), N,N-dimethylformamide (DMF), CH₂CN, toluene, AcOEt, and CH₂Cl₂ were purified by passing through a solvent purification system (Glass Contour). Dry 1,4-dioxane, MeOH, dimethyl sulfoxide (DMSO), and pyridine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used as received.

**Methyl (S)-5-(Acetoxy(tert-butoxycarbonyl)amino)-2-(((benzyloxy)carbonyl)amino) Pentanoate (5)** To a solution of 4 (125 mg, 0.444 mmol) in 4.44 mL of toluene were added BocNHOAc (84.8 mg, 0.484 mmol), PPh₃ (150 mg, 0.572 mmol), and DEAD (diethyl azodicarboxylate, 2.2 mol/L in toluene, DMSO-δ₆) 2.49). For 1³C-NMR, chemical shifts were reported in the scale relative to the NMR solvent (CDCl₃: δ 7.26 ppm, CD₂OD: δ 3.30 ppm, DMSO-δ₆: δ 49.0 ppm, DMSO-δ₂: 39.7 ppm) as an internal reference. NMR data are reported as follows: chemical shifts, multiplicity (s: singlet, d: doublet, t: triplet, m: multiplet, br: broad signal), coupling constant (Hz), and integration. Optical rotation was measured using a 2 mL cell with a 1.0 dm path length on a JASCO polarimeter P-1030. High-resolution mass spectra (HR-MS) (ESI) Anal. Calcd for C₁₇H₃₀N₄NaO₇ m/z 425.1992; 1³C-NMR (100 MHz, CD₂OD) δ: 174.7, 173.7, 170.2, 158.3, 156.2, 138.2, 129.5, 129.0, 128.8, 67.6, 55.6, 53.1, 52.7, 50.5, 30.4, 29.4, 28.4, 24.6, 24.2, 20.2, 18.3, 18.2.

**Methyl (S)-5-(Acetoxy(tert-butoxycarbonyl)amino)-2-(((benzyloxy)carbonyl)amino) Pentanoate (5)** To a solution of 8 (19 mg, 30.5 µmol) in 0.44 mL of MeOH was added 10% Pd/C (2 mg), and the mixture was stirred at room temperature for 3 h under atmospheric pressure of H₂. The catalyst was filtered off with a pad of Celite, and the filtrate was concentrated under reduced pressure to give a crude material containing 9, which was used for the succeeding step without further purification.

**t-Butyl Hydroxy-3-((2S,5S)-5-(3-(N-hydroxyaminopropyl)-3,6-dioxopiperazin-2-yl)propyl) carbamate (10)** To a solution of 9 (19.9 mg, 49.7 µmol), DIPEA (14.9 µL, 85.5 µmol) were added to the solution at 0°C, and the mixture was stirred at room temperature for 4 h. The reaction mixture was concentrated in vacuo, after which the resultant residue was dissolved in AcOEt, washed with 1 m HCl, saturated NaHCO₃, and brine successively. The organic layer was dried over Na₂SO₄, and was concentrated to dryness. The residue was purified with preparative TLC (CHCl₃/MeOH=10/1) to give 10 (2.9 mg, 7.21 µmol, 24% yield over 2 steps) as a colorless oil (15.2 mg, 23.3 µmol). 

**N-Hydroxy-N-(3-((2S,5S)-5-(3-(hydroxyamino)propyl)-3,6-dioxopiperazin-2-yl)propyl)acetamide (11)** To a mixture of 0.5 mL of TFA and 0.5 mL of CH₂Cl₂ was added 10 (20.0 mg, 49.7 µmol), and the solution was stirred at room temperature for 1.5 h at 0°C and was concentrated to dryness. The residue was suspended in toluene and concentrated, which was repeated 5 times. The resultant residue was used for the next reaction without further purification.

**NBR116716B (2)** To a solution of 12 (36.6 mg, 99.4 µmol) in 0.25 mL of DMF were added DIPEA (28.0 µL, 0.159 mmol) and COMU* (43.0 mg, 99.4 µmol) at 0°C, and the resultant mixture was stirred at the same temperature for 5 min. Then, 10 obtained from the previous step was added to the solution and stirring continued at room temperature for 3.5 h. After evaporation of the solvent, the residue was partitioned between H₂O and AcOEt. After concentrating the aqueous layer, the crude sample thus obtained was purified with silica gel column chromatography (CHCl₃/MeOH=6/1) and the suc-
ceeding CPC (CHCl₃/MeOH/H₂O=5/6/4, ascending mode) to give 2 (4.4 mg, 21% yield over 2 steps) as a white powder; mp 156–158°C; [α]D₂⁰=−29.0 (c=0.11, MeOH) (lit. [α]D₂⁰=−21.3 (c=0.4, MeOH)); IR (KBr) ν 3421, 1684, 1457 cm⁻¹; HR-MS (ESI) Anal. Calcd for C₇₁H₆₂N₄O₁₈Sι m/z 2478.6920 [M+Na]⁺. Found 2478.6928.

To a solution of diethylamine (4.8 mg, 9.00 mmol) in 1 mL of toluene and 2 mL of THF were added CbzNHTBOS (381 mg, 1.35 mmol), PPh₃ (461 mg, 1.76 mmol), and DEAD (0.78 mL, 1.69 mmol), at room temperature successively. The mixture was stirred at the temperature for 18 h, and concentrated. The resultant residue was purified with silica gel column chromatography (n-hexane/AcOEt=1/1) to give 14 as a colorless oil (437 mg, 0.691 mmol) in 51% yield; [α]D₂⁰=8.22 (c=0.13, MeOH); IR (KBr) ν 3339, 3295, 2925, 1725, 1522, 1450, 1252, 1213 cm⁻¹; HR-MS (ESI) Anal. Calcd for C₃₂H₄₀N₄O₁₈Sι m/z 633.2996 [M+H]⁺. Found 633.2991; ¹H-NMR (400 MHz, CDCl₃) δ: 7.77 (2H, d, J=7.3 Hz), 7.60 (2H, d, J=6.9 Hz), 7.42–7.30 (9H, m), 5.32 (1H, d, J=7.8 Hz), 5.15 (2H, s), 4.44–4.34 (3H, m), 4.23 (1H, t, J=6.9 Hz), 3.73 (3H, s), 3.53 (2H, m), 1.88–1.80 (4H, m), 0.90 (9H, s), 0.10 (3H, s); ¹³C-NMR (100 MHz, CDCl₃) δ: 172.8, 158.4, 156.0, 144.0, 143.8, 141.4, 136.0, 128.6, 128.5, 128.4, 127.8, 127.2, 125.2, 120.1, 68.0, 67.2, 53.7, 52.5, 51.6, 47.2, 29.7, 25.8, 22.2, 17.9, −5.0.

Methyl (S)-2-((3-(((9H-Fluoren-9-yl)ethoxy)carbonyl)amino)-5-(((benzoxyl)carbonyl)((tert-butylidimethylsilyloxy)carbonyl)amino)pentanoate (15) To a solution of 14 (96.0 mg, 0.152 mmol) in 1 mL of DMF was added piperidine (17.0 mg, 0.165, MeOH) and HATU (27.3 mg, 71.8 mmol) and DIPEA (32.0 mg, 0.182 mmol), at room temperature for 1 h and then at 40°C for 12 h. The solution was concentrated in vacuo, and Et₂O and hexane was added to the resultant residue to give a white solid. After thorough washing with Et₂O and n-hexane, 15 was obtained as a colorless amorphous (4.8 mg, 9.00 mmol) in 71% yield; [α]D₂⁰=−35.7 (c=0.23, CHCl₃); IR (KBr) ν 3209, 3069, 2954, 1672, 1456, 1338, 1258 cm⁻¹; HR-MS (ESI) Anal. Calcd for C₃₂H₄₂N₄NaO₁₈Si m/z 557.2771 [M+Na]⁺. Found 557.2766; ¹H-NMR (400 MHz, CDCl₃) δ: 7.36–7.30 (5H, m), 6.78 (1H, br), 6.45 (1H, br), 6.11 (1H, br), 5.13 (2H, s), 4.03 (1H, m), 3.96 (1H, m), 3.52 (2H, t, J=6.4 Hz), 3.22 (2H, m), 1.96 (3H, s), 1.89–1.83 (5H, m), 0.88 (9H, s), 0.08 (3H, s); ¹³C-NMR (125 MHz, CDCl₃) δ: 170.6, 167.92, 167.88, 158.5, 135.8, 128.6, 128.7, 128.5, 128.4, 86.1, 54.6, 54.4, 51.4, 38.9, 31.0, 30.8, 25.7, 24.8, 23.2, 21.8, 17.8, −5.1.

N-(3-(((25,55)-5-(((tert-butylidimethylsilyloxy)carbonyl)propyl)-3,6-dioxopiperazin-2-yl)propyl)acetamide (19) To a solution of 18 (40 mg, 74.8 µmol) in 1 mL of MeOH was added 10% Pd/C (0.4 mg), and the mixture was stirred at room temperature for 1 h under atmospheric pressure of H₂. The catalyst was filtered off with a pad of Celite, and the filtrate was concentrated under reduced pressure to give a crude material containing 19, which was used for the succeeding step without further purification.

(E)-N-(3-(((25,55)-5-(((tert-butylidimethylsilyloxy)carbonyl)propyl)-3,6-dioxopiperazin-2-yl)propyl)-N,5-dihydroxy-3-methylpent-2-enamide (21) To a solution of 20 (16.1 mg, 65.9 µmol) in 0.5 mL of DMF were added DIPEA 12.5 µL, 71.8 µmol and HATU (0.7-azabenzotriazol-1-yl)-N,N',N'-tetramethyluronium hexafluorophosphate, 27.3 µg, 71.8 µmol) at 0°C, and the mixture was stirred for 30 min at room temperature. Then, 19 (all the material obtained above), was added to the solution at 0°C, and the mixture was stirred at room temperature for 10h. The reaction mixture was concentrated in vacuo, and the residue was purified with silica gel column chromatography (CHCl₃/MeOH=4/1) to give 21 as a white powder (7.2 mg, 18.1 µmol) in 30% yield over 2 steps; mp 149–151°C; [α]D₂⁰=−16.0 (c=0.165, MeOH); IR (KBr) ν 3242, 1654, 1457 cm⁻¹; HR-MS (ESI) Anal. Calcd for C₃₂H₄₂N₄NaO₁₈Si m/z 421.2063 [M+Na]⁺. Found 421.2058; ¹H-NMR (400 MHz, CDCl₃) δ: 6.29 (1H, br), 3.98 (2H, m), 3.70–3.64 (2H, m), 3.67–3.61 (2H, m), 3.17 (2H, m), 2.34 (2H, t, J=6.5 Hz), 2.05 (2H, br), 1.91 (3H, s), 1.84–1.50 (8H, m); ¹³C-NMR (125 MHz, CDCl₃) δ: 173.4, 170.4, 169.6, 152.6,
respectively, as a background value. The residual was dissolved in 0.5 mL of CH$_3$CN, and NaHCO$_3$ (18.4 mg, 219 µmol) AcCl (3.9 µL, 54.7 µmol) was added to the solution at 0°C, then the solution was stirred for 1 h at the temperature. A white powder of 22 was precipitated out as the reaction proceeded. The white powder was corrected on funnel, which was thoroughly washed with CH$_3$CN and MeOH to give pure sample of 22 (14.9 µg, 43.3 µmol) in 79% yield; mp 207–209°C; [α]$_D^{25}$ −12.3 (c=0.165, MeOH); IR (KBr) ν 3430, 3191, 2937, 1682, 1598, 1468, 1444, 1206 cm$^{-1}$; HR-MS (ESI) Anal. Calcd for C$_{14}$H$_2$_N$_2$Na$_6$O$_6$ m/z 367.1594 [M$^+$Na]$^+$; Found 367.1588; $^1$H-NMR (600 MHz, DMSO-$d_6$) δ: 9.71 (2H, s), 8.09 (2H, s), 7.38 (2H, t, J=6.4 Hz), 1.93 (6H, s), 1.18–1.51 (8H, m); $^{13}$C-NMR (150 MHz, DMSO-$d_6$) δ: 170.7, 168.4, 54.3, 47.3, 43.3, 30.8, 22.5, 20.9.

**Cells and Reagents** Human lung cancer cell line A549 was obtained as described.$^{29}$ NHLF normal human lung fibroblasts were obtained from BioWhittaker. The stromal cells were maintained in Dulbecco’s modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, 100 µg/mL streptomycin, ITS (5 µg/mL insulin, 5 µg/mL transferrin, and 1.4 µM hydrocortisone), and 5 nM FGF (Pepro Tech, NJ, U.S.A.) at 37°C with 5% CO$_2$ as described.$^8$

**Cell Growth and Coculture Experiment** For coculture experiment, stromal cells were first inoculated in 96-well plates at 5x10$^3$ cells per well in 0.1 mL of DMEM supplemented with 1% n-FBS and ITS. Test samples were added into the well and the stromal cells were cultured for 2 d. Then 10 µL of cancer cell suspension (5x10$^3$) in serum-free DMEM were inoculated onto a monolayer of the stromal cells and the cells were further cultured for 3 d. For monoculture of cancer cells, only assay medium with test samples was first incubated for 2 d, and then cancer cells were inoculated as described above and further cultured for 3 d. The growth of the cancer cells was determined using rhodamine blue dye as described.$^3$ Cells were fixed for 15 min by adding 50 µL of 5% glutaraldehyde in phosphate-buffered saline. After washing three times with tap water, the plate was dried. Then the cells were stained for 15 min by adding 10 µL of 0.2% rhodamine blue dye in distilled water. After washing seven times with tap water and drying, the dye was eluted with 100 µL of 50% ethanol and absorbance at 550 nm measured using a microplate reader. The absorbance at 550 nm of the medium alone and the NHLF alone was subtracted from the values in monoculture and coculture, respectively, as a background value.

**Acknowledgment** We thank Dr. Ryuichi Sawa, Ms. Yumiko Kubota, and Ms. Yuko Takahashi (BIKAKEN) for collection of spectral data.

**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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