

Structure of Tyroscherin, an Antitumor Antibiotic against IGF-1-dependent Cells from *Pseudallescheria* sp.

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An antitumor antibiotic, tyroscherin, was isolated from the culture of a fungus identified as *Pseudallescheria* sp. The structure of tyroscherin including the absolute stereochemistry was determined as shown in Fig. 1 by NMR and degradation studies. Tyroscherin selectively inhibited IGF-1-dependent growth of MCF-7 human breast cancer cells with an IC₅₀ of 9.7 ng/ml.

Insulin-like growth factors (IGFs) play a key role in human cancer progression¹⁾. IGF signals through IGF-1 receptor are known to be significant for tumor cell growth and survival. Thus, selective inhibitors of IGF signal transduction are expected to be new anticancer agents against IGF-dependent tumor cells. In the course of our screening for inhibitors of IGF-dependent cell growth or survival, a fungal strain identified as *Pseudallescheria* sp. was found to produce an active substance designated tyroscherin (Fig. 1).

Fermentation and Isolation

The producing organism was cultured in 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of dextrin 4.0%, glucose 1.0%, malt extract 0.5%, Polypepton 0.5%, soybean meal 0.5%, yeast extract 0.2% and K₂HPO₄ (pH 6.5) on a rotary shaker at 24°C for 6 days.

The whole broth (2 liters) was centrifuged and the mycelium was extracted with acetone. The extract was

evaporated to an aqueous concentrate and then partitioned between ethyl acetate and water. The organic layer was subjected to silica gel column chromatography with chloroform-methanol (5 : 1). The active eluate was purified by HPLC using a Senshu Pak PEGASIL ODS column with 65% methanol containing 0.1% trifluoroacetic acid to give a colorless powder of tyroscherin (13.5 mg).

Physico-chemical Properties

The physico-chemical properties of tyroscherin are summarized in Table 1. The molecular formula of

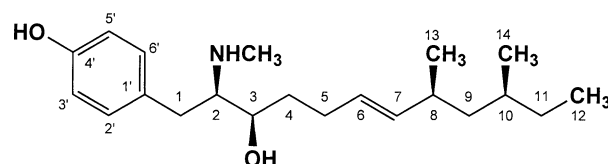


Fig. 1. Structure of tyroscherin.

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Table 1. Physico-chemical properties of tyroscherin.

Appearance	colorless powder
MP	123 ~ 127°C
$[\alpha]_D^{24}$	-21°(c 0.35, MeOH)
Molecular formula	C ₂₁ H ₃₅ NO ₂
FAB-MS (<i>m/z</i>)	334.2740 (M+H) ⁺ 334.2746 (calcd.)
UV λ_{\max} nm(ϵ)	
MeOH	225 (5,500), 278 (1,100)
0.01 M HCl-MeOH	225 (5,500), 278 (1,100)
0.01 M NaOH-MeOH	227 (4,300), 240 (3,600), 286 (1,100)
IR ν_{\max} (KBr) cm ⁻¹	3200, 1680, 1210, 1140

tyroscherin was established to be C₂₁H₃₅NO₂ by high-resolution FAB-MS.

Structure Elucidation

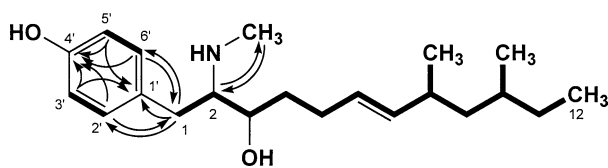
The ¹³C NMR spectrum of tyroscherin confirmed the presence of 21 carbons and a heteronuclear multiple-quantum coherency (HMQC)²⁾ experiment established all one-bond ¹H-¹³C connectivities (Table 2). A COSY experiment revealed two spin networks including a *trans* olefin (*J*=15.0 Hz) as shown in Fig. 2. Long-range couplings observed in the heteronuclear multiple-bond correlation (HMBC)³⁾ spectrum established the connection of the two partial structures (Fig. 2). A ¹H-¹³C long-range correlation between a singlet methyl (δ_H 2.62, δ_C 32.4) and a methine (δ_H 3.34, δ_C 66.7) revealed the presence of a methylamino group at C-2. Low-field ¹³C chemical shifts for C-3 (δ 68.7) and C-4' (δ 157.9) indicated the substitution of a hydroxyl group. These results established the planar structure of tyroscherin (Fig. 2), which is identical with that of an antifungal antibiotic, JM971B⁴⁾. However, neither stereochemistry nor spectral data have been reported for JM971B.

The relative stereochemistry of tyroscherin was analyzed by ¹H-¹H and ¹H-¹³C coupling constants⁵⁾. Both 2-H and C-1 exhibited small three-bond couplings with 3-H (*J*_{2H-3H} = 3.0 Hz, *J*_{C1-3H} < 3.0 Hz) and were required to be *gauche* to 3-H. A small two-bond coupling constant between 2-H and C-3 (< 3.0 Hz) revealed an *anti* relationship between 2-H and 3-O (Fig. 3). Each *anti* orientation between 9-Ha and 8-

Table 2. ¹³C and ¹H NMR data summary for tyroscherin in methanol-*d*₄.

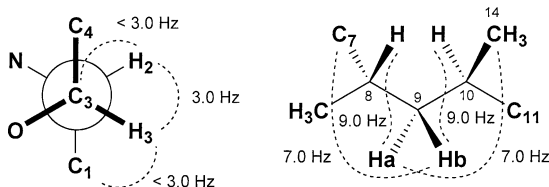
No.	δ_C	δ_H (multiplicity, <i>J</i> = Hz)
1	32.4	2.86 (dd, 14.0, 7.0) 2.91 (dd, 14.0, 7.0)
2	66.7	3.34 (ddd, 7.0, 7.0, 3.0)
3	68.7	3.83 (dt, 10.0, 3.0)
4	33.0	1.52 (m)
5	29.8	2.18 (m) 1.97 (m)
6	128.4	5.33 (dt, 15.0, 7.0)
7	138.8	5.22 (dd, 15.0, 8.0)
8	35.8	2.16 (m)
9	45.5	1.22 (ddd, 14.0, 9.0, 4.0) 0.98 (ddd, 14.0, 9.0, 4.0)
10	33.2	1.30 (m)
11	31.1	1.28 (m) 1.12 (m)
12	11.6	0.84 (3H, t, 7.0)
13	22.3	0.91 (3H, d, 6.5)
14	17.3	0.82 (3H, d, 6.5)
1'	127.6	
2', 6'	131.8	7.10 (2H, d, 8.0)
3', 5'	116.9	6.76 (2H, d, 8.0)
4'	157.9	
N-Me	32.4	2.62 (3H, s)

Fig. 2. COSY and HMBC data summary for tyroscherin.



Bold lines show proton spin networks and arrows indicate ^1H - ^{13}C long-range correlations.

Fig. 3. Stereochemical assignments from ^1H - ^1H and ^1H - ^{13}C coupling constants.

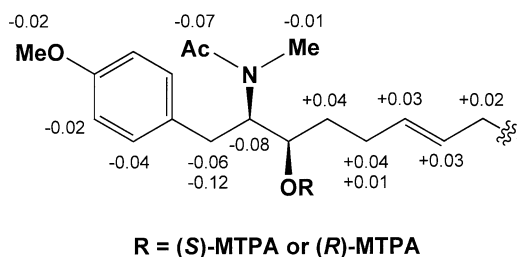


H, between 9-Hb and 10-H, between 9-Ha and C-14, and between 9-Hb and C-7 was determined from large three-bond couplings ($J_{9\text{Ha}-8\text{H}}=9.0$ Hz, $J_{9\text{Hb}-10\text{H}}=9.0$ Hz, $^3J_{9\text{Ha}-\text{C}14}=7.0$ Hz, $^3J_{9\text{Hb}-\text{C}7}=7.0$ Hz). These relationships assigned the relative stereochemistry of tyroscherin to ($2R^*,3R^*,8R^*,10R^*$) or ($2R^*,3R^*,8S^*,10S^*$) as shown in Fig. 3.

The absolute stereochemistry at C-3 was elucidated by the modified Mosher method⁶⁾. Both the (*S*)- and (*R*)-2-methoxy-2-(trifluoromethyl)phenyl acetate (MTPA) esters of *N*-acetyl-4'-*O*-methyltyroscherin were prepared and subjected to ^1H NMR analysis. In the (*R*)-derivative spectrum, 4-H₂, 5-H₂, 6-H, 7-H and 8-H appeared upfield relative to the (*S*)-derivative one, while 1-H₂, 2-H, 2'-H, 3'-H, 5'-H, 6'-H, 4'-*O*-CH₃, *N*-CH₃ and *N*-COCH₃ exhibited downfield shifts in the (*R*)-derivative spectrum (Fig. 4). These data allowed the absolute configuration at C-3 to be assigned as *R*.

To determine the absolute stereochemistry at C-8, 2,4-dimethylhexanol was prepared by ozonolysis of tyroscherin. Its 3,5-dinitrobenzoate displayed a positive optical rotation ($[\alpha]_{\text{D}}^{23} +4.6^\circ$ (c 0.044, CH₂Cl₂)) and was required to be (*2S,4S*)-2,4-dimethylhexyl 3,5-dinitrobenzoate (literature value⁷⁾: $[\alpha]_{\text{D}}^{20} +5.71^\circ$ (c 2.1, CH₂Cl₂)). These results identified the absolute

Fig. 4. Stereochemical analysis by the modified Mosher method.



R = (*S*)-MTPA or (*R*)-MTPA

$\Delta\delta$ ($\delta_{S\text{-MTPA}} - \delta_{R\text{-MTPA}}$) values are expressed in ppm.

configurations of tyroscherin as $2R$, $3R$, $8S$ and $10S$ (Fig. 1).

Tyroscherin inhibited the growth of MCF-7 human breast cancer cells in a serum-free medium containing IGF-1 (30 ng/ml) with an IC₅₀ of 9.7 ng/ml. In the presence of 0.5% fetal bovine serum instead of IGF-1, tyroscherin exhibited no activity against MCF-7 cells at less than 1 $\mu\text{g}/\text{ml}$, although serum pretreatment did not reduce the activity of tyroscherin. This substance showed a similar effect on IGF-dependent T47D human breast cancer cells (IC₅₀ 32 ng/ml). Such a selective activity of tyroscherin was not observed in other human cell lines including MDA-MB-231 breast cancer, HBC-4 breast cancer, Colo320DM colon cancer, HeLa cervical cancer and Saos-2 osteosarcoma (IC₅₀ 2.2~9.7 $\mu\text{g}/\text{ml}$). Further biological studies on tyroscherin are in progress.

Experimental

General

UV and IR spectra were measured on Hitachi U-3210 and JASCO FT/IR-470 spectrometers, respectively. Mass spectra were obtained on a JEOL HX-110 spectrometer in the FAB mode using *m*-nitrobenzyl alcohol as matrix and polyethylene glycol as internal standard. Optical rotations were recorded on a JASCO DIP-1000 spectropolarimeter. ^1H and ^{13}C NMR spectra were measured on a JEOL JNM-A500 spectrometer with ^1H NMR at 500 MHz and ^{13}C NMR at 125 MHz. Chemical shifts are given in ppm using TMS as internal standard. ^1H - ^{13}C long-range coupling constants were obtained by a *J*-resolved HMBC experiment⁸⁾.

Preparation of the MTPA Esters of *N*-Acetyl-4'-*O*-metyltyroscherin

Acetic anhydride (1 ml) was added to a solution of tyroscherin (5 mg) in methanol (1 ml), and the mixture was stirred at room temperature for 10 hours. After evaporation, the residue was treated with 2.0 M (trimethylsilyl)diazomethane in diethyl ether (1 ml) at room temperature for 4 hours. The reaction mixture was evaporated and dissolved in pyridine (1 ml). To half of the solution was added (*R*)-(-)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride (25 mg). The mixture was allowed to stand at room temperature for 1 hour. After evaporation, the residue was purified by HPLC using a Senshu Pak PEGASIL ODS column to give the (*S*)-MTPA ester of *N*-acetyl-4'-*O*-metyltyroscherin (1.0 mg). Similarly, the (*R*)-MTPA ester of *N*-acetyl-4'-*O*-metyltyroscherin (0.8 mg) was obtained using (*S*)-(+)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride.

(*S*)-MTPA Ester of *N*-Acetyl-4'-*O*-metyltyroscherin

$^1\text{H NMR}$ (CDCl_3): δ 7.60 (2H, m, MTPA), 7.39 (3H, m, MTPA), 6.92 (2H, d, 2'-H, 6'-H), 6.74 (2H, d, 3'-H, 5'-H), 5.26 (m, 3-H), 5.21 (m, 6-H), 5.16 (dd, 7-H), 3.73 (3H, s, 4'-OMe), 3.67 (m, 2-H), 3.58 (3H, s, MTPA), 2.79 (3H, s, *N*-Me), 2.54 (3H, s, *N*-Ac), 2.53 (dd, 1-Ha), 2.47 (dd, 1-Hb), 2.12 (m, 8-H), 1.99 (m, 5-Ha), 1.94 (m, 5-Hb), 1.66 (2H, m, 4-H₂), 1.26 (3H, m, 10-H₂, 11-Ha), 1.19 (m, 9-Ha), 1.10 (m, 11-Hb), 0.97 (m, 9-Hb), 0.90 (3H, d, 13-H₃), 0.82 (3H, t, 12-H₃), 0.79 (3H, d, 14-H₃).

(*R*)-MTPA Ester of *N*-Acetyl-4'-*O*-metyltyroscherin

$^1\text{H NMR}$ (CDCl_3): δ 7.58 (2H, m, MTPA), 7.42 (3H, m, MTPA), 6.96 (2H, d, 2'-H, 6'-H), 6.76 (2H, d, 3'-H, 5'-H), 5.22 (m, 3-H), 5.18 (m, 6-H), 5.13 (dd, 7-H), 3.75 (3H, s, 4'-OMe), 3.75 (m, 2-H), 3.53 (3H, s, MTPA), 2.80 (3H, s, *N*-Me), 2.61 (3H, s, *N*-Ac), 2.59 (2H, m, 1-H₂), 2.10 (m, 8-H), 1.95 (m, 5-Ha), 1.93 (m, 5-Hb), 1.62 (2H, m, 4-H₂), 1.26 (3H, m, 10-H₂, 11-Ha), 1.19 (m, 9-Ha), 1.10 (m, 11-Hb), 0.97 (m, 9-Hb), 0.90 (3H, d, 13-H₃), 0.82 (3H, t, 12-H₃), 0.79 (3H, d, 14-H₃).

Preparation of 2,4-Dimethylhexaol 3,5-Dinitrobenzoate

Under dry-ice cooling, a solution of tyroscherin (9.2 mg) in dichloromethane (10 ml) was treated with ozone for 30 minutes. An aqueous solution (1 ml) of NaBH_4 (10 mg) was added to the reaction mixture, which was stirred at room temperature overnight. To the organic layer were added pyridine (0.3 ml) and 3,5-dinitrobenzoyl chloride (200 mg), and the mixture was stirred at room temperature for 1 hour. After evaporation, the residue was purified by HPLC using

a Senshu-Pak PEGASIL-ODS column with 80% methanol to give 2,4-dimethylhexanol 3,5-dinitrobenzoate (0.88 mg). $^1\text{H NMR}$ (CDCl_3): δ 9.22 (d), 9.14 (2H, m), 4.33 (dd), 4.19 (dd), 2.09 (m), 1.50 (m), 1.39 (2H, m), 1.13 (m), 1.08 (m), 1.03 (3H, d), 0.91 (3H, d), 0.87 (3H, t).

Cells and Cell Culture

Colo320DM and Saos-2 cell lines were obtained from the Japanese Cancer Research Resources Bank (JCRB). MCF-7, MDA-MB-231 and HBC-4 cell lines were provided by Dr. TAKASHI TSURUO (Institute of Molecular and Cellular Biosciences, The University of Tokyo). HBC-4 and MDA-MB-231 cells were maintained in RPMI-1640 medium and the other cell lines were cultured in DULBECCO'S modified EAGLE'S medium. These culture media were supplemented with 10% heat-inactivated fetal bovine serum. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2 . Serum-free media were supplemented with 0.1% bovine serum albumin. Cells were plated in each well of 96-well plates at the density of 2×10^4 to 2×10^5 cells/ml. After incubation with various concentrations of a sample at 37°C for 72 hours, the cells were treated with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 37°C for 1 or 2 hours. Relative cell number was measured with formazan formation at 570 nm using a multilabel counter (Wallac 1420 ARVOSx, Perkin Elmer, Inc.).

Acknowledgements

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