Tanzawaic Acids A, B, C, and D: Inhibitors of Superoxide Anion Production from *Penicillium citrinum*

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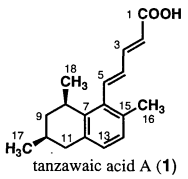
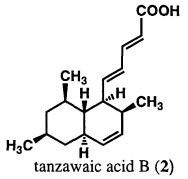
Tanzawaic acids have been isolated from *Penicillium citrinum* and their structures were elucidated by spectroscopic analysis. Their relative stereochemistries were also clarified by detailed analyses of ^{1}H-^{1}H coupling constants and NOE data. Tanzawaic acid B (GS-1302-1) significantly inhibits superoxide anion production in human neutrophils.

In our ongoing search for biologically active compounds from microorganisms,[1] we isolated four compounds, called tanzawaic acids A (GS-1302-3) (**1**), B (GS-1302-1) (**2**), C (**3**), and D (**4**), from *Penicillium citrinum* SCRC-SA124 which was collected in the Tanzawa area of Japan. Tanzawaic acids A (**1**) and B (**2**) inhibited the induction of superoxide anion (O_2^-)[2] in human neutrophils (IC_{50}: 80 μg/ml and 26 μg/ml, respectively). Superoxide anion may be closely related to inflammation, cancer and aging. Therefore, compounds that reduce the expression of O_2^- may be useful as anti-inflammatory agents.[2]

The lipophilic extract ($CHCl_3/CH_3OH$= 2/1) of whole broth of *P. citrinum* (2 L) was fractionated by column chromatography on SiO_2, using a gradient elution of chloroform and methanol. The concentrate of chloroform eluate was separated by reversed phase chromatography on ODS, using a gradient elution of methanol and water. The 75% CH_3OH/H_2O eluate was concentrated under reduced pressure and the residue was purified by preparative TLC on SiO_2 with 30% AcOEt/hexane to give **3** (1.9 mg) and **4** (5.7 mg), and a mixture of **1** and **2**. Finally, the mixture was purified by reversed phase HPLC (ODS) using a solution of 0.1% TFA in 75% CH_3OH/H_2O to afford **1** (4.3 mg) and **2** (1.4 mg). Based on extensive 1-D and 2-D NMR experiments and HREIMS spectral data, tanzawaic acids A and B were identified as GS-1302-3 and GS-1302-1, respectively, which exhibited antibacterial activity.[3] We report here the isolation and structural elucidation of new carboxylic acids, tanzawaic acids C (**3**) and D (**4**).[4,5]



tanzawaic acid A (**1**)



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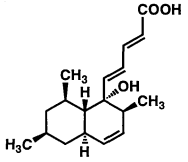
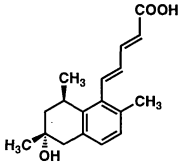


tanzawaic acid C (**3**)



tanzawaic acid D (**4**)

The molecular formula of tanzawaic acid D (**4**) was determined to be $C_{18}H_{22}O_3$ based on the HREIMS (*m/z* 286.1566, Δ - 0.1 mmu)[6] and NMR spectral data. The ^{1}H and ^{13}C NMR spectral data of tanzawaic acid D are shown in Table 1. Extensive NMR experiments (^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY and DEPT) and a detailed analysis of the results indicate that **4** has three methyl groups, two methylenes, seven methines, six quaternary carbons and two exchangeable protons.

The ^{1}H-^{1}H coupling network in the ^{1}H-NMR spectrum of this compound could not be readily revealed due to the presence of quaternary carbons. Therefore, the structural assignment was based on a detailed analysis of the ^{1}H-^{1}H COSY and HMBC spectra, which gave partial structural units **a**-**c**, as shown in Figure 1. The carbon network among C1-C5 was revealed by observation of cross peaks in the ^{1}H-^{1}H COSY spectrum (H2-H5), and the HMBC spectrum: H2/C1 (δ_C 175.9). The configurations of both double bonds (C2-C3 and C4-C5) were determined to be *E* based on the large coupling constants of H2/H3 (15.1 Hz) and H4/H5 (15.6 Hz). Tanzawaic acid D was treated with CH_2N_2/CH_2Cl_2 to give a methyl ester.[4] The presence of a carboxylic group at C1 was consistent with partial structure **a**. The carbon network among C8-C11, C8/C18, and C10/C17 in partial structure **b** was clarified by ^{1}H-^{1}H COSY cross peaks for H8/H18 and H8/H9, and HMBC cross peaks. Insertion of quaternary carbon C10 (δ_C 70.9) between C9 and C11 was indicated by the HMBC cross peaks for H9/C10, H9/C10, H11/C9, H11/C10 H17/C9, H17/C10, and H17/C11. These data indicated partial structure **b**.

The carbon network among C13-C16 was revealed by ^{1}H-^{1}H COSY cross peaks between H13 and H14, and HMBC cross peaks. The location of quaternary carbon C15 (δ_C 135.8) between C14 and C16 was verified by HMBC cross peaks for H14/C15, H14/C16, H16/C14, and H16/C15. The double bond (C13-C14) was determined to be located in the benzene ring based on the coupling constants of H13/H14 (7.3 Hz). These data suggested partial structure **c**. Three quaternary carbons were deduced to be aromatic based on their chemical shifts: δ 139.1 (C6), 141.7 (C7), and 134.6 (C12). The positions of these quaternary carbons and the connectivity of units **a**-**c** were verified by further extensive HMBC experiments. Insertion of a quaternary carbon (C6) between C5 and C15 was suggested by the HMBC cross peaks for H4/C6, H5/C6, and H16/C6. Furthermore, the HMBC cross peaks for H5/C7, H9/C7, and H18/C7 indicated that C7 was located between C6 and C8. Finally, the HMBC cross peaks for H11/C7, H11/C12, H11/C13, H13/C11, H13/C12, and H13/C7 indicated that C12 was located between C11 and C13. Eventually, the planar structure of tanzawaic acid D was assigned to be **4**.

Table 1. NMR spectral data of tanzawaic acids C and D[a]

position	tanzawaic acid (**3**) ^{1}H (J in Hz)	^{13}C (mult.)[b]	tanzawaic acid D (**4**) ^{1}H (J in Hz)	^{13}C (mult.)[b]	HMBC
1		170.8 (s)		175.9 (s)	
2	5.80(d, 15.4)	129.7 (d)	5.96 (d, 15.1)	129.3 (d)	C1, C4
3	7.09(dd, 15.4, 10.6)	141.7 (d)	7.26 (dd, 15.1, 10.7)	142.9 (d)	C1, C4, C5
4	6.38(dd, 15.4, 10.6)	125.3 (d)	6.36 (dd, 15.6, 10.7)	134.3 (d)	C2, C3, C6
5	6.30(d, 15.4)	151.0 (d)	6.96 (d, 15.6)	139.1 (d)	C3, C4, C6, C7
6		78.0 (s)		139.1 (s)	
7	1.10(dd, 10.2, 9.8,)	51.5 (d)		141.7 (s)	
8	1.60(qddd, 6.2, 12.3, 10.2, 3.6)	34.8 (d)	3.29 (qdd, 7.3, 7.8, 6.3)	30.9 (d)	C18
9 9a	0.82(ddd, 12.3, 12.3, 12.3)	50.0 (t)	1.55 (dd, 13.7, 6.3)	47.1 (t)	C7, C10, C17, C18
9b	1.63(ddd, 12.3, 5.2, 3.6)		2.04 (ddd, 13.7, 7.8, 2.8)		
10	1.57(qdddd, 6.6, 12.3, 12.3, 5.2, 3.2)	35.4 (d)		70.9 (s)	
11 11a	0.83(ddd, 12.3, 12.3, 12.3)	44.4 (t)	2.65 (d, 13.0)	45.6 (t)	C7, C9, C10, C12,
11b	1.76(ddd, 12.3, 4.8, 3.2)		2.81 (dd, 13.0, 2.8)		C13, C17
12	2.12(ddddd, 12.3, 9.8, 4.8, 2.9, 1.5)	40.7 (d)		134.6 (d)	
13	5.36(ddd, 9.9, 1.5, 1.5)	132.5 (d)	6.85 (d, 7.3)	129.5 (d)	C7, C11, C12, C15
14	5.47(ddd, 9.9, 4.8, 2.9)	131.2 (d)	6.93 (d, 7.3)	130.7 (d)	C12, C15, C16
15	1.90(qdd, 7.0, 1.5, 4.8)	48.0 (d)		135.8 (s)	
16	0.95(d, 7.0)	20.4 (q)	2.23 (s)	22.0 (q)	C6, C14, C15
17	0.82(d, 6.6)	23.5 (q)	1.34 (s)	31.7 (q)	C9, C10, C11
18	0.93(d, 6.2)	25.5 (q)	1.13 (d, 7.3)	24.1 (q)	C7, C8, C9

a) Spectra were recorded in CD_3OD on JEOL JNM-GSX400 NMR and JEOL JNM-EX400 spectrometers. b) Multiplicity was determined by DEPT experiments.

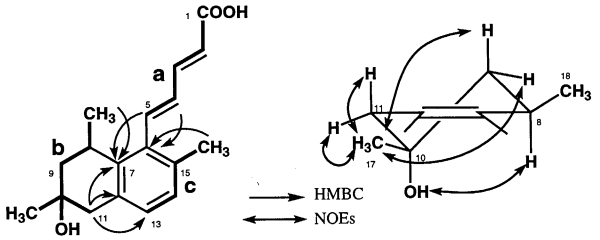


Figure 1. Planar structure and the relative stereochemistry at C_8 and C_{11} of tanzawaic acid D (**4**).

The relative stereochemistry at C8 and C10 in tanzawaic acid D (**4**) was deduced by NOE experiments of methyl tanzawaate D in the ^{1}H NMR spectrum (DMSO-d_6). The NOE cross peaks for 17-CH_3/H9, 17-CH_3/H11, and H8/10-OH undoubtedly indicated stereochemistry at C8 and C10 as shown in Figure 1. Therefore, the relative stereochemistry of tanzawaic acid D is suggested to be **4**.

The molecular formula of tanzawaic acid C (**3**) was determined to be $C_{18}H_{26}O_3$ based on the HREIMS of methyl tanzawaate C ($C_{19}H_{28}O_3$, m/z 304.2044, Δ + 0.7 mmu).[6] The ^{1}H NMR spectral data of **3** (Table 1) resembled those of **4**. All of the signals were assigned by a detailed comparison of the NMR spectral data with those of **4** and by ^{1}H-^{1}H COSY spectra. The chemical shift at C6 (δ_C 78.0 ppm) and the molecular formula of **3** indicated the presence of a hydroxyl group at C6. As a result, the planar structure of tanzawaic acid C was proposed to be **3**.

The relative stereochemistry of **3** (Figure 2) was clarified by ^{1}H-^{1}H coupling constants and NOE experiments. The stereochemistry of the cyclohexane moiety (C7-C12) was established by the large coupling constants ($J_{7,8}$ = 10.2 Hz, $J_{8,9a}$ = 12.3 Hz, $J_{9a,10}$ = 12.3 Hz, $J_{10,11a}$ = 12.3 Hz, $J_{11a,12}$ = 12.3 Hz, $J_{12,7}$ = 9.8 Hz). The NOE cross peaks among 16-CH_3 , H7 and H5 suggested the stereochemistry of the hydroxyl group at C6. Furthermore, the carbon chemical shifts of 16-CH_3 (δ_C 20.4: axial), 17-CH_3 (δ_C 23.5: equatorial), and 18-CH_3 (δ_C 25.5: equatorial) revealed the stereochemistries at C8, C10 and

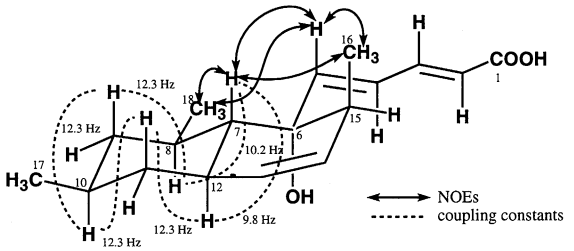


Figure 2. Relative stereochemistry of tanzawaic acid C (**3**).

C15.[7] Therefore, the relative stereochemistry of tanzawaic acid C is believed to be **3**.

Tanzawaic acids A (**1**) and B (**2**) inhibited superoxide anion production in human neutrophils induced by TPA (12-*O*-tetradecanoylphorbol-13-acetate),[8] whereas tanzawaic acids C (**3**) and D (**4**) did not. The mechanism of action and *in vivo* behavior of the samples are currently under investigation. Further studies on the detailed chemistry of tanzawaic acids, including their absolute configurations, biogenetic pathways and structure-activity relationships, are currently under way in our laboratory.

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References and Notes

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4 The methyl esters of the tanzawaic acids were obtained by treating the corresponding acid with CH_2N_2/CH_2Cl_2 at r.t. for 30 min. The resulting esters was purified by preparative TLC on SiO_2 with 50% AcOEt/hexane. The ^{1}H-NMR spectrum was measured in CD_3OD. The chemical shifts of H1' ($COOCH_3$) in the ^{1}H-NMR spectral data of the methyl esters of the tanzawaic acids are as follows:methy tanzawaate A (3.74 ppm); methyl tanzawaate B (3.73 ppm); methyl tanzawaate C (3.71 ppm); methyl tanzawaate D (3.74 ppm).

5 **1**: IR ($CHCl_3$) 3400-3100, 1690, 1625 cm^{-1}, ($CHCl_3$+Et_3N) 3400-3100, 1625 cm^{-1}, $[\alpha]_D$= + 53^o (*c* 0.43, CH_3OH); **2**: IR ($CHCl_3$) 3400-3100, 1690, 1635, 1605 cm^{-1}, $[\alpha]_D$= + 16^o (*c* 0.21, CH_3OH); **3**: IR ($CHCl_3$) 3500-3200, 1700, 1640, 1605 cm^{-1}, $[\alpha]_D$= + 2^o (*c* 0.17, CH_3OH); **4**: IR ($CHCl_3$) 3500-3100, 1640, 1570, 1400 cm^{-1}, $[\alpha]_D$= + 118^o (*c* 0.57, CH_3OH).

6 Mass spectra were recorded on an M-80B mass spectrometer.

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