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Tanzawaic acids from a marine-derived fungus of the genus *Penicillium* with cytotoxic activities

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Tanzawaic acids M (**1**), N (**2**), O (**3**) and P (**4**) and the known tanzawaic acids B (**5**) and E (**6**), have been isolated from an extract of a cultured marine-derived fungus (strain CF07370) identified as a member of the genus *Penicillium*. The structures of **1**-**4** were determined based on spectroscopic evidences. Antimicrobial and cytotoxic activities of compounds **1**-**6** were evaluated.

Introduction

Fungi synthesize some of the simplest and most complex polyketides known, many of which possess biological activities of importance to medicine and agriculture.[1] The family of tanzawaic acids is a relatively small class of polyketides isolated from fungi, mostly of the genus *Penicillium*.[2-8]

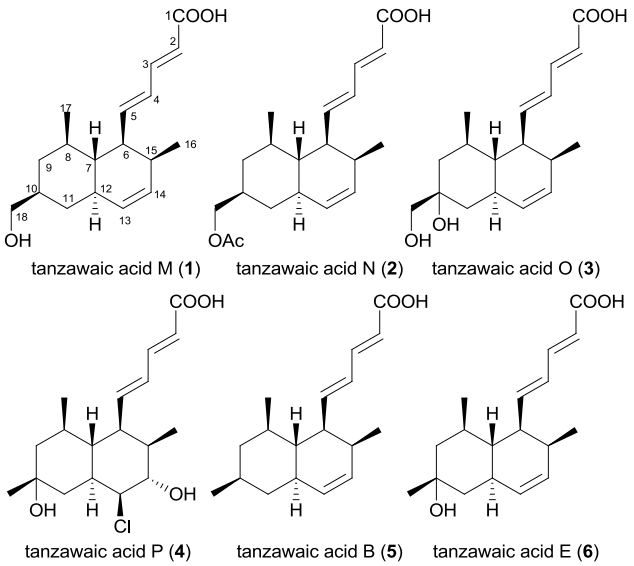

Fig. 1 Tanzawaic acids-derived metabolites from CF07370.

Structurally, these compounds possess a highly reduced polyunsaturated octaketide backbone derived from the condensation of 8 mol of acetate, plus the addition of two methyl groups at C-8 and C-10, predictably from methionine, as frequently occurs in fungal metabolism.[9] It is worth to note that they all possess a conserved configuration at C-6, C-7, C-8, C-10, C-12 and C-15, as far as determined. Some of these compounds display anticoccidial activity, inhibition of superoxide anion production, inhibition of NO production or inhibition of protein tyrosine phosphatase 1B (PTP1B).[2,5,7,8]

Here we describe the chemical structure and relative stereochemistry, propose a biogenetic hypothesis and investigate antimicrobial and cytotoxic activities of four novel tanzawaic acids M-P (**1**-**4**) (Fig. 1). Compounds were obtained from the extract of a fungal isolate strain, CF07370, isolated from a marine sediment. Along with compounds **1**-**4**, two known polyketides identified as tanzawaic acids B and E were isolated. Their structures were established based on NMR data and by comparison with those reported in the literature.[2,3]

Results and discussion

Penicillium sp., strain CF07370, Gulf of California, Mexico, was cultured in sea-water-based medium. The mycelium and the broth were extracted with ethyl acetate and the crude extract was sequentially purified by flash chromatography, Sephadex LH-20 chromatography and normal phase HPLC to yield tanzawaic acids M-P (**1**-**4**) in 4.3, 24.2, 4.6 and 2.5 mg yields, respectively (0.8 mg to 8.1 mg/L)

The molecular formula of tanzawaic acid M (**1**) was determined to be $C_{18}H_{26}O_3$ on the basis of HRESIMS measurements (*m/z* 289.1800 $[M - 1]^-$) indicating six degrees of unsaturation. The structure of **1** was established from NMR analysis using one– and two–dimensional NMR techniques including proton, carbon, COSY, HMQC, HMBC, DEPT, and NOESY. The ^{13}C NMR and DEPT spectra (Table 1) showed 18 signals assigned to 2 x CH_3, 3 x CH_2 (one geminal to hydroxyl group), 12 x CH (six olefinic), and one carbonyl quaternary carbon. The ^1H NMR spectrum displayed signals for six olefinic protons at δ

5.77 (*d*, 15.4), 7.36 (*dd*, *J* = 10.9, 15.4), 6.26 (*dd*, *J* = 10.1, 15.1), 6.12 (*dd*, *J* = 10.9, 15.1), 5.58 (*ddd*, *J* = 2.7, 4.5, 9.5) and 5.45 (*ddd*, *J* = 1.9, 1.9, 9.5); a methylene geminal to hydroxyl group at δ 3.45 (*d*, *J* = 6.4); ten aliphatic protons at δ 2.44-0.75 ppm; and two methyl groups at δ 0.93 (*d*, *J* = 7.2) and 0.92 (*d*, *J* = 6.4). Therefore the presence of one carboxylic group, three disubstituted double bonds and a hydroxyl methylene group was certain, implying a bicyclic ring system.

The carbon skeleton for **1** could be deduced almost exclusively from the ^{1}H-^{1}H COSY experiment, which delineated a continuous spin system of a branched chain that includes part of the bicycle core from C-13 to C-18, linked to the unsaturated side chain and methyl groups at C-6, C-8 and C-15, respectively (Fig. 2). The bicyclic core and the position of the carboxylic acid were established by HMBC correlations. The HMBC cross peaks between H-2/C-1 and H-3/C-1 established the presence of the pentadienyl carboxylic acid side chain, typical of tanzawaic acids family. HMBC correlation of H-13/H-14/H-6 with C-12, and correlation of H-13/C-7, secured ring B of the molecule, and HMBC correlation of H_2-18/C-11 secured linkage C-10/C-11 and correlations of H-11a (δ_H 0.80 ppm) with C-12 allowed us to establish the planar structure of compound **1** as a member of the tanzawaic acids family.

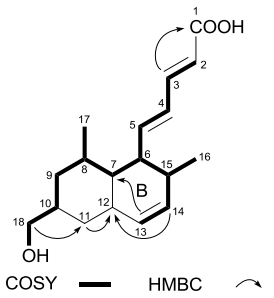

Fig. 2 COSY and selected HMBC correlations for compound **1**.

From accurate mass measurement, tanzawaic acid N (**2**) was found to have a molecular formula of $C_{20}H_{28}O_4$ (*m/z* 331.1897 $[M - 1]^-$) with seven degrees of unsaturation. The spectroscopic data of **2** were very similar to those of **1**, the most significant differences being the presence of signals indicative of an acetate group (δ_H 2.04 (s); δ_C 20.9 and δ_C 171.2) and the chemical shift of C-18 (δ_H 3.87 (*d*, *J* = 6.6); δ_C 69.2). These differences can be explained by the presence of an acetoxymethyl instead of the hydroxmethyl group found in compound **1**. ^{1}H-^{1}H COSY correlation of H_2-18 (δ_H 3.87) to H-10 (δ_H 1.81) along with the HMBC correlation of H_2-18 with the carbonyl of the acetate group further supported the above deduction. ^{1}H-^{1}H COSY, HSQC, and HMBC experiments allowed the complete assignment for structure **2**.

HRESIMS analysis of tanzawaic acid O (**3**) indicated a molecular formula of $C_{18}H_{26}O_4$ (*m/z* 305.1753 $[M - 1]^-$). The ^{1}H NMR data of **3** are very similar to those of **1**. The most significant difference was the absence of methine H-10. A signal observed at δ_C 72.1 in the ^{13}C NMR, and its HMBC correlation with methylene H_2-18 indicated the substitution of C-10 with a hydroxyl group and confirmed the structure of compound **3**.

Tanzawaic acid P (**4**) was isolated as a white powder. Its ESIMS spectrum showed peaks *m/z* 341/343 with relative intensities suggestive of one chlorine atom, which corresponds to the empirical formula $C_{18}H_{27}O_4Cl$ (HRESIMS $[M - 1]^-$ *m/z* 341.1526 (calcd for $C_{18}H_{26}O_4{}^{35}Cl$, 341.1520), 343.1487 (calcd for $C_{18}H_{26}O_4{}^{37}Cl$, 343.1490)). Analysis of the spectroscopic data of **4** (Table 1) indicates that the 2*E*,4*E*-pentadienyl carboxylic acid side chain, characteristic of the tanzawaic acids family, is also present in **4**. Therefore, according to its molecular formula, the decalin system of **4** must contain two hydroxyl groups and one chlorine atom with no double bonds. HMBC correlations of H_3-18 with C-9, C-10 and C-11 confirm the position of the methyl group at C-10 and its chemical shift ($\delta_{H\text{-}18}$ 1.18 ppm) indicates that a hydroxyl group must be attached to C-10. COSY correlation of H-13 with H-14 and HMBC correlations of H_3-16 with C-14 secure the chlorohydrin system on ring B. ^{1}H and ^{13}C chemical shifts of C-13 ($\delta_{C\text{-}13}$ 69.7 ppm) C-14 ($\delta_{C\text{-}14}$ 78.2 ppm) and H-13 ($\delta_{H\text{-}13}$ 3.97 ppm) and H-14 ($\delta_{H\text{-}14}$ 3.75 ppm) indicate that the hydroxyl group must be placed at C-14 and the chlorine atom at C-13.[10] Compound **4** is the first member of the tanzawaic acids family that incorporates a halogen atom in its structure.

Relative configuration of tanzawaic acids M-P (1-4)

Configuration of the double bonds of the pentadienyl side chains of tanzawaic acids M-O (**1**–**3**) were deduced as 2*E*, 4*E* on the basis of the large coupling constants ($J_{H2/H3} \approx 15.4$ Hz; $J_{H4/H5}$ = 15.1 Hz). The relative configuration of the six stereogenic centers on the dehydrodecaline ring (Fig. 3) was assigned on the basis of ^{1}H-NMR coupling constants, and NOESY experiments. NOEs observed of H-6 with H-8, H-12 and H-15 indicated a *trans* fusion of the rings and placed Me-16, Me-17 and the pentadienyl chain on the same side of the molecule and placed H-6, H-7, H-8 and H-12 on axial disposition. This evidence is in agreement with the observed coupling constants for H-6 [*J* = 5.5-5.7 Hz (H-6ax/H-15eq); 10.1 Hz (H-6ax/H-7ax) and 10.1 Hz (H-6ax/H-5)]. Additionally, the NOEs observed between H_2-18 and both methylene, H_2-9 and H_2-11, indicate that the hydroxymethylene at C-10 is located equatorial. Thus, the relative configurations at C-6, C-7, C-8, C-10, C-12 and C-15 were established for compounds **1**–**3** to be the same as those for tanzawaic acids B[3] and E,[4] compounds also isolated in this work.

Relative configuration of compound **4** was deduced from ^{1}H-NMR coupling constants and NOESY experiments. The large coupling constants, $J_{H2/H3}$ = 15.4 Hz; $J_{H4/H5}$ = 15.1 Hz, established the 2*E*,4*E*-pentadienyl side chain. The NOEs observed between H-8 with H-6 and H-12 and the NOE of H-6 and H-15 as well as the NOEs observed between H_3-18 and both H_2-9, and H_2-11 established the same configuration for C-6, C-7, C-8, C-10, C-12 and C-15 as that assigned for compounds **1**-**3**. Finally, the NOEs observed between H-14 and

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Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for compounds **1-4** in $CDCl_3$

	1		**2**		**3**		**4**	
Pos.	δ_H, mult (*J* in Hz)	δ_C	δ_H, mult (*J* in Hz)	δ_C	δ_H, mult (*J* in Hz)	δ_C	δ_H, mult (*J* in Hz)	
1	---	171.9	---	171.6	---	171.3	---	169.3
2	5.77, d (15.4)	118.2	5.77, d (15.5)	118.0	5.77, d (15.5)	118.1	5.72, d (15.4)	119.2
3	7.36, dd (10.9, 15.4)	147.3	7.36, dd (11.0, 15.5)	147.3	7.36, dd (11.0, 15.5)	147.2	7.23, dd (10.4, 15.4)	145.8
4	6.12, dd (10.9, 15.1)	126.5	6.12, dd (11.0, 15.1)	126.5	6.14, dd (11.0 ,15.1)	126.6	6.09, dd (10.4, 15.1)	128.2
5	6.26, dd (10.1, 15.1)	151.4	6.26, dd (10.1, 15.1)	151.4	6.26, dd (10.1, 15.1)	151.3	6.17, dd (9.8, 15.1)	148.0
6	2.44, ddd (5.6, 10.1, 10.1)	49.3	2.44, ddd (5.5, 10.1, 10.1)	49.2	2.50, ddd (5.7, 10.1, 10.1)	48.9	2.40, m	46.3
7	0.96, m	47.3	0.96, (m)	47.0	0.97, m	47.1	1.13, m	42.2
8	1.38, m	36.1	1.39, (m)	36.0	1.73, m	31.9	1.66, m	33.0
9	α 1.70, m	40.5	1.70, ddd (3.2, 5.4, 13.3)	40.5	a 1.66, ddd (3.2, 3.2, 14.2)	44.6	1.57, m	48.2
	β 0.75, m		0.81, m		b 1.07, m		1.07, dd (11.9, 13.6)	
10	1.66, m	40.2	1.81, m	36.9	---	72.1	---	69.6
11	α 1.82, m	35.9	1.82, m	35.9	a 1.76, ddd (3.2, 3.2, 13.6)	40.2	1.53, d (11.6)	42.6
	β 0.80, m		0.85, m		b 1.12, m		1.43, ddd (2.8, 2.8, 13.6)	
12	1.80, m	42.1	1.81, m	42.0	2.28, m	36.9*	2.34, m	35.1
13	5.45 ddd (1.9, 1.9, 9.5)	131.9	5.44, ddd (1.6, 1.6, 9.5)	131.5	5.40, ddd (1.6, 1.6, 9.5)	131.3	3.97, dd (2.5, 2.5)	69.7
14	5.58 ddd (2.7, 4.5, 9.5)	132.4	5.57, ddd (2.5, 4.4, 9.5)	132.5	5.61, ddd (2.5,4.4, 9.5)	132.7	3.75, dd (1.9, 8.2)	78.2
15	2.18, m	36.9	2.18, m	36.8	2.21, m	37.0*	1.83, m	38.2
16	0.93, d (7.2)	16.4	0.94, d (7.3)	16.4	0.94, d (7.2)	16.4	0.96, d (6.9)	15.6
17	0.92, d (6.4)	22.7	0.92, d (7.3)	22.7	0.92, d (6.3)	22.4	0.85, d (6.3)	20.8
18	3.45, d (6.4)	68.3	3.87, d (6.6)	69.2	3.41, s	71.5	1.18, s	31.1
CO			---	171.2				
CH$_3$CO			2.04, s	20.9				

*interchangeable

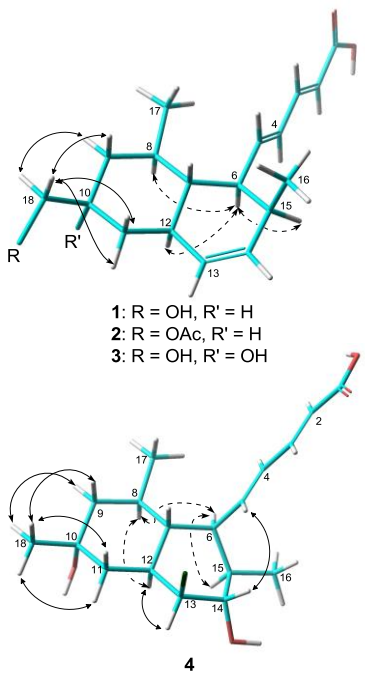


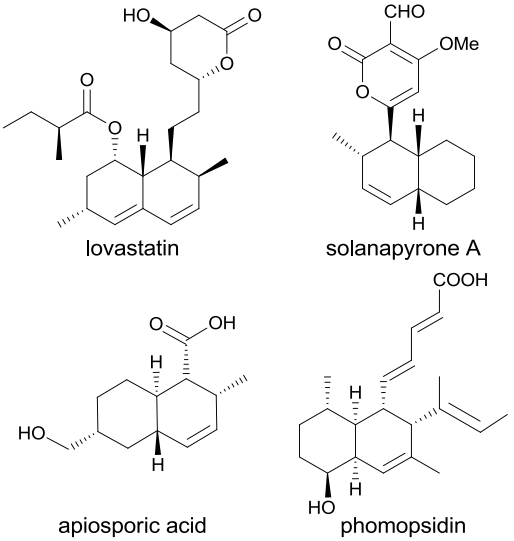
Fig. 3 Selected NOEs for compounds **1-4**.

H-5 and the NOE of H-12 and H-13 indicated that the chlorine must be on the same side of the molecule as Me-15, Me-17, Me-18 and the side chain and that the hydroxyl group at C-14 must be on the opposite side. This diaxial chlorohydrin probably results from the regio and stereoselective opening of an epoxide at C-13/C-14 according to the Fürst-Plattner rule.[11-14] The Optical rotation of tanzawaic acid E isolated in this work is coincident with that established previously.[4] Since the absolute stereochemistry of tanzawaic acid E was determined by X-ray,[6] we assume the absolute configuration of compounds **1**–**4** to match that of tanzawaic acid E.

Possible biogenesis of tanzawaic acids

The extraordinary structural diversity of fungal polyketides is generated by the enzymes due to differences in their PKS protein sequence, structure and programming encoded by PKSs.[15] There is a striking fraction of highly reduced fungal polyketides in which the linear PKS-assembled unit is cyclized by an intramolecular Diels-Alder addition.[16,17] In fungal decalin-based metabolites, a Diels-Alderase catalysed step has been established for lovastatin,[18] and solanapyrone A.[19] Similar polyketide synthase (PKS) enzymes are assumed to be involved

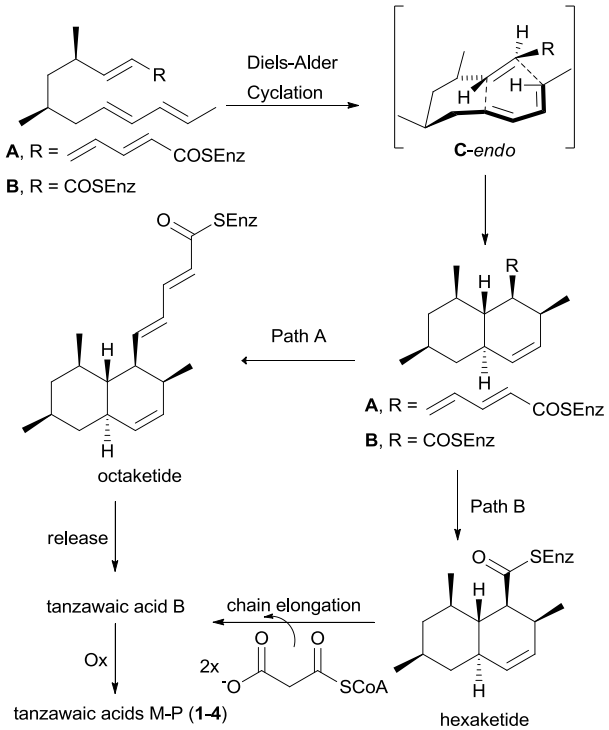
in the biosynthesis of the decalin skeleton of apiosporic acid[20] and phomopsidin,[21] among others.[22]



The chemical structures of the tanzawaic acids family suggest that intramolecular cyclization *via* a [4+2] cycloaddition, may be a biosynthetically reasonable step for the decalin ring system formation as it is outlined in Scheme 1. There may be two pathways leading to tanzawaic acids biogenesis through a biological Diels-Alder. By path A, the entire length of the carbon chain of octaketide participates in the Diels-Alder reaction. Such ring formation processes would be similar to the biosynthesis of the solanapyrones family.[23,24] However, the identification of the naturally occurring apiosporic acid[20] also suggests a path B that would be similar to the biosynthesis of lovastatin.[22] In this case, the PKS may use its dehydratase domain selectively, to produce a suitably activated intermediate hexaketide dienophile, in which the Diels-Alder reaction occurs first, followed by chain elongation until octaketide level.

Attending to the *trans* configuration of the decalin system of the tanzawaic acids family and the conserved configurations at C-6 and C-15 it seems that the active site of the putative enzyme that catalyzes the Diels Alder reaction, holds the diene tightly stabilized in an *endo* transition state to allow intramolecular cycloaddition to form one out of the four possible Diels-Alder adducts: the **C**-*endo* adduct, Scheme 1. This is in contrast to the stereochemical outcomes of formation of the decalin system of solanapyrones. In this family there is concurrence of optically active diastereoisomers arising from *endo* and *exo* cyclization in a biological Diels Alder reaction.

The stereochemical outcomes of tanzawaic acids family may be a proof for the existence of biological Diels-Alder reaction and of the corresponding enzyme in the biosynthesis. However, questions on the control of the tanzawaic acids biosynthesis such as: the chemistry of ring closure, formation of the double bonds, reductions of the carbonyls and hydroxyl groups, incorporation of the methyl groups, as well as side chain organization, will be definitely answered when the proposed tanzawaic acid synthase is isolated and characterized.



Scheme 1 Possible biogenesis of tanzawaic acids family.

Bioactivity of tanzawaic acids M-P (1-4)

The biological activities of compounds **1-6** were tested *in vitro* by antimicrobial and cytotoxicity bioassays. In the initial antimicrobial screening against a panel of microorganisms, the AcOEt crude extract displayed weak antibacterial activity against Gram-negative *Klebsiella pneumoniae* and Gram-positive *Staphylococcus aureus* (MIC $\geq$ 100 μg/mL) whereas purified compounds **1-6** were found to be practically inactive against all bacterial strains under study (data not shown).

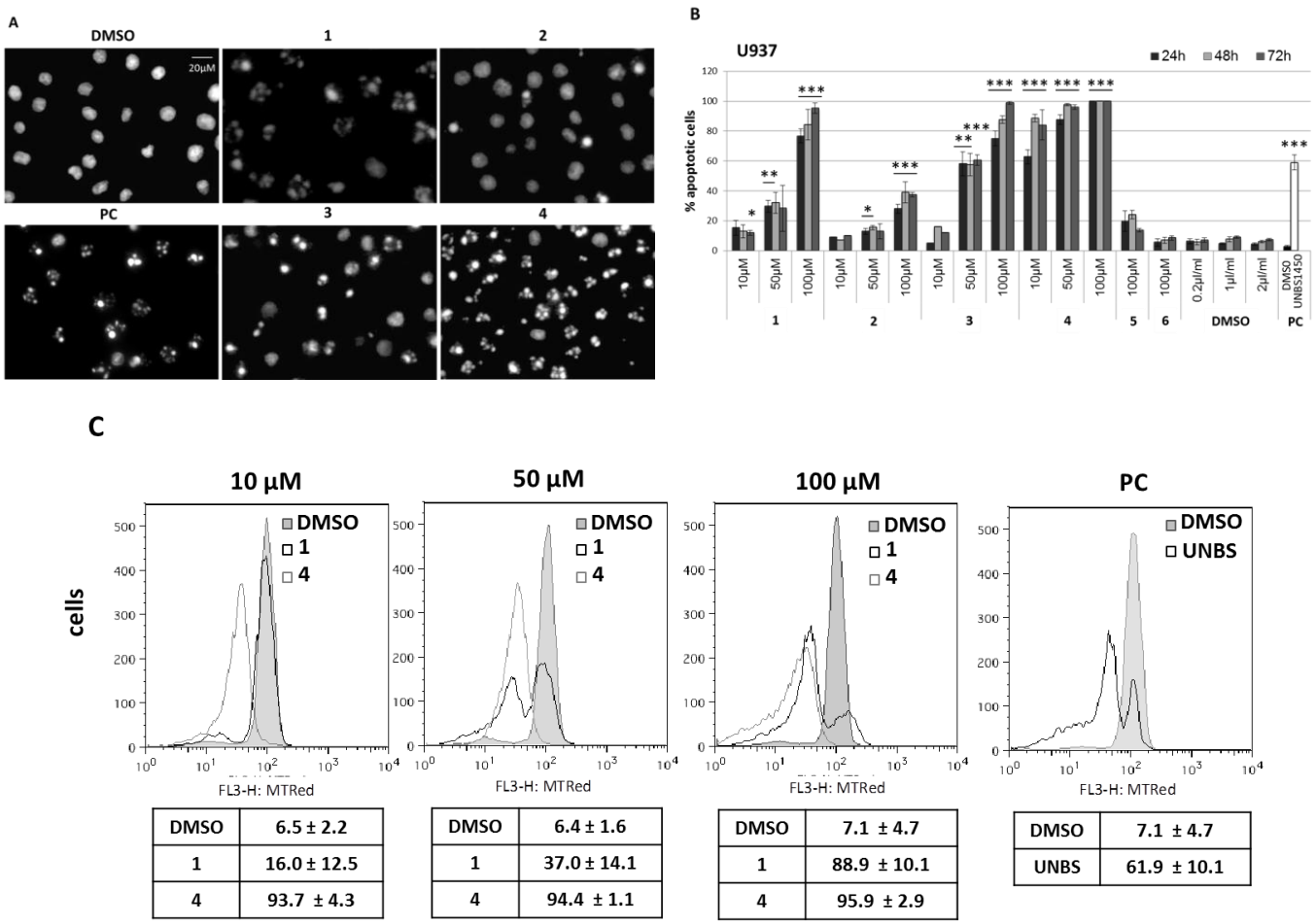
Table 2. Cytotoxic activity of compounds **1-6** in U937 cells

		IC50 (μM)		
	Compd.	**24 h**	**48 h**	**72 h**
U937	**1**	74.5 ± 3.5	58.2 ± 4.9	58.2± 34.2
	2	> 100	83.9 ± 8.6	86.4 ± 4.8
	3	69.4 ± 11.4	44.9 ± 12.1	18.2 ± 9.4
	4	7.3 ± 0.4	5.7 ± 0.3	5.9 ± 0.8
	5	> 100	> 100	> 100
	6	> 100	> 100	> 100

Table 3. Cytotoxic activity of compound **4** in Jurkat, K562 and Raji cell lines

		IC50 (μM)		
	Compd.	**24 h**	**48 h**	**72 h**
Jurkat	**4**	28.6 ± 0.2	25.7 ± 2.53	22.0 ± 2.2
K562	**4**	30.2 ± 7.6	20.0 ± 3.8	16.7 ± 7.6
Raji	**4**	20.3 ± 10.5	27.9 ± 14.2	28.7 ± 14.7

Cytotoxicity of compounds **1-6** was investigated using a cell viability XTT assay against a panel of leukemic and lymphoblastic cell lines (K562, U937, Jurkat and Raji). Compounds were added in a range of concentrations up to 100 µM, and the cells were incubated at 37 °C for 24, 48 and 72 h. U937 proved to be the most sensitive cell line to tested compounds. The most active compound was tanzawaic acid P (**4**) with an IC_{50} of 5.9 ± 0.8 µM in U937 cells after 72 h of exposure. The least active was tanzawaic acid N (**2**) which exhibited an IC_{50} of 86.4 ± 4.8 µM in U937 after 72 h. Tanzawaic acids B (**5**) and E (**6**) did not significantly impact cell metabolism. Compound **4** was the most active compound in all cell lines (Table 3) already after 24 h of incubation. To assess selectivity towards cancer cell lines, we investigated the impact of compounds **1-4** on peripheral blood mononuclear cells (PBMCs) from healthy donors. Compounds **1** and **2** did not affect PBMCs viability (≈ 100% at 10, 50 and 100 µM) whereas compounds **3** and **4** showed no cytotoxic effect against PBMCs at 10 µM. Only at elevated concentrations of 50 and 100 µM a 75% inhibition on viability was measured (not shown). These results underline an important differential toxicity of compounds **1** and **4**.



DMSO	6.5 ± 2.2
1	16.0 ± 12.5
4	93.7 ± 4.3

DMSO	6.4 ± 1.6
1	37.0 ± 14.1
4	94.4 ± 1.1

DMSO	7.1 ± 4.7
1	88.9 ± 10.1
4	95.9 ± 2.9

DMSO	7.1 ± 4.7
UNBS	61.9 ± 10.1

Fig. 5. Tanzawaic acid derivatives 1-4 induced apoptosis in U937 cells in a time and dose-dependent manner. A) Detection of typical apoptotic fragmented nuclei in U937 cells after 48 h of treatment with compounds **1-4** (100 µM) in comparison with cells treated only with an equal volume of vehicle (DMSO) or the positive control (PC; 20 nM UNBS1450 for 24 h). Immunofluorescence microscopy of nuclei after Hoechst 33342 staining. **B**) Percentage of apoptotic cells assessed by analysis of nuclear morphology after 24, 48 and 72 h treatment with 10, 50 or 100 µM of compounds **1-4**. Compounds **5** and **6** were used at a concentration of 100 µM, taking into account their effect on cell metabolism/viability (Table 2). **C**) Analysis of mitochondrial membrane potential for the selected compounds **1** and **4** vs. vehicle-treated cells in comparison to the positive control UNBS1450, upon staining with the fluorescent probe MitoTracker® Red. A representative flow cytometric profile and the percentage of cells displaying loss of the mitochondrial membrane potential are reported. Results are representative or the mean of three independent experiments ± standard deviation (significant difference compared to untreated cells: *p <0.05; ** <0.01, *** <0.001).

To determine whether the growth inhibitory activity exerted by **1-4** on tumor cell lines was related to the induction of cell death by apoptosis, the nuclear morphology of cells was monitored by fluorescence microscopy upon staining with the DNA-specific dye Hoechst 33342. As positive control, we incubated cells for 24 h with the hemi-synthetic cardenolide UNBS1450 (used at the IC_{50}

concentration of 20 nM).[25] U937 cells showed typical apoptotic nuclear fragmentation (Fig. 5A) and apoptosis was estimated by the percentage of cells with apoptotic fragmented nuclei and, in parallel, by assessing decrease of mitochondrial potential (Fig. 5B) after MitoTrackerRed staining.[26,27] Fig. 5C documents the dose-dependent effect of compound **1** (showing the best selectivity towards PBMCs vs. U937 cells), compound **4** (the only one that is active in all cancer cell lines tested) on the mitochondrial membrane potential. Our results indicate activation of the mitochondrial apoptotic pathway by both compounds. Similar effects were confirmed in the other cell lines after treatment with compound **4** (Fig. 6).

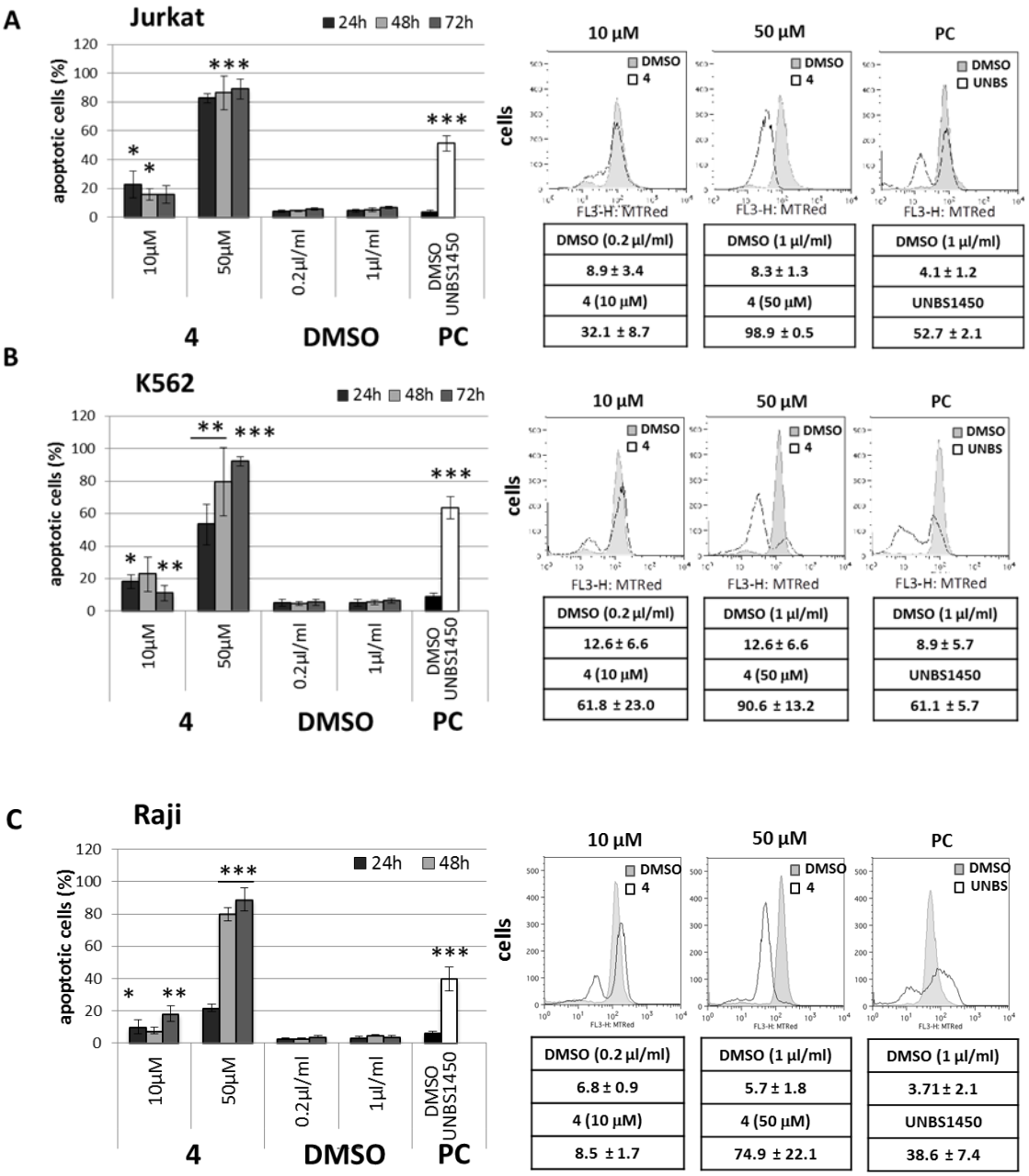


Fig. 6. Comparative effect of compound 4 on apoptosis in Jurkat, K562 and Raji cells. Jurkat (**A**), K562 (**B**) and Raji (**C**) treated for 24, 48 and 72 h with 10 or 50 µM compound **4**. In parallel cells were treated for 24h with UNBS1450 as positive control (PC; used at the respective IC_{50} concentrations of 20 nM for Jurkat and 50 nM for K562 and Raji cells).[25] Percentage of apoptosis was estimated by analysis of nuclear morphology (left panels) and confirmed by flow cytometric estimation of the sub-population of cells exhibiting mitochondrial membrane potential loss. Results are representative or the mean of three independent experiments ± standard deviation (significant difference compared to untreated cells: * $p < 0.05$; ** < 0.01, *** < 0.001).

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Based on the results described above a structure-activity relationship can be proposed. While the presence of a hydroxyl group at C-10 it is not sufficient to produce a cytotoxic effect on the tested cell lines, a hydroxyl group at C-18 increases the activity. The cytotoxic activity also improves when C-10 and C-18 are simultaneously hydroxylated. The best activity was obtained when there is a chlorohydrin on ring B instead of the double bond. The regeneration of the epoxide from the chlorohydrin could be the responsible of the strong biological activity. Therefore chemical structure of tanzawaic acids could be further optimized to increase their potency.

Experimental

General experimental procedures

Optical rotations were measured on a Perkin-Elmer model 343 Plus polarimeter using a Na lamp at 20 ºC. IR spectra were recorded on a Perkin-Elmer 1650/FTIR spectrometer. ^{1}H NMR and ^{13}C NMR, HSQC, HMBC and COSY spectra were measured employing a Bruker AMX 500 instrument operating at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR. All ^{13}C and ^{1}H NMR spectra were internally referenced to the residual solvent signal ($CDCl_3$: δ_C 77.0 ppm, δ_H 7.25 ppm). Two-dimensional NMR spectra were obtained using the standard Bruker software. ESIMS and HRESI MS (negative-ion mode) data were taken on a Micromass LCT Premier XE. The gel filtration column (Sephadex LH-20) used Hexane/CH_2Cl_2/MeOH (3:1:1) as eluent. HPLC separations were performed on an Agilent 1200 Series Quaternary LC system using a Jaigel-Sil-043-10 semipreparative column (10µm, 20×250 mm) eluted with hexane/EtOAc mixtures. The spray reagent for TLC was H_2SO_4/H_2O/AcOH (1:4:20).

Fungal isolation and identification

Strain CF07370 was isolated from a sediment sample collected at ~100 m depth at Bahia de Los Angeles (Gulf of California, Mexico), in winter 2007. The strain was identified as a member of the genus *Penicillium* by macro and microscopic characters.

Fermentation conditions and extraction

Penicillium strain CF07370 was cultivated in 3 L Erlenmeyer flask at room temperature on a rotatory shaker (150 rpm/min) in a sea-water based medium [SYP broth medium yeast extract (4 g/L, Sigma, Y1001), starch (10 g/L, Sigma, S9765), fish peptone (2 g/L, Fluka, 93490)]. After 21 days the culture filtrate was extracted with AcOEt (3 × 700 ml), and the combined organic layers were concentrated under reduced pressure to obtain 462.8 mg of crude extract.

Purification

Crude extract was fractionated by reversed-phase C-18 silica flash chromatography using step-gradient elution (100% H_2O to 100% MeOH). Fraction eluted with H_2O:MeOH (1:4) was subjected to filtration chromatography (Sephadex LH-20) followed by HPLC to afford tanzawaic acids E (5.8 mg), M (4.3 mg), N (24.2 mg), O (4.6 mg) and P (2.5 mg). Following the same procedure tanzawaic acid B (2.1 mg) was isolated from fraction eluted with MeOH (100%).

Characterisation of compounds

Tanzawaic acid M (1): Isolated as a colorless oil; $[\alpha]^{20}_{D}$ + 104 (*c* 0.91, CH_2Cl_2); IR (film) ν_{max} 3438, 1656 cm^{-1}; ESIMS *m/z* 298 [M – 1]$^-$ (100); HRESIMS [M]$^-$ *m/z* 289.1800 (calcd for $C_{18}H_{25}O_3$, 289.1804); ^{1}H and ^{13}C NMR see Table 1.

Tanzawaic acid N (2): Isolated as a white powder; $[\alpha]^{20}_{D}$ + 60 (*c* 0.28, CH_2Cl_2); IR (film) ν_{max} 3249, 1653 cm^{-1}; ESIMS *m/z* 331 [M – 1]$^-$ (100); HRESIMS [M]$^-$ *m/z* 331.1897 (calcd for $C_{20}H_{27}O_4$, 331.1909); ^{1}H and ^{13}C NMR see Table 1.

Tanzawaic acid O (3): Isolated as a white powder; $[\alpha]^{20}_{D}$ – 12 (*c* 0.25, CH_2Cl_2); IR (film) ν_{max} 3446, 1656 cm^{-1}; ESIMS *m/z* 305 [M – 1]$^-$ (100); HRESIMS [M]$^-$ *m/z* 305.17560 (calcd for $C_{18}H_{25}O_4$, 305.1753); ^{1}H and ^{13}C NMR see Table 1.

Tanzawaic acid P (4): Isolated as a white powder; $[\alpha]^{20}_{D}$ – 37 (*c* 0.14, CH_2Cl_2); IR (film) ν_{max} 3434, 1660 cm^{-1}; ESIMS *m/z* 341 [M – 1]$^-$ (100), 343 [M – 1]$^-$ (50); HRESIMS [M – 1]$^-$ *m/z* 341.1526 (calcd for $C_{18}H_{26}O_4{}^{35}Cl$, 341.1520), 343.1487 (calcd for $C_{18}H_{26}O_4{}^{37}Cl$, 343.1490); ^{1}H and ^{13}C NMR see Table 1.

Antimicrobial activity

Antimicrobial susceptibility test was performed by the broth macrodilution method (within the range 10-100 µg/ml) against the following strains obtained from the Spanish Collection of Type Cultures (CECT; Faculty of Biological Sciences, University of Valencia, Spain) and American Type Culture Collection (ATCC, USA): *Staphylococcus aureus* (ATCC 6538), *Salmonella* sp. (CECT 456), *Klebsiella pneumoniae* (ATCC 23357), *Escherichia coli* (ATCC 9637), *Bacillus cereus* (ATCC 21772), *Proteus mirabilis* (CECT 170), *Enterococcus faecalis* (ATCC 29212), and *Candida albicans* (ATCCMYA-2876) as described elsewhere.[28] In the case of the yeast *C. albicans* the tryptic soy medium was replaced by the non-filament-inducing medium; YPD [2% (w/v) Bacto peptone, 1% (w/v) yeast extract and 2% (w/v) glucose]. The selected strains

were chosen for key characteristics, in terms of ecology, physiology, metabolism and for ease of screening. Minimum inhibitory concentrations (MIC), was defined as the lowest substance concentration that completely inhibits microbial growth. MIC was determined from two independent experiments performed in triplicate for each concentration. All compounds were previously dissolved in DMSO.

Cell proliferation assay

Cytotoxic assays were performed against leukemic and lymphoblastic cell lines: U937 (monocyte-like histiocytic lymphoma cells), K562 (human erythromyeloblastoid leukemia cells), Jurkat (human T lymphoblast-like cells) and Raji (human Burkitt's lymphoma cells). Cell lines were purchased from DSMZ (Braunschweig, Germany). Peripheral blood mononuclear cells (PBMCs) were used as controls to assess the effect on the proliferation of normal cells. Cancer cell lines were maintained in RPMI 1640 medium (Bio-Whittaker, Verviers, Belgium) supplemented with 10% (v/v) fetal bovine serum (FCS, Lonza, Verviers, Belgium), 1% (v/v) antimitotic agent (Lonza, Verviers, Belgium) and 2mM L-glutamine (Lonza, Verviers, Belgium) for 24 h at 37 °C in humidified atmosphere with 5% CO_2. PBMCs were isolated from the non-coagulated peripheral blood of healthy adult human donors (Red Cross, Luxembourg), using the standard Ficoll-Hypaque density separation method (GE Healthcare, Roosendaal, The Netherlands). After collection, PBMCs were washed, counted, re-suspended at a cell density of 2 x 10^6 cells/mL with the same conditions described above except that 0.1% (v/v) FCS was used.[25,29] Before screening all compounds were pre-dissolved in DMSO at appropriate concentrations. Cell viability after incubation with the test compounds was evaluated by Promega Cell Proliferation Kit II (XTT; Roche, Luxembourg, Luxembourg). The XTT Kit detects formazan dye produced from XTT conversion by mitochondrial enzymes in cells. Because these mitochondrial enzymes are inactivated shortly after cell death, the orange colored formazan dye only appears in viable cells. For all the cell lines 2 x 10^5 cells/mL and different concentrations of compounds (up to 100 µM) were used during 24, 48 and 72 h. Data were normalized to the DMSO control and reported as percentage of metabolically active cells.

Analysis of apoptosis

Percentage of apoptotic cells was quantified as the fraction of apoptotic nuclei (different stages of nuclear fragmentation) assessed by fluorescence microscopy (Leica-DM IRB microscope, Lecuit, Luxembourg) upon staining with the DNA-specific dye Hoechst 33342 (Sigma, Bornem, Belgium), as described elsewhere.[25,27,30] The fraction of cells with nuclear apoptotic morphology was counted (at least 300 cells in at least three independent fields). Mitochondrial membrane potential loss was estimated by flow cytometry upon incubation of treated/untreated cells for 20 min at 37 °C with 50 nM MitoTracker® Red (Invitrogen/Molecular Probes, Merelbeke, Belgium). As positive control, cells were incubated for 24 h with the hemi-synthetic cardenolide UNBS1450, used at the respective IC50 concentrations as assessed by viability assay (U937 and Jurkat: 20 nM; K562 and Raji: 50 nM).[25]

Statistical analysis

Data are presented as mean of at least three independent experiments ± standard deviation. Significance was estimated by using Student T-test or one-way ANOVA test. Post-hoc analyses where performed using Prism 6 software, GraphPad Software (La Jolla California, USA). P-values < 0.05 were considered as significant.

Conclusions

During the course of our chemical investigation of *Penicillium* strain CF07370 we identified four new members of the tanzawaic acids family, being tanzawaic acid P (**4**) the first member of this family that incorporates a halogen atom in its structure. The *trans* configuration of the decalin system of the tanzawaic acids family and the conserved configurations at C-6 and C-15 indicates that cyclization of the diene precursor may occur via an intramolecular Diels-Alder addition through a unique **C**-*endo* transition state. The cytotoxic activity of tanzawaic acids improves when the double bond of ring A is saturated and by the presence of hydroxyl groups simultaneously at C-10 and C-18.

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