Tyrosine Derivatives Isolated from *Streptomyces* sp. IFM 10937 in a Screening Program for TRAIL-Resistance-Overcoming Activity

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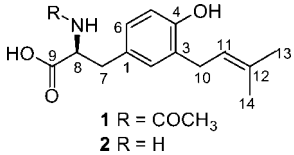
Received July 12, 2008

Exploration of actinomycetes for isolation of natural products for abrogating TRAIL resistance led to the isolation of two new tyrosine derivatives (**1** and **2**) along with novobiocin (**3**). The structures of **1** and **2** were determined by spectroscopic methods, while the absolute configuration was determined by analyzing CD spectra and by a modified Marfey's method. Compounds **1** (150 μM) and **3** (37.5 and 75 μM) in combination with TRAIL showed synergistic activity in sensitizing TRAIL-resistant human gastric adenocarcinoma cells.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) has emerged as an attractive antineoplastic agent because of its ability to selectively kill tumoral cells.[1] TRAIL-induced apoptosis initiated by the death-receptor pathway involves death receptor (DR4/TRAIL-R1 and DR5/TRAIL-R2) engagement, death-inducing signaling complex (DISC) formation, proteolytic activation of caspase-8, and, consequently, activation of effector caspase-3.[2] However, it has become a problem that considerable numbers of cancer cells, especially some highly malignant tumors, are resistant to apoptosis induction by TRAIL.[3] A search for compounds capable of abrogating TRAIL resistance has, thus, become an important strategy for anticancer drug discovery.[4] Different chemotherapeutic agents including some natural products are reported to overcome TRAIL resistance.[1,5] Epigallocatechin-3-gallate, resveratrol, and curcumin are able to sensitize TRAIL-resistant prostate cancer LNCaP cells and can be used in combination with TRAIL for prostate cancer prevention and therapy.[5,6] BB-1, a dihydroflavonol, sensitizes TRAIL-resistant leukemia cells and may be used for the treatment of leukemia.[7] Combined treatment with tunicamycin and TRAIL may be a promising candidate for prostate cancer therapy.[8] Sulforaphane in combination with TRAIL may be a safe strategy for treating resistant hepatomas, as it induces rapid apoptosis in TRAIL-resistant hepatoma cells.[9] Since actinomycetes are a potent and prolific source of secondary metabolites,[10] we explored active strains to isolate compounds for abrogating TRAIL resistance. After an initial screening of the actinomycetes, collected from different areas of Japan, the active strains were selected for further separation. TRAIL-resistance-overcoming activity of the extracts and isolates was assessed by comparison of cell growth inhibitory activity in the presence and absence of TRAIL against TRAIL-resistant human gastric adenocarcinoma (AGS) cell lines. Bioassay-guided fractionation of the fermentation broths of the soil actinomycetes, identified as *Streptomyces* sp. IFM 10937, led to the isolation of two new tyrosine derivatives, **1** and **2**, from its *n*-BuOH extract, and novobiocin (**3**)[11] from an EtOAc extract. Here, we report the structure elucidation of **1** and **2** along with the activity of the isolates (**1**−**3**) in overcoming TRAIL resistance in AGS cells.



1 R = $COCH_3$
2 R = H

Compound **1** was isolated as optically active, colorless solid and was assigned the molecular formula $C_{16}H_{21}NO_4$ on the basis of

Table 1. NMR Spectroscopic Data (CD_3OD) for **1** and **2**

	1[a]		**2**[b]	
position	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)
1	130.2, qC		129.8, qC	
2	131.5, CH	6.88, d (2.0)	131.5, CH	6.99, d (2.0)
3	128.9, qC		128.6, qC	
4	154.6, qC		155.6, qC	
5	115.7, CH	6.62, d (8.3)	116.2, CH	6.71, d (8.3)
6	128.4, CH	6.86, dd (8.3, 2.0)	127.6, CH	6.93, dd (8.3, 2.0)
7	38.2, CH_2	3.09, dd (13.7, 4.4) 2.79, dd (13.7, 8.5)	37.6, CH_2	3.20, dd (14.4, 4.1) 2.83, dd (14.4, 9.0)
8	57.8, CH	4.39, dd (8.5, 4.4)	57.9, CH	3.67, dd (9.0, 4.1)
9	172.7, qC		174.0, qC	
10	29.3, CH_2	3.23, d (7.1)	29.4, CH_2	3.27, d (7.1)
11	124.3, CH	5.19, t (7.4)	123.9, CH	5.32, t-like (7.4)
12	132.6, qC		132.9, qC	
13	25.9, CH_3	1.71, s	26.0, CH_3	1.71, br s
14	17.9, CH_3	1.69, s	17.8, CH_3	1.71, br s
-CO	179.8, qC			
$-COCH_3$	22.7, CH_3	1.88, s		

[a] Recorded at 600 MHz. [b] Recorded at 400 MHz.

Figure 1. Key HMBC (black arrows) and COSY (bold lines) correlations of **1**.

(−)-HRFABMS and ^1H and ^{13}C NMR spectroscopic data. The IR spectrum showed a strong absorption band at 1651 cm^{-1}, suggesting the presence of one or more carbonyl groups. The ^1H NMR spectrum (CD_3OD) exhibited signals of four sp^2 methine (δ_H 6.88, 6.86, 6.62, and 5.19), one aliphatic methine (δ_H 4.39), two sp^3 methylene [δ_H 3.23 (2H); 3.09 and 2.79], and three methyl carbon singlets [δ_H 1.88, 1.71, and 1.69 (each 3H)]. The ^{13}C NMR (CD_3OD) spectrum exhibited 16 signals, of which 10 resonated between δ_C 115.7 and 179.8. The other six included one aliphatic methine (δ_C 57.8), two methylenes (δ_C 38.2 and 29.3), and three methyls (δ_C 25.9, 22.7, and 17.9) (Table 1). Carbons with their attached protons were assigned by the HMQC experiment. The chemical shifts of the quaternary carbons represented two carbonyls (δ_C 179.8 and 172.7) and four sp^2 carbons at δ_C 154.6, 132.6, 130.2, and 128.9. From HMBC and COSY correlations (Figure 1) and from their coupling constants, three methine signals at δ_H 6.88 (d, J = 2.0, 1H), 6.86 (dd, J = 8.3, 2.0, 1H), and 6.62 (d, J = 8.3, 1H) with their corresponding carbons (δ_C 131.5, 128.4, and 115.7) were assigned to a 1,2,4-trisubstituted benzene ring. This system was oxygenated at C-4 on the basis of HMBC correlations from both H-2 (δ_H 6.88) and H-6 (δ_H 6.86) to a carbon at δ_C 154.6. It

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10.1021/np8004248 CCC: $40.75
Published on Web 10/11/2008

was further supported by the proton signal of a phenolic hydroxy group at δ_H 9.00 in DMSO-d_6. H-2 and H-6 showed HMBC correlations to a carbon at δ_C 38.2 (C-7), to which the diastereotopic protons δ_H 2.79 (H-7a) and 3.09 (H-7b) were attached. Both H-7a and H-7b showed HMBC correlations with the carbons at δ_C 130.2 (C-1), 131.5 (C-2), and 128.4 (C-6). These findings confirmed that C-7 was a benzylic carbon attached to C-1. The COSY spectrum and $^1H-^1H$ coupling constants revealed that the diastereotopic protons H-7a [δ_H 2.79 (dd, J = 13.7, 8.5 Hz, 1H)] and H-7b [δ_H 3.09 (dd, J = 13.7, 4.4 Hz, 1H)] were positioned next to the aliphatic methine proton H-8 [δ_H 4.39 (dd, J = 8.5, 4.4 Hz, 1H)], which was attached to the carbon at δ_C 57.8 (C-8). These characteristic data suggested that compound **1** was a derivative of the α-amino acid tyrosine.[12] HMBC correlations from H-7a, H-7b, and H-8 to a carboxylic acid carbonyl at δ_C 172.3 provided further proof for this assignment. From HMBC and COSY correlations the methine signal at δ_H 5.19 (t-like, J = 7.4 Hz, 1H) attached to δ_C 124.3, the methylene signal at δ_H 3.23 (d, J = 7.1 Hz, 2H) attached to δ_C 29.3, and two methyl singlets at δ_H 1.71 and 1.69 with respective carbons at δ_C 25.9 and 17.9 were assigned to a dimethylallyl group. The allylic methylene protons H-10 (δ_H 3.23, 2H) attached to the carbon at δ_C 29.3 showed HMBC correlations with C-2, C-3, and C-4, suggesting its attachment to C-3 of the benzene ring. This assignment was further supported by the HMBC correlation of the allylic methine H-11 (δ_H 5.19) to C-3. The methyl singlet at δ_H 1.88 (3H) showed HMBC correlation only with the carbonyl carbon at δ 179.8 and was thus assigned to an acetyl moiety. Although no 2D NMR correlations were observed between the acetyl moiety and the amino acid skeleton, the best possibility of its linkage was to the amino group of the substance forming an amide linkage (−NHCO−). This was supported by the relatively lower field (δ_H 4.39) H-8 signal of **1**, compared to that of 3-chlorotyrosine, where the same proton resonated at δ_H 3.37,[13] and that of compound **2** resonated at δ_H 3.67 (*vide infra*).

Compound **2** was isolated as an optically active, colorless solid and was assigned the molecular formula $C_{14}H_{19}NO_3$ on the basis of (−)-HRFABMS and ^1H and ^{13}C NMR spectroscopic data. The IR spectrum showed a strong absorption band at 1606 cm^{-1} indicating the presence of a carbonyl group. The ^1H NMR spectrum (CD_3OD) exhibited signals of four sp^2 methine (δ_H 6.99, 6.93, 6.71, and 5.32), one aliphatic methine (δ_H 3.67), two sp^3 methylene [δ_H 3.27 (2H); 3.20 and 2.83], and two methyl (δ_H 1.71, br s, 6H) carbons. The ^{13}C NMR (CD_3OD) spectrum exhibited 14 signals, of which nine resonated between δ_C 116.2 and 174.0. The remaining five carbons included one aliphatic methine (δ_C 57.8), two methylenes (δ_C 38.2 and 29.3), and two methyls (δ_C 26.0 and 17.8) (Table 1). These data were similar to those of compound **1** except for the absence of signals due to an acetyl moiety (carbonyl carbon at δ_C 179.8, a methyl singlet at δ_C 22.7 with its corresponding proton at δ_H 1.88 observed for **1**) and an upfield shift of H-8 at δ_H 3.67 (dd, J = 9.0, 4.1 Hz, 1H). This further supported the assignment of the acetyl moiety as attached to the amino group of **1**, where the H-8 proton signal resonated at δ_H 4.39. The absence of the acetyl moiety in compound **2** conferred a free amino group, which caused an upfield shift of proton H-8 at δ_H 3.67.[12,13]

The absolute configuration of compounds **1** and **2** was first proposed by comparing their CD spectra with that of 3-chloro-L-tyrosine.[14] The CD of **1** showed positive Cotton effects at 210 nm ([θ] +32 477) and 235 nm ([θ] +10 981), and **2** showed Cotton effects at 208 nm ([θ] +22 213) and 234 nm ([θ] +4796). 3-Chloro-L-tyrosine showed Cotton effects at 208 nm ([θ] +27 477) and 232 nm ([θ] +10 852). These observations suggested that the absolute configurations of compounds **1** and **2** were L-tyrosine related, and thus these compounds were assigned an 8*S* configuration.

The absolute configuration of **2** was further confirmed by a modification of Marfey's method,[15a] using D-FDLA (1-fluoro-2,4-dinitrophenyl-5-D-leucinamide) and L-FDLA as derivatizing agents.[15b] In this method, the resolution between the L- and D-amino acid derivatives was due to the difference in their hydrophobicity, which was derived from the *cis*- or *trans*-type arrangement of two more hydrophobic substituents at both α-carbons of an amino acid and leucinamide.[15c] The FDLA derivative of the *cis* (*Z*)-type arrangement interacts more strongly with ODS silica gel and has a longer retention time than that of the *trans* (*E*)-type arrangement. Generally, L-FDLA derivatives of L-amino acids (**L-L** type) confer *trans*-type arrangements and the D-FDLA derivatives (**D-L** type) confer *cis*-type arrangements. Therefore, the **L-L**-type complex is eluted earlier than the **D-L** type. When **2** was derivatized with L- and D-FDLA, respectively, the L-FDLA derivative (**L-2**) was eluted earlier (61.72 min) than its D-FDLA derivative, **D-2** (66.12 min). These results indicated that **L-2** had a *trans*-type arrangement and **D-2** had a *cis*-type arrangement. These results suggested the configuration of tyrosine in **2** was L,[15d] and thus its absolute configuration was confirmed as 8*S*.

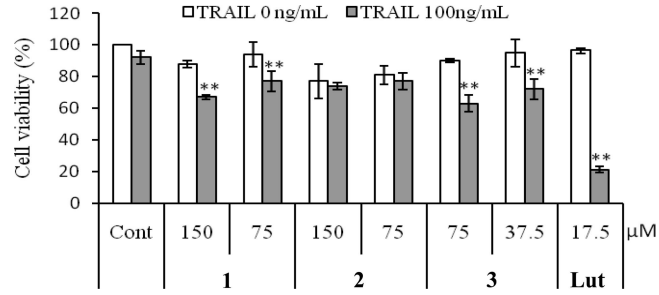

Figure 2. Effect of the isolated compounds **1**−**3** and luteolin (positive control: Lut) and TRAIL treatment, alone and in combination, on the viability of AGS cells. Cells were seeded in a 96-well culture plate (6×10^3 cells per well) for 24 h and then treated with indicated concentrations (μM) of the isolates and/or TRAIL for 24 h. Cell viability was determined by fluorometric microculture cytotoxicity assay (FMCA). The bar represents the means ($n = 3 \pm$ SD). Significance was determined by Student's t-test, $p < 0.01$ (**) vs control (Cont).

The isolated compounds (**1**−**3**) were evaluated for their activity in overcoming TRAIL resistance in AGS cells. Recently, this cell line has been widely used as a model system for evaluating cancer cell apoptosis[16] and is reported to be refractory to apoptosis induction by TRAIL.[17] To assess the effects of **1**−**3**, TRAIL, or their combined treatment on cell viability, AGS cells were treated with the indicated agents and subjected to FMCA.[18] As shown in Figure 2, treatment with 100 ng/mL TRAIL for 24 h resulted in only a slight decrease in cell viability (92 ± 4%), which was similar to the effects of **1** and **3** alone. Luteolin, used as positive control, produced about 70% more inhibition along with TRAIL than the TRAIL alone. Treating cells with 100 ng/mL TRAIL and 75 or 150 μM **1** reduced cell viability to 77 ± 7% and 67 ± 5% of control levels ($p < 0.01$), respectively, which was 15 and 25% more than the TRAIL alone, suggesting a possible synergism between the two agents. Combined treatment with TRAIL and **2** did not produce any significant reduction in the cell viability, suggesting its inactivity in overcoming TRAIL resistance. On the other hand, treatment with TRAIL and 37.5 or 75 μM **3** reduced cell viability to 72 ± 6% and 63 ± 5% of controls ($p < 0.01$), respectively, which was 20 and 29% more than the TRAIL alone. These results tend to suggest its possible synergistic activity in combination with TRAIL against AGS cells.

Resistance of cancer cells toward TRAIL may occur at different points in the TRAIL-induced apoptotic pathways. Understanding the mechanisms of such resistance and developing strategies to overcome it are important for the successful use of TRAIL in cancer therapy.[19] Combined treatment of TRAIL and chemotherapeutic agents including some natural products can overcome such resistance and sensitize TRAIL-resistant cells to enhance the therapeutic potential of TRAIL against the cancer cells. Therefore, a natural product producing

synergistic activity with TRAIL would be a new tool for cancer therapy.[20] In this study, **1** along with TRAIL showed synergistic activity against AGS cells at a higher concentration of 150 μM. A cyclic peptide, isolated from the mold *Aspergillus flavipes*, containing 3-prenyl-β-hydroxytyrosine, a compound structurally related to **1**, has been reported as a competitive antagonist of NK1 and NK2 receptors.[21] No other structurally similar compounds to **1** are, however, reported so far. Compound **3** (novobiocin) along with TRAIL produced synergistic activity against AGS cells at concentrations of 37.5 and 75 μM. Novobiocin, a topoisomerase II inhibitor, has been reported to potentiate the cytotoxicity of some chemotherapeutic agents such as etoposide and teniposide,[22] epirubicin and doxorubicin,[23] and *cis*-platin.[24] Here, we report for the first time the synergistic activity of **3** in sensitizing TRAIL-resistant AGS cells, thereby suggesting its possible use in combination with TRAIL against human gastric adenocarcinoma.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were measured on ATR in a JASCO FT-IR 230 spectrophotometer. UV spectra were obtained on a Shimadzu UV mini-1240 spectrometer. CD spectra were obtained in a JASCO J-720WI spectropolarimeter. The NMR spectra were recorded on JEOL A 400 and ECP 600 spectrometers with deuterated solvents, the chemical shift of which was used as an internal standard. (−)-HRFABMS was measured on a JEOL HX-110A spectrometer.

Microbial Strain. *Streptomyces* sp. IFM 10937 was separated on humic acid-vitamin (HV) agar,[25] a medium for the selective isolation of soil actinomycetes, from a soil sample collected at Chiba City, Japan, in 2007. The identification was carried out by Professor Yuzuru Mikami, Medical Mycology Research Center, Chiba University, Japan, where a voucher specimen was deposited with the code IFM 10937.

Fermentation. Spores of the strain growing on solid Waksman medium were inoculated into a 1 L round-bottom flask containing 100 mL of liquid medium[26] and then incubated at 28 °C for 5 days with reciprocal shaking at 200 rpm to produce seed culture. The seed culture (10 mL) was then inoculated into each of 10 3-L flasks, each containing 500 mL of liquid Waksman medium, and incubated at 28 °C for 7 days with reciprocal shaking at 200 rpm to get 5 L of fermentation broths.

Extraction and Isolation. Fermentation broths (5 L) thus obtained were centrifuged, and the supernatant was concentrated to 1 L and partitioned between EtOAc and *n*-BuOH. The EtOAc extract (50 μg/mL produced 30% more inhibition than the TRAIL alone), subjected to ODS flash column chromatography (65% MeOH), yielded 153 mg of novobiocin (**3**). The *n*-BuOH extract (200 μg/mL produced 25% more inhibition), subjected to ODS flash column chromatography (75% MeOH), afforded active fractions A (produced 23% more inhibition at 200 μg/mL) and B (produced 35% more inhibition at 200 μg/mL). Fraction A (3 g) was subjected to silica gel column chromatography ($CHCl_3$−MeOH, 3:1 → 0:1) followed by ODS HPLC (50% MeOH, Develosil ODS HG-5, 10 × 250 mm, flow rate 1.5 mL/min) to yield compound **1** (5 mg, t_R 15 min). Fraction B (200 mg) was subjected to ODS HPLC (65% MeOH, Develosil ODS HG-5, 10 × 250 mm, flow rate 1.2 mL/min) to yield compound **2** (3.5 mg, t_R 20 min).

Marfey Analysis. To 50 μL of aqueous solution (2 mg/mL) of **2** were added 20 μL of 1 M $NaHCO_3$ and then 100 μL of 1% (w/v) FDLA (Tokyo Chemical Industry Co. Ltd., Japan) in acetone. The solution was vortexed and incubated at 37 °C for 1 h, after which the reaction was quenched with 20 μL of 1 M HCl. Samples were diluted with 810 μL of acetonitrile, and 10 μL of this solution was analyzed by reversed-phase HPLC systems (Shimadzu, Japan). Separations were carried out on a Capcell Pak C18-ACR (250 mm × 4.6 mm) column, heated at 40 °C. For gradient elution in HPLC, mobile phase A was prepared from 0.1 M NH_4OAc in aqueous solution, adjusted to pH 3−4 by addition of TFA, and the mobile phase B was acetonitrile. In all cases, linear gradients started with 15% B and finished with 100% B in 85 min. The system was allowed to equilibrate for 10 min at 15% B prior to the next analysis. The flow rate was 0.75 mL/min, with UV detection at an absorbance of 340 nm by photodiode array detection.

Fluorometric Microculture Cytotoxicity Assay (FMCA). AGS cells were seeded in a 96-well culture plate (6×10^3 cells per well) in 200 μL of RPMI medium containing 10% FBS. Cells were incubated at 37 °C in a 5% CO_2 incubator for 24 h. Then the test samples with or without TRAIL (100 ng/mL) at different doses were added to each well. After 24 h incubation, the cells were washed with PBS, and 200 μL of PBS containing fluorescein diacetate (10 μg/mL) was added to each well. The plates were then incubated at 37 °C for 1 h, and fluorescence was measured in a 96-well scanning spectrofluorometer at 538 nm, following excitation at 485 nm.

Compound 1: colorless solid; $[\alpha]^{17}_D$ −8.7 (*c* 0.28, MeOH); UV (MeOH) λ_{max} (log ε) 211 (3.9) and 263 (3.2) nm; CD (*c* 0.48 μM, CH_3OH) nm $\Delta_{\varepsilon 210}$ +32 477, $\Delta_{\varepsilon 235}$ +10 981; IR (ATR) ν_{max} 3281 (br), 2929, 1651, 1437, and 1266 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; (−)-FABMS *m/z* 290 $[M - H]^-$; (−)-HRFABMS *m/z* 290.1388 $[M - H]^-$ (calcd for $C_{16}H_{20}NO_4$, 290.1392).

Compound 2: colorless solid; $[\alpha]^{17}_D$ −43.8 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.7) and 279 (2.8) nm; CD (*c* 0.75 μM, CH_3OH) nm $\Delta_{\varepsilon 208}$ +22 213, $\Delta_{\varepsilon 234}$ +4796; IR (ATR) ν_{max} 3252(br), 2928, 1606, 1508, 1399, and 1267 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; (−)-FABMS *m/z* 248 $[M - H]^-$; (−)-HRFABMS *m/z* 248.1290 $[M - H]^-$ (calcd for $C_{14}H_{18}NO_3$, 248.1287).

Acknowledgment. We are thankful to Professor Yuzuru Mikami (Medical Mycology Research Center, Chiba University) for the identification of *Streptomyces* sp. IFM 10937. This work was partly supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Futaba Electronics Memorial Foundation, and the Japan Science and Technology Agency.

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(26) Liquid Waksman medium (100 mL): glucose (2 g), meat extract (0.5 g), peptone (0.5 g), dried yeast (0.3 g), NaCl (0.5 g), and $CaCO_3$ (0.3 g); agar (1.5 g) was added for solidification.

NP8004248