Regular Article

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

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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

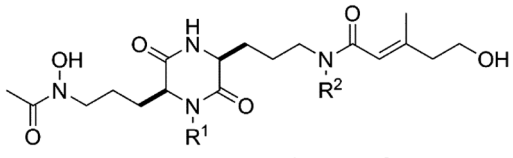
Tumor tissues comprise not only of tumor cells, but also the surrounding stroma[1] made up of normal cells, including endothelial cells, fibroblast-like cells (termed stromal cells), and extracellular matrix.[2] Stromal cells regulate the growth of adjacent tumor cells either positively or negatively *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] NBRI23477 A and B,[11] leucinostatins,[12] and intervenolin.[13,14] Two of NBRI16716s[15] (Fig. 1), having a diketopiperazine moiety as the core structure, are also among this class.

The structures of NBRI16716A (**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 *Perisporiopsis melioloides* 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, NBRI16716B had the most potent and selective *in vitro* activity under the cocultured conditions described above, whereas NBRI16716C exhibited weak activity, indicating that the OH group at the R^2 position (Fig. 1) is crucial. Notably, NBRI16716A 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 NBRI16716s has been obtained. Herein we disclose a synthetic route to NBRI16716B (**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



NBRI16716A (**1**): R^1 = OH, R^2 = OH
NBRI16716B (**2**): R^1 = H, R^2 = OH
NBRI16716C (**3**): R^1 = OH, R^2 = H

Fig. 1. Structure of NBRI16716s

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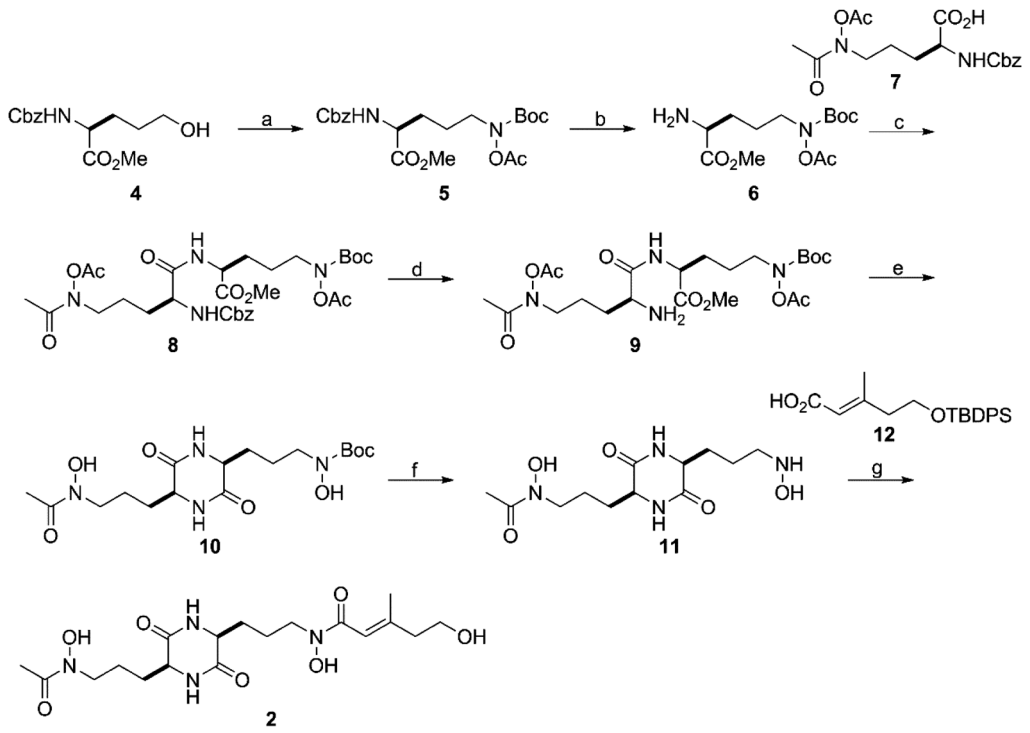


Reagents and conditions: (a) BocHNOAc, DEAD, PPh_3, toluene rt, 20 h, 72%; (b) H_2, 10% Pd/C, MeOH, rt, 1 h; (c) **7**, WSC·HCl, HOBt, DIPEA, CH_2Cl_2, rt, 4 h, 41% for 2 steps; (d) H_2, 10% Pd/C, MeOH, rt, 3 h; (e) DIPEA, MeOH, rt, 48 h, 24% for 2 steps; (f) TFA, CH_2Cl_2, 0°C, 1.5 h; (g) **12**, COMU, DIPEA, DMF, rt, 3.5 h, 21% for 2 steps.

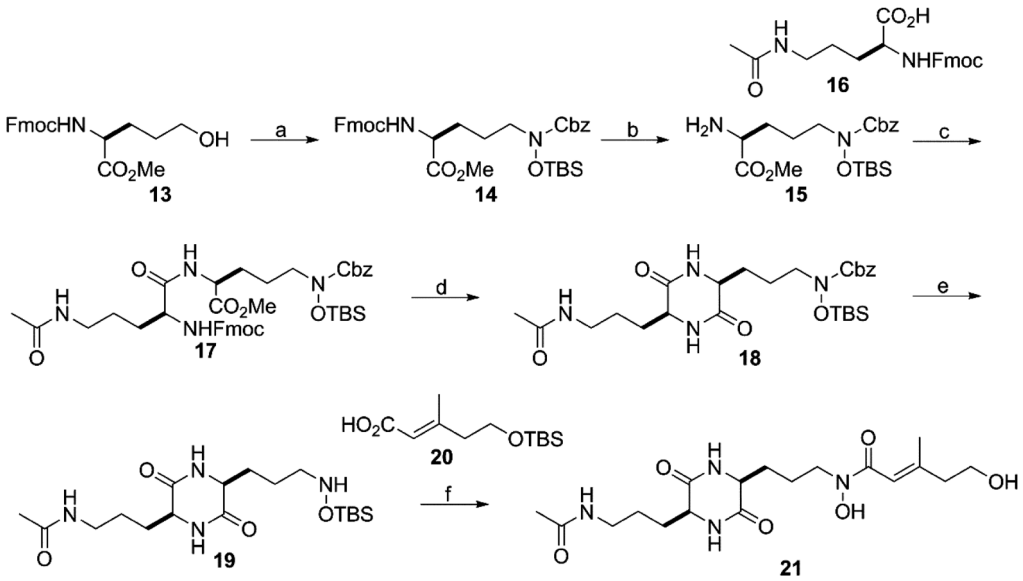
Chart 1. Total Synthesis of NBRI16716B

natural product produced by *Epicoccus purpurascens*.[19] It was also found as a natural product by us,[15] 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 **8** 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 by-products. 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)[20] as a coupling reagent, while at the same time the *tert*-butyldiphenylsilyl (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 Fmoc-norvalin 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 $EtNH_2$ 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.

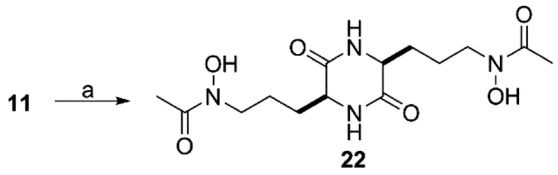
To evaluate the importance of the side chain moiety, the diacetyl analog **22** was also synthesized by simple acetylation of **11** (Chart 3).

Biological Activity of NBRI16716B Derivatives In the next study, the selective inhibitory activity of the synthesized



Reagents and conditions: (a) CbzHNOTBS, DEAD, PPh_3, toluene, THF, rt, 18 h, 51%; (b) Piperidine, DMF, rt, 2.5 h; (c) **16**, WSC·HCl, HOBt, DIPEA, CH_2Cl_2, rt, 6 h, 38% for 2 steps; (d) Et_2NH, THF, rt for 5 h, 40°C for 12 h, 71%; (e) H_2, 10% Pd/C, MeOH, rt, 1 h; (f) **20**, HATU, HOBt, DIPEA, DMF, 0°C, 10 h, 30% for 2 steps.

Chart 2. Synthesis of a Dehydroxy Derivative of NBRI16716B (**21**)



Reagents and conditions: (a) AcCl, $NaHCO_3$, CH_3CN, 0°C, 1 h, 79%.

Chart 3. Synthesis of a Diacetyl Derivative of NBRI16716B (**22**)

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_{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_{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.

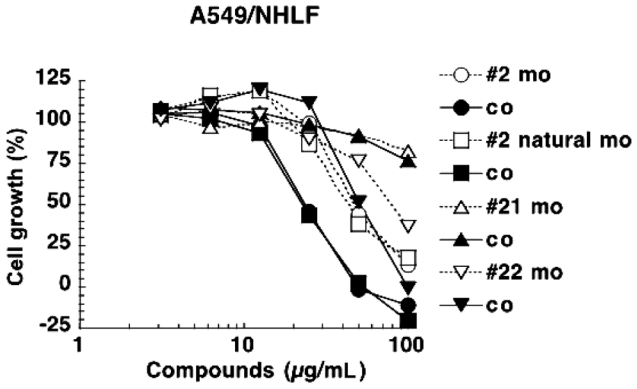


Fig. 2. Effect of NBRI16716B (**2**) and Its Derivatives (**21** and **22**) on Coculture of Tumor Cells and Stromal Cells

The growth of tumor cells cocultured with stromal cells (filled) or that of tumor cells alone (open) in the presence of the indicated concentrations of the test compounds (**2** synthetic: circle, **2** natural: square, **21**: triangle, **22**: inverted triangle) was determined by measuring fluorescence intensity of green fluorescent protein (GFP). A549 human lung cancer cell lines were cocultured with NHLF lung stromal cells. Data are means of duplicate determination. S.E. is less than 10%.

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

(IR) spectra were recorded on a JASCO FT/IR 4100 Fourier transform infrared spectrophotometer. NMR was recorded on JEOL ECS-400 (^{1}H-NMR: 400 MHz, ^{13}C-NMR: 100 MHz) or on JEOL ECA-600 (^{13}C-NMR: 150 MHz) or on Bruker AVANCE 500 (^{13}C-NMR: 125 MHz) spectrometers. Chemical shifts for proton are reported in parts per million downfield from tetramethylsilane and are referenced to residual protium in the NMR solvent ($CDCl_3$: δ 7.26 ppm, CD_3OD: δ 3.30 ppm, DMSO-d_6: 2.49). For ^{13}C-NMR, chemical shifts were reported in the scale relative to NMR solvent ($CDCl_3$: δ 77.0 ppm, CD_3OD: δ 49.0 ppm, DMSO-d_6: 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) (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), CH_3CN, toluene, AcOEt, and CH_2Cl_2 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), PPh_3 (150 mg, 0.572 mmol), and DEAD (diethyl azodicarboxylate, 2.2 mol/L in toluene, 250 μL, 0.550 mmol) at 0°C. The mixture was stirred at room temperature for 20 h and concentrated *in vacuo*. The resultant residue was purified with silica gel column chromatography (*n*-hexane/AcOEt=2/1 to 3/2) to give **5** as a colorless oil (140 mg, 0.319 mmol) in 72% yield; $[\alpha]_D^{26}$ 6.75 (c=0.150, $CHCl_3$); IR (neat) ν 3349, 2978, 1792, 1716, 1527, 1455, 1366, 1254 cm^{-1}; HR-MS (ESI) *Anal.* Calcd for $C_{21}H_{30}N_2NaO_8$ m/z 461.1900 $[M+Na]^+$, Found 461.1894; ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.37–7.26 (m, 5H), 6.55 (1H, d, J=16.0 Hz), 5.09 (2H, s), 4.21 (1H, dd, J=8.6, 5.1 Hz), 3.71 (3H, s), 3.58 (2H, m), 1.65 (1H, m), 1.74–1.58 (3H, m), 1.41 (9H, s); ^{13}C-NMR (100 MHz, CD_3OD) δ: 174.4, 170.1, 158.6, 156.2, 138.2, 129.5, 129.0, 128.8, 83.7, 67.6, 54.9, 52.7, 50.6, 29.6, 28.4, 24.6, 18.2.

Methyl (*S*)-5-(Acetoxy(*tert*-butoxycarbonyl)amino)-2-aminopentanoate (6) To a solution of **5** (25.0 mg, 57.0 μmol) in 1.2 mL of MeOH was added 10% Pd/C (2.5 mg), and the mixture was stirred at room temperature for 4 h under atmospheric pressure of H_2. The catalyst was filtered off with a pad of Celite, and the filtrate was concentrated under reduced pressure to give a crude material containing **6**, which was used for the succeeding reaction without further purification.

Methyl (*S*)-5-(Acetoxy(*tert*-butoxycarbonyl)amino)-2-((*S*)-5-(*N*-acetoxyacetamido)-2-(((benzyloxy)carbonyl)amino)pentanamido)pentanoate (8) To a solution of **7** (25.0 mg, 68.2 μmol) in 0.5 mL of CH_2Cl_2 was added HOBt (1-hydroxybenzotriazole, 11.5 mg, 85.1 μmol) at 0°C, and the mixture was stirred for 30 min at room temperature. Then, **6** (all the material obtained above), WSC·HCl (13.1 mg, 68.3 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 $NaHCO_3$, and brine successively. The organic layer was died over Na_2SO_4, and was concentrated to dryness. The residue was purified with preparative TLC ($CHCl_3$/MeOH=10/1) to give **8** as a colorless oil (15.2 mg, 23.3 μmol) in 41% yield over 2 steps; $[\alpha]_D^{26}$ −10.9 (c=1.43, MeOH); IR (neat) ν 3310, 2937, 1793, 1716, 1671, 1526, 1368 cm^{-1}; HR-MS (ESI) *Anal.* Calcd for $C_{30}H_{44}N_4NaO_{12}$ m/z 675.2853 $[M+Na]^+$, Found 675.2837; ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.37–7.25 (m, 5H), 5.06 (2H, s), 4.42 (1H, dd, J=9.1, 4.7 Hz), 4.14 (1H, m), 3.78–3.50 (4H, m), 3.68 (3H, s), 2.18 (3H, s), 2.10 (3H, s), 1.98–1.58 (8H, m), 1.96 (3H, s), 1.43 (9H, s); ^{13}C-NMR (100 MHz, CD_3OD) δ: 174.7, 173.7, 170.2, 158.3, 156.2, 138.2, 129.5, 129.0, 128.8, 83.6, 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-((*S*)-5-(*N*-acetoxyacetamido)-2-aminopentanamido)pentanoate (9) To a solution of **8** (19.9 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 H_2. 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.

***tert*-Butyl Hydroxy(3-((2*S*,5*S*)-5-(3-(*N*-hydroxyacetamido)propyl)-3,6-dioxopiperazin-2-yl)propyl)carbamate (10)** To a solution of **9** (all the material obtained above) in 0.15 mL of MeOH was added DIPEA (8.0 μL, 45.9 μmol). The mixture was stirred at room temperature for 48 h. The resultant white precipitates were collected, and washed with MeOH thoroughly to give **10** (2.9 mg, 7.21 μmol, 24% yield over 2 steps) as a white powder; mp 174–176°C; $[\alpha]_D^{25}$ –13.2 (c=0.155, DMSO); IR (neat) ν 3199, 2938, 1793, 1684, 1608, 1457, 1169 cm^{-1}; HR-MS (ESI) *Anal.* Calcd for $C_{17}H_{30}N_4NaO_7$ m/z 425.2012 $[M+Na]^+$, Found 425.1997; ^{1}H-NMR (600 MHz, DMSO-d_6) δ: 9.64 (1H, s), 9.11 (1H, s), 8.09 (1H, s), 8.08 (1H, s), 3.78 (2H, m), 3.43 (2H, t, J=6.5 Hz), 3.30 (2H, t, J=6.6 Hz), 1.93 (3H, s), 1.69–1.50 (6H, m), 1.37 (9H, s) 1.23 (2H, m); ^{13}C-NMR (150 MHz, DMSO-d_6) δ: 170.8, 168.4, 156.4, 79.9, 54.4, 54.3, 50.6, 47.3, 30.9, 28.6, 22.8, 22.6, 20.9.

***N*-Hydroxy-*N*-(3-((2*S*,5*S*)-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 CH_2Cl_2 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.

NBRI16716B (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 pemperature for 3.5 h. After evaporation of the solvent, the residue was partitioned between H_2O and AcOEt. After concentrating the aqueous layer, the crude sample thus obtained was purified with silica gel column chromatography ($CHCl_3$/MeOH=6/1) and the suc-

ceeding CPC ($CHCl_3$/MeOH/H_2O=5/6/4, ascending mode) to give **2** (4.4 mg, 21% yield over 2 steps) as a white powder; mp 156–158°C; $[\alpha]_D^{19}$ −29.0 (c=0.11, MeOH) (lit. $[\alpha]_D$ −21.3 (c=0.4, MeOH)); IR (KBr) ν 3421, 1684, 1457 cm^{-1}; HR-MS (ESI) *Anal.* Calcd for $C_{18}H_{30}N_4NaO_7$ m/z 437.2012 $[M+Na]^+$, Found 425.2007; ^{1}H-NMR (400 MHz, CD_3OD) δ: 6.28 (1H, s), 3.99 (2H, t, J=5.2 Hz), 3.68 (2H, t, J=6.6 Hz), 3.67–3.60 (6H, m), 2.34 (2H, t, J=6.4 Hz), 2.08 (3H, s), 2.05 (2H, br), 1.85–1.68 (8H, m); ^{13}C-NMR (150 MHz, CD_3OD) δ: 172.4, 169.0, 168.2, 151.2, 116.5, 59.6, 54.4, 43.5, 31.0, 30.9, 29.3, 22.2, 22.0, 18.9, 17.5.

NMR data from a natural sample: ^{1}H-NMR (400 MHz, CD_3OD) δ: 6.28 (1H, s), 3.99 (2H, t, J=5.2 Hz), 3.68 (2H, t, J=6.6 Hz), 3.67–3.60 (6H, m), 2.34 (2H, t, J=6.5 Hz), 2.09 (3H, s), 2.05 (2H, br), 1.85–1.68 (8H, m); ^{13}C-NMR (150 MHz, CD_3OD) δ: 172.5, 169.1, 168.3, 151.3, 116.5, 59.6, 54.4, 43.5, 31.0, 30.9, 29.3, 22.2, 22.0, 18.9, 17.5.

Methyl (*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-5-(((benzyloxy)carbonyl)((*tert*-butyldimethylsilyl)oxy)-amino)pentanoate (14) To a solution of **13** (500 mg, 1.35 mmol) in 10 mL of toluene and 2 mL of THF were added CbzNHOTBS (381 mg, 1.35 mmol), PPh_3 (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; $[\alpha]_D^{26}$ 8.22 (c=0.13, MeOH); IR (KBr) ν 3339, 2952, 1725, 1522, 1450, 1252, 1213 cm^{-1}; HR-MS (ESI) *Anal.* Calcd for $C_{35}H_{45}N_2O_7Si$ m/z 633.2996 $[M+H]^+$, Found 633.2991; ^{1}H-NMR (400 MHz, $CDCl_3$) 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), 1.88–1.60 (4H, m), 0.90 (9H, s), 0.10 (3H, s); ^{13}C-NMR (100 MHz, $CDCl_3$) 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-Amino-5-(((benzyloxy)carbonyl)((*tert*-butyldimethylsilyl)oxy)amino)pentanoate (15) To a solution of **14** (96.0 mg, 0.152 mmol) in 1 mL of DMF was added piperidine (17.0 μL, 0.167 mmol). The mixture was stirred at room temperature for 2.5 h. Then the mixture was concentrated to dryness to give a crude material containing **15**, which was used for the succeeding step after short column chromatography ($CHCl_3$/MeOH=10/1).

Methyl (*S*)-2-((*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-5-acetamidopentanamido)-5-(((benzyloxy)-carbonyl)((*tert*-butyldimethylsilyl)oxy)amino)pentanoate (17) To a solution of **16** (72.3 mg, 0.182 mmol) in 0.9 mL of CH_2Cl_2 was added HOBt (31.0 mg, 0.229 mmol) at 0°C, and the solution was stirred at room temperature for 0.5 h. After successive addition of **15** (all the material obtained above), WSC·HCl (35.0 mg, 0.182 mmol), and DIPEA (32.0 μL, 0.184 mmol) at 0°C, the mixture was stirred at room temperature for 6 h. Then, the mixture was diluted with CH_2Cl_2 and washed with 1 M HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, and was concentrated *in vacuo*. The resultant residue was purified with silica gel column chromatography (*n*-hexane/AcOEt=1/1) to give **17** as a colorless amorphous (41.3 mg, 52.3 μmol) in 38% yield over 2 steps; $[\alpha]_D^{26}$ 6.85 (c=0.18, $CHCl_3$); IR (KBr) ν 3424, 2953, 1708, 1658, 1532, 1450, 1253 cm^{-1}; HR-MS (ESI) *Anal.* Calcd for $C_{42}H_{57}N_4O_9Si$ m/z 789.3895 $[M+H]^+$, Found 789.3889; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.75 (2H, d, J=7.5 Hz), 7.59 (2H, d, m), 7.40–7.16 (9H, m), 5.80 (1H, br), 5.65 (1H, brd, J=8.0 Hz), 5.12 (2H, s), 4.54–4.46 (3H, m), 4.36 (1H, d, J=6.7 Hz), 4.20 (1H, t, J=7.1 Hz), 3.68 (3H, s), 3.68–3.48 (3H, m), 3.11 (2H, m), 1.97 (3H, s), 1.93–1.55 (8H, m), 0.87 (9H, s), 0.07 (3H, s); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 172.6, 172.0, 171.1, 158.3, 156.2, 144.0, 143.8, 141.3, 135.9, 128.5, 128.3, 127.7, 127.1, 125.2, 120.0, 67.9, 67.0, 52.9, 52.3, 52.1, 51.5, 47.2, 37.9, 30.9, 28.8, 25.6, 23.3, 22.5, 17.8, −5.1.

Benzyl (3-((2*S*,5*S*)-5-(3-Acetamidopropyl)-3,6-dioxopiperazin-2-yl)propyl)((*tert*-butyldimethylsilyl)oxy)carbamate (18) To a solution of **17** (10.0 mg, 12.7 μmol) in 0.5 mL of THF was added 0.2 mL of Et_2NH. The mixture was stirred at room temperature for 5 h and at 40°C for 12 h. The solution was concentrated *in vacuo*, and Et_2O and hexane was added to the resultant residue to give a white solid. After through wash with Et_2O and *n*-hexane, **18** was obtained as a colorless amorphous (4.8 mg, 9.00 μmol) in 71% yield; $[\alpha]_D^{26}$ −35.7 (c=0.23, $CHCl_3$); IR (KBr) ν 3209, 3069, 2954, 1672, 1456, 1338, 1258 cm^{-1}; HR-MS (ESI) *Anal.* Calcd for $C_{26}H_{42}N_4NaO_6Si$ m/z 557.2771 $[M+Na]^+$, Found 557.2766; ^{1}H-NMR (400 MHz, $CDCl_3$) 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), 1.89–1.53 (8H, m), 0.88 (9H, s), 0.08 (3H, s); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 170.6, 167.92, 167.88, 158.5, 135.8, 128.6, 1287.5, 128.4, 68.1, 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-((2*S*,5*S*)-5-(3-(((*tert*-Butyldimethylsilyl)oxy)amino)-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 (4.0 mg), and the mixture was stirred at room temperature for 1 h under atmospheric pressure of H_2. 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-((2*S*,5*S*)-5-(3-Acetamidopropyl)-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 (*O*-7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, 27.3 mg, 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 10 h. The reaction mixture was concentrated *in vacuo*, and the residue was purified with silica gel column chromatography ($CHCl_3$/MeOH=3/1) and repeated CPC (ascending mode, $CHCl_3$/MeOH/H_2O=5/6/4; descending mode, $CHCl_3$/MeOH/H_2O=5/6/4; descending mode, $CHCl_3$/MeOH/H_2O=2/2/1) to give **21** as a white powder (7.2 mg, 18.1 μmol) in 30% yield over 2 steps; mp 149–151°C; $[\alpha]_D^{23}$ −16.0 (c=0.165, MeOH); IR (KBr) ν 3242, 1654, 1457 cm^{-1}; HR-MS (ESI) *Anal.* Calcd for $C_{18}H_{30}N_4NaO_6$ m/z 421.2063 $[M+Na]^+$, Found 421.2058; ^{1}H-NMR (400 MHz, CD_3OD) 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); ^{13}C-NMR (125 MHz, CD_3OD) δ: 173.4, 170.4, 169.6, 152.6,

120.9, 117.8, 60.9, 55.7, 44.8, 44.6, 40.0, 32.5, 25.9, 23.4, 22.6, 18.8.

***N*,*N*′-(((2*S*,5*S*)-3,6-Dioxopiperazine-2,5-diyl)bis(propane-3,1-diyl))bis(*N*-hydroxyacetamide) (22)**[21] To a mixture of TFA (0.55 mL) in CH_2Cl_2 (0.55 mL) was added **11** (20.5 mg, 54.7 μmol) at 0°C, then the solution was stirred for 2 h at the temperature. A crude mixture containing **21** was obtained after concentration, which was used for the next reaction without further purification. The residue was dissolved in 0.5 mL of CH_3CN, 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_3CN and MeOH to give pure sample of **22** (14.9 mg, 43.3 μmol) in 79% yield; mp 207–209°C; $[\alpha]_D^{25}$ −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 $C_{14}H_{24}N_4NaO_6$ 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), 3.78 (2H, m), 3.43 (4H, t, J=6.4 Hz), 1.93 (6H, s), 1.68–1.51 (8H, m); ^{13}C-NMR (150 MHz, DMSO-d_6) δ: 170.7, 168.4, 54.3, 47.3, 30.8, 22.5, 20.9.

Cells and Reagents Human lung cancer cell line A549 was obtained as described.[22] 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, ITH (5 μg/mL insulin, 5 μg/mL transferrin, and 1.4 μM hydrocortisone), and 5 ng/mL basic-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 5×10^3 cells per well in 0.1 mL of DMEM supplemented with 1% D-FBS and ITH. Test samples were added into the well and the stromal cells were cultured for 2 d. Then 10 μL of cancer cell suspension (5×10^3) 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 rhodanile blue dye as described.[8] 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% rhodanile 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.

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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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