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Targeted solid phase fermentation of the soil dwelling fungus *Gymnascella dankaliensis* yields new brominated tyrosine-derived alkaloids†

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Seven new brominated tyrosine-derived alkaloids, gymnastatins T–Y (**1–6**) and dankastatin D (**7**), together with three known likewise brominated analogues gymnastatins I–K (**8–10**) were isolated from the soil fungus *Gymnascella dankaliensis* through fermentation on solid rice medium following addition of NaBr. None of these compounds were detected when the fungus was cultured on rice that either lacked NaBr or that contained NaCl instead, indicating a remarkable plasticity of the fungal secondary metabolism. All structures were elucidated on the basis of one and two dimensional NMR spectroscopic analyses and MS data. The absolute configuration of the new gymnastatin T (**1**) was determined by X-ray crystallographic data. All isolated alkaloids showed potent to moderate cytotoxicity against the L5178Y mouse lymphoma cell line with IC_{50} values ranging from 0.078 to 14.1 μM.

Introduction

More than 5000 halogenated natural products have been reported so far with many of them exhibiting a variety of biological activities including antibacterial, antifungal, antiviral, antitumor, anti-inflammatory, and antioxidant activity.[1–3] Fungi are known as rich sources of numerous bioactive metabolites and receive increasing attention in the quest for new drug candidates[4] as exemplified by plinabulin[5] which is currently in clinical trials as a new anticancer drug or by the antifungal echinocandins.[6] Numerous fungal metabolites are chlorinated, however, only few brominated fungal metabolites have been isolated so far.[7] Two classes of halogenating enzymes including halogenases and haloperoxidases have been discovered from a broad range of organisms and the mechanisms of enzymatic halogenation were described as well.[8] In recent years several halogenated natural products have been obtained by culturing fungi in media containing halogen salts.[9–11] The plasticity of halogenating enzymes with regard to accepting different halogens can be applied for a targeted fermentation since adding different halogens (*e.g.* NaCl or NaBr) to the medium will direct the fungal metabolism in producing mainly chlorinated[12] or alternatively brominated metabolites as shown in this study.

The soil dwelling fungus *Gymnascella dankaliensis* that was isolated from desert sand close to the pyramids of Giza provides an excellent example for the application of this experimental approach. This fungus which has also been reported from a marine *Halichondria* sponge is known to produce a variety of mainly chlorinated tyrosine derived alkaloids.[13–17] In a previous study[12] we could show that addition of KBr to rice medium resulted in bromination of fungal metabolites as indicated by the typical isotope peaks during LC-MS analysis of the respective extracts. The nature of the brominated compounds could, however, not be elucidated. We have now obtained seven new brominated tyrosine-derived alkaloids, gymnastatins T–Y (**1–6**) and dankastatin D (**7**), as well as three likewise brominated known analogues following fermentation of *G. dankaliensis* on solid rice medium containing NaBr as halogen source. All isolated alkaloids were tested against the L5178Y mouse lymphoma cells and showed significant to moderate activity (Fig. 1).

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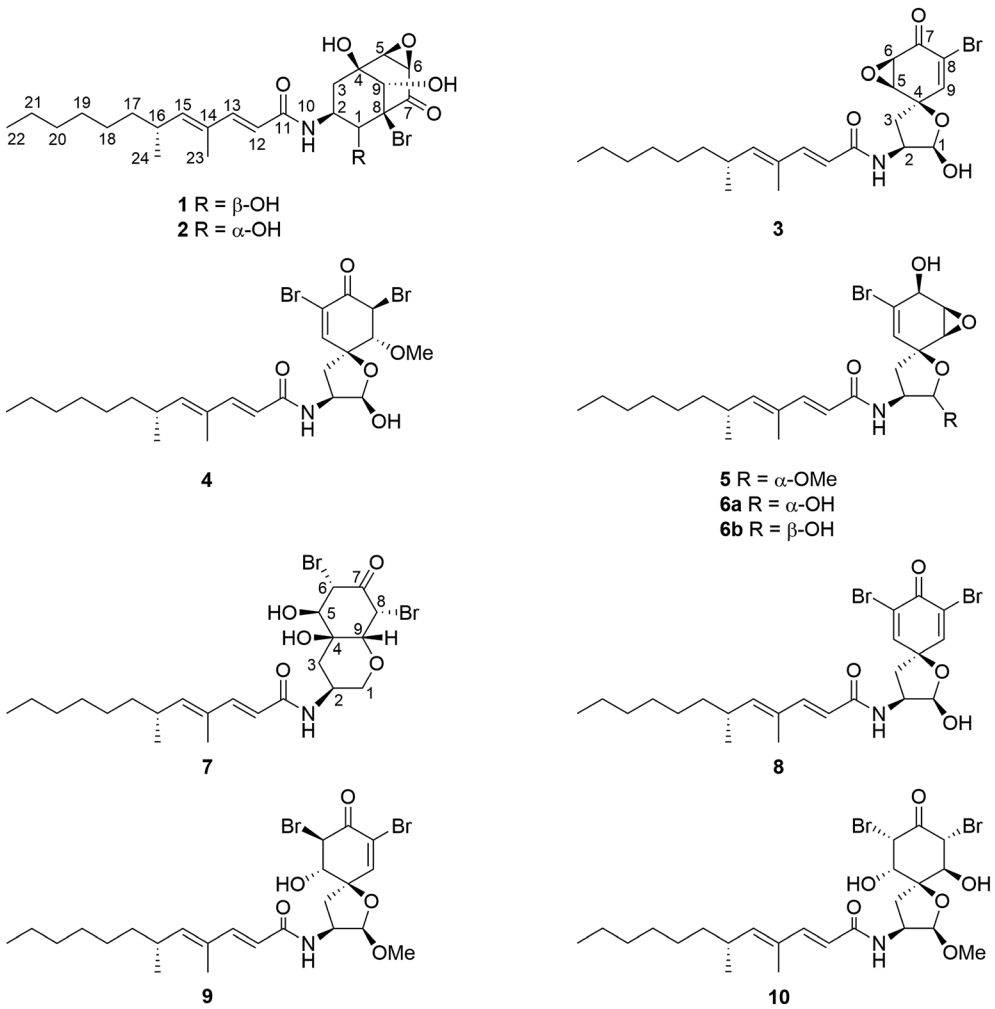

Fig. 1 Structures of compounds isolated from *G. dankaliensis*.

Results and discussion

Gymnastatin T (**1**) possessed the molecular formula $C_{23}H_{34}BrNO_6$ as evident from its HRESIMS. Comparison of its ^{1}H and ^{13}C NMR data (Table 1) with those of the known brominated gymnastatins I–K (**8–10**)[16] indicated the presence of a branched aliphatic chain from C-11 to C-22. This was further confirmed by the COSY correlations between H-12/H-13, H-15/H-16, H-16/H_{ab}-17, H-16/H_3-24, and H_2-21/H_3-22, in addition to the HMBC correlations from H-10 and H-12 to C-11, from H_3-23 to C-13, C-14, and C-15, from H_3-24 to C-15, C-16, and C-17, from H-17a (δ_H 1.32) to C-18 and C-19, and from H_3-22 to C-20 and C-21 (Fig. 2). The remaining ^{1}H and ^{13}C signals of **1** were comparable to those of gymnastatin G.[14] The nature of the bicyclo[3.3.1]nonane ring was established by the COSY correlations between 1-OH/H-1, H-1/H-2, H-2/H-3α, H-2/H-3β, H-2/NH-10, H-5/H-6, and 9-OH/H-9, as well as by key HMBC correlations from 1-OH to C-1, C-2, and C-8, from H-3α and H-3β to C-4, C-5, and C-9, from 4-OH to C-3, C-4, C-5, and C-9, from H-6 and H-9 to C-7, and from 9-OH to C-4, C-8, and C-9. Assignments of the remaining oxygen atom linked to C-5 and C-6, and of the bromine atom at C-8 were indicated by the chemical shifts of the respective carbons [δ_C 59.1 (C-5), 53.7 (C-6), and 78.7 (C-8)]. The HMBC correlation from NH-10 to C-11 indicated the linkage between the aliphatic chain and the bicyclononane ring *via* an amide bond. Thus, the planar structure of **1** was elucidated as shown. The coupling constants ($^3J_{1,2}$ = 2.8 Hz, $^3J_{2,3\alpha}$ = 5.5 Hz, and $^3J_{2,3\beta}$ = 12.8 Hz) together with the NOE correlations between H-1/H-2, H-2/H-3α, H-3α/H-5, H-5/H-6, and between 1-OH/NH-10, NH-10/H-3β, H-3β/H-9, H-9/1-OH suggested a chair conformation of the cyclohexane ring (C-1 to C-4, C-9, C-8) with H-1, H-2, H-3α, H-5 and H-6 being on the same face of the ring while 1-OH, NH-10, H-3β, and H-9 were oriented to the opposite face (Fig. 3). The large coupling constants ($^3J_{12,13}$ = 15.5 Hz) and the NOE correlations between H-12/H_3-23, and H-13/H-15 indicated 12*E*, 14*E* configuration. The absolute configuration of **1** was determined as 1*S*, 2*S*, 4*R*, 5*S*, 6*R*, 8*S*, 9*S*, and 16*R* by X-ray single crystal analyses (Fig. 4).

The molecular formula of gymnastatin U (**2**) was identical to that of **1** as indicated by HRESIMS. Detailed analysis of the ^{1}H, ^{13}C NMR and of the 2D NMR data indicated that both compounds shared the same gross structure. The $^3J_{1,2}$ value of **2**

Table 1 ^{1}H and ^{13}C NMR data of compounds **1** and **2**

	1[a]		**1**[b]		**2**[b]	
No.	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	74.6, CH	3.67, dd (5.9, 2.8)	76.8, CH	3.83, d (2.8)	80.2, CH	3.92, d (10.7)
2	46.3, CH	3.84, dddd (12.8, 7.7, 5.5, 2.8)	47.8, CH	4.09, ddd (11.0, 7.1, 2.8)	50.9, CH	4.12, ddd (12.7, 10.7, 5.5)
3α	35.6, CH_2	1.88, dd (12.8, 5.5)	36.5, CH_2	2.09, dd (13.0, 7.1)	39.0, CH_2	2.34, dd (13.4, 5.5)
3β		1.92, dd (12.8, 12.8)		2.07, dd (13.0, 11.0)		1.74, dd (13.4, 12.7)
4	69.4, C		71.0, C		70.9, C	
5	59.1, CH	3.60, dd (3.8, 2.2)	60.9, CH	3.66, dd (3.8, 2.3)	60.8, CH	3.63, dd (3.7, 2.0)
6	53.7, CH	3.79, d (3.8)	55.9, CH	3.80, d (3.8)	54.3, CH	3.68, d (3.7)
7	199.8, C		199.7, C		195.1, C	
8	78.7, C		77.9, C		80.3, C	
9	73.5, CH	3.97, dd (8.6, 2.2)	75.8, CH	4.15, d (2.3)	79.8, CH	3.85, d (2.0)
10		8.02, d (7.7)				
11	164.7, C		168.5, C		169.3, C	
12	119.5, CH	5.99, d (15.5)	118.8, CH	5.99, d (15.4)	119.0, CH	5.91, d (15.4)
13	144.0, CH	6.97, d (15.5)	147.6, CH	7.16, d (15.4)	147.5, CH	7.18, d (15.4)
14	130.9, C		132.7, C		132.6, C	
15	145.6, CH	5.59, d (9.8)	148.5, CH	5.62, d (9.8)	148.5, CH	5.64, d (9.7)
16	32.4, CH	2.49, m	34.3, CH	2.56, m	34.3, CH	2.56, m
17	36.7, CH_2	1.32, m; 1.21, m	38.4, CH_2	1.38, m; 1.27, m	38.4, CH_2	1.39, m; 1.27, m
18	26.9, CH_2	1.19, m	28.6, CH_2	1.26, m	28.6, CH_2	1.26, m
19	28.7, CH_2	1.22, m	30.5, CH_2	1.27, m	30.5, CH_2	1.28, m
20	31.2, CH_2	1.22, m	33.0, CH_2	1.27, m	33.0, CH_2	1.27, m
21	22.0, CH_2	1.24, m	23.7, CH_2	1.28, m	23.7, CH_2	1.30, m
22	13.9, CH_3	0.84, t (7.0)	14.4, CH_3	0.89, t (7.0)	14.4, CH_3	0.89, t (7.1)
23	12.4, CH_3	1.70, s	12.7, CH_3	1.79, s	12.7, CH_3	1.80, s
24	20.5, CH_3	0.93, d (6.6)	20.9, CH_3	0.98, d (6.7)	20.9, CH_3	0.99, d (6.7)
1-OH		6.49, d (5.9)				
4-OH		5.94, s				
9-OH		4.62, d (8.6)				

[a] Measured in DMSO-d_6. [b] Measured in methanol-d_4.

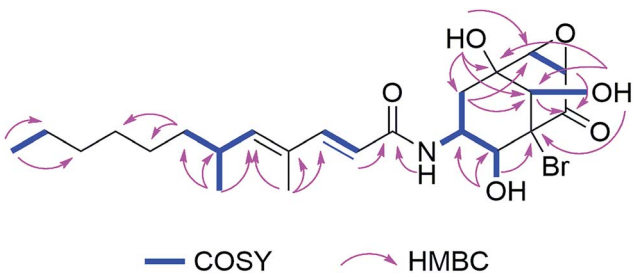

Fig. 2 Key COSY and HMBC correlations for **1**.

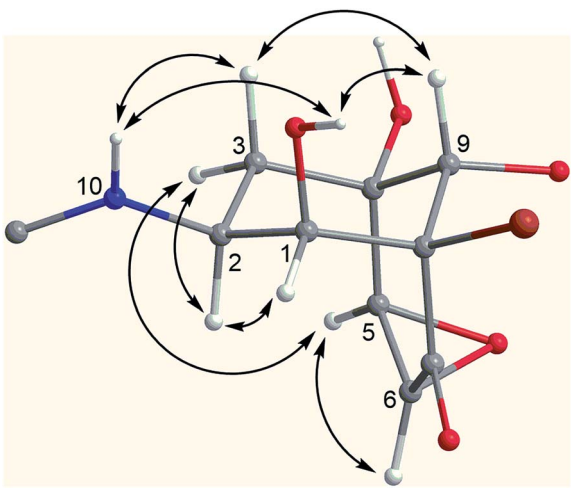

Fig. 3 Key NOE correlations and computer-generated model using MM2 force field calculations for **1**.

(10.7 Hz), however, was much larger than that of **1** (2.8 Hz), suggesting **2** to be the 1-epimer of **1**. In the ROESY spectrum of **2**, the observed correlations between H-2/H-3α, H-3α/H-5, and between H-1/H-3β, H-3β/H-9, H-9/H-1 confirmed this assumption. Based on the close biogenetic similarity of **1** and **2** as well as the X-ray structure of **1**, the absolute configuration of **2** can be assumed to be 1*R*, 2*S*, 4*R*, 5*S*, 6*R*, 8*S*, 9*S*, and 16*R*.

Gymnastatin V (**3**) had the molecular formula $C_{23}H_{32}BrNO_5$ as established by HRESIMS, indicating the loss of one Br atom and addition of one further oxygen and one proton compared to the known gymnastatin I (**8**).[16] The ^{1}H and ^{13}C NMR data of **3** (Table 2) were very similar to those of **8**, except for the replacement of the C-5/C-6 double bond of **8** by an epoxy ring as indicated by the characteristic resonances at δ_C 58.2 (C-5), 52.4 (C-6), and δ_H 3.80 (H-5), 3.63 (H-6), respectively. The HMBC correlations from H-5 to C-4 (δ_C 81.2) and C-9 (δ_C 147.4), from H-6 to C-7 (δ_C 186.3) and C-8 (δ_C 119.0), as well as from H-3α (δ_H

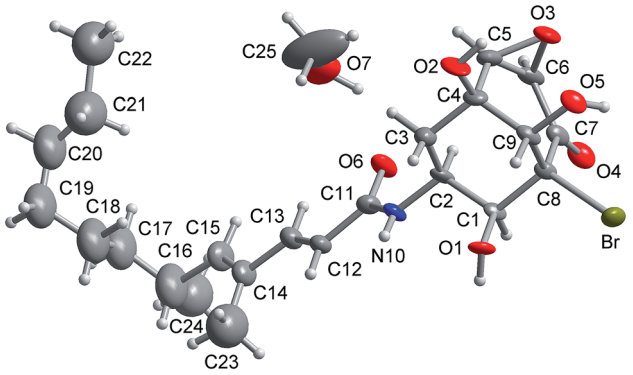

Fig. 4 Molecular structure of **1** (with a methanol molecule) from single-crystal X-ray diffractometry.

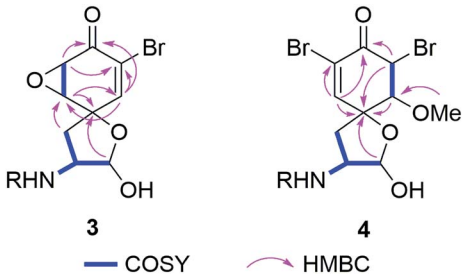

Fig. 5 Key COSY and HMBC correlations for **3** and **4**.

2.55), H-3β (δ_H 2.16) and H-9 (δ_H 6.94) to C-5 confirmed the location of this epoxy group at the C-5/C-6 position (Fig. 5). The remaining structure of **3** was identical to that of **8** as indicated by 2D NMR. The NOE relationships between H-1/H-2, H-1/H-9, H-2/H-3α, H-2/H-9, and H-3α/H-9 of **3** indicated that these protons were oriented on the same face, whereas the NOE correlations between NH-10/H-3β, H-3β/H-5, and H-5/H-6 suggested that these latter protons were oriented towards the opposite side (Fig. 6). The geometries of the double bonds in the aliphatic side chain were determined as 12*E* and 14*E*, respectively based on their coupling constants and NOE correlations as described for **1**. Thus, the structure of **3** was elucidated as shown.

Gymnastatin W (**4**) shared the same molecular formula as gymnastatin J (**9**).[16] Both compounds differed only by the positions of their methoxy and hydroxy groups as indicated by detailed analysis of their 2D NMR spectra. The assignment for the methoxy group at C-9 in **4** was evident from the COSY correlation between H-8 (δ_H 4.57) and H-9 (δ_H 3.68) in addition to the HMBC correlations from 9-OMe (δ_H 3.67) to C-9 (δ_C 85.9) and from H-8 to C-7 (δ_C 183.1). Analysis of the COSY and HMBC spectra led to the planar structure of **4** (Fig. 5). The large

Table 2 ^{1}H and ^{13}C NMR data of compounds **3**–**5**

	3[a]		**4**[a]		**5**[b]	
No.	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	96.4, CH	5.56, d (4.4)	96.4, CH	5.55, d (4.4)	110.0, CH	4.96, s
2	51.9, CH	4.78, m	52.6, CH	4.76, m	57.5, CH	4.40, dd (7.3, 2.2)
3α	38.8, CH_2	2.55, dd (13.0, 8.5)	33.6, CH_2	2.83, dd (12.4, 8.6)	40.6, CH_2	2.56, dd (14.1, 7.3)
3β		2.16, dd (13.0, 10.8)		1.80, dd (12.4, 10.6)		1.87, dd (14.1, 2.2)
4	81.2, C		86.4, C		84.4, C	
5	58.2, CH	3.80, dd (3.8, 2.6)	153.9, CH	7.46, s	133.2, CH	6.09, dd (2.4, 1.7)
6	52.4, CH	3.63, d (3.8)	119.9, C		125.0, C	
7	186.3, C		183.1, C		67.6, CH	4.35, dd (2.8, 1.7)
8	119.0, C		54.7, CH	4.57, d (11.5)	55.7, CH	3.54, dd (4.1, 2.8)
9	147.4, CH	6.94, d (2.6)	85.9, CH	3.68, d (11.5)	59.3, CH	3.40, dd (4.1, 2.4)
10		6.12, d (8.1)		5.95, d (8.4)		
11	166.8, C		166.6, C		169.2, C	
12	116.7, CH	5.76, d (15.3)	117.0, CH	5.75, d (15.3)	118.6, CH	6.01, d (15.4)
13	147.7, CH	7.26, d (15.3)	147.3, CH	7.24, d (15.3)	147.7, CH	7.19, d (15.4)
14	130.7, C		130.8, C		132.7, C	
15	148.8, CH	5.68, d (9.8)	148.4, CH	5.66, d (9.7)	148.4, CH	5.65, d (9.8)
16	33.2, CH	2.50, m	33.2, CH	2.50, m	34.3, CH	2.57, m
17	37.2, CH_2	1.35, m; 1.25, m	37.2, CH_2	1.35, m; 1.26, m	38.4, CH_2	1.40, m; 1.27, m
18	27.4, CH_2	1.21, m	27.5, CH_2	1.21, m	28.3, CH_2	1.28, m
19	29.4, CH_2	1.23, m	29.4, CH_2	1.24, m	30.4, CH_2	1.28, m
20	31.8, CH_2	1.22, m	31.8, CH_2	1.23, m	33.0, CH_2	1.27, m
21	22.6, CH_2	1.26, m	22.6, CH_2	1.27, m	23.3, CH_2	1.30, m
22	14.1, CH_3	0.86, t (7.1)	14.1, CH_3	0.87, t (7.0)	14.4, CH_3	0.89, t (7.0)
23	12.5, CH_3	1.76, s	12.5, CH_3	1.76, s	12.5, CH_3	1.81, s
24	20.5, CH_3	0.97, d (6.6)	20.5, CH_3	0.97, d (6.6)	20.8, CH_3	1.00, d (6.7)
1-OMe					54.9, CH_3	3.41, s
9-OMe			62.6, CH_3	3.67, s		

[a] Measured in $CDCl_3$. [b] Measured in methanol-d_4.

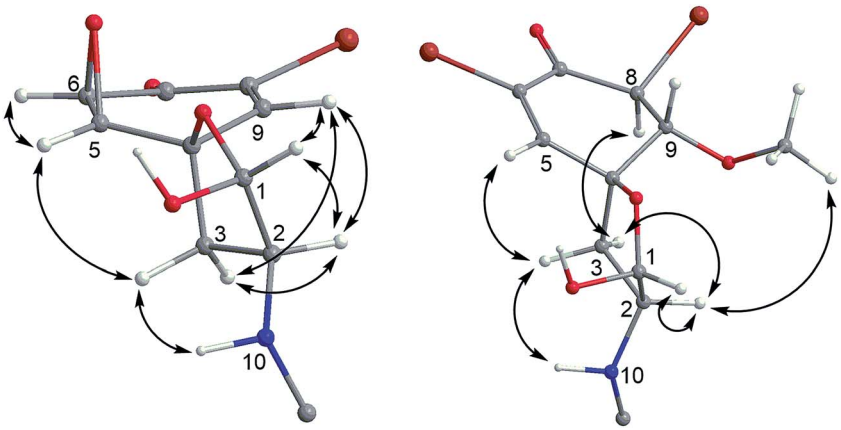

Fig. 6 Key NOE correlations and computer-generated models using MM2 force field calculations for **3** (left) and **4** (right).

coupling constant between H-8 and H-9 (11.5 Hz) along with the NOE correlations between H-1/H-2, H-2/H-3α, H-2/9-OMe, H-3α/H-8, and between NH-10/H-3β, H-3β/H-5 revealed that H-1, H-2, H-8, and 9-OMe were oriented at the same face of the ring as H-3α whereas H-5 and NH-10 were oriented to the opposite face as found also for H-3β. The above evidence led to assignment of the relative configuration of **4** as shown in Fig. 6.

The molecular formula of gymnastatin X (**5**) was established as $C_{24}H_{36}BrNO_5$ as indicated by HRESIMS. The 1 : 1 isotopic peaks at *m*/*z* 498 and 500 indicated the presence of one bromine atom in the structure. Its NMR data closely resembled those of the known chlorinated compound gymnastatin D.[13] Inspection of 2D NMR spectra of **5** indicated the replacement of chlorine by bromine at C-6 and the appearance of an additional methoxy group (δ_C 54.9 and δ_H 3.41) at C-1, which was evident from the HMBC correlation from the methoxy protons to C-1 (δ_C 110.0) and in turn from H-1 (δ_H 4.96) to the methoxy carbon (Fig. 7). When comparing the NMR data of **5** with those of the reported C-1 isomers of gymnastatin D, the coupling constants $^3J_{1,2}$ (close to zero), $^3J_{2,3\alpha}$ (7.3 Hz), $^3J_{2,3\beta}$= (2.2 Hz), $^4J_{5,7}$ (1.7 Hz), $^4J_{5,9}$= (2.4 Hz), $^3J_{7,8}$ (2.8 Hz), $^3J_{8,9}$= (4.1 Hz) and the correlations between H-2/H-3α, H-3α/H-9, H-1/H-3β and H-3β/H-5 detected in the ROESY spectrum of **5** indicated its relative configuration as shown (Fig. 8).

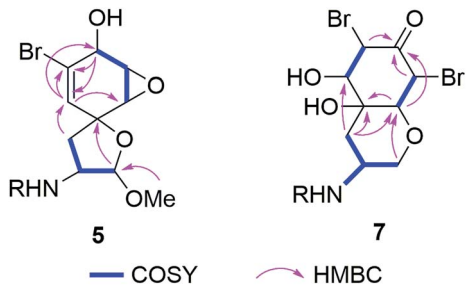

Fig. 7 Key COSY and HMBC correlations for **5** and **7**.

Gymnastatin Y (**6**) had the molecular formula $C_{23}H_{34}BrNO_5$ as determined by HRESIMS, lacking a methyl group compared to **5**, which was confirmed by the disappearance of the methoxy group signal in the NMR spectra of **6**. HPLC analysis and NMR spectra (Table 3) of **6** suggested that it existed as a 3 : 1 mixture of two stereoisomers (**6a** and **6b**) as previously also reported for gymnastatin D.[13] The ^{1}H NMR data of **6a** were very similar to those of **5**, except for the absence of the methoxy group at C-1. Interpretation of 2D NMR indicated that **6a** and **6b** were stereoisomers of 1-demethoxy gymnastatin X (**5**). Compound **6a**

Fig. 8 Key NOE correlations and computer-generated model using MM2 force field calculations for **5** (left) and **7** (right).

Table 3 ^{1}H and ^{13}C NMR data of compounds **6** and **7**

	6a[a]		**6b**[a]		**7**[b]	
No.	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1α			97.0, CH	5.37, d (4.3)	71.2, CH_2	4.18, m
1β	103.3, CH	5.38, s				3.15, t (11.0)
2	58.2, CH	4.33, m	53.3, CH	4.55, ddd (11.6, 8.1, 4.3)	43.4, CH	4.27, m
3α	40.8, CH_2	2.59, m	37.5, CH_2	2.37, dd (12.7, 8.1)	39.7, CH_2	2.72, ddd (12.4, 4.7, 1.5)
3β		1.90, m		2.04, dd (12.7, 11.6)		1.49, dd (12.4, 12.4)
4	83.6, C		81.6, C		72.6, C	
5	134.9, CH	6.06, m	134.9, CH	6.06, m	73.8, CH	4.19, m
6	124.4, C		124.4, C		58.8, CH	4.89, d (9.9)
7	67.7, CH	4.34, m	67.7, CH	4.34, m	188.1, C	
8	55.4, CH	3.53, m	55.4, CH	3.53, m	52.2, CH	5.29, dd (3.3, 1.0)
9	59.6, CH	3.52, m	58.1, CH	3.40, m	83.5, CH	3.90, d (3.3)
10						5.22, d (8.3)
11	169.3, C		169.3, C		166.2, C	
12	119.0, CH	6.04, d (15.4)	119.0, CH	6.04, d (15.4)	116.2, CH	5.67, d (15.2)
13	147.5, CH	7.19, d (15.4)	147.5, CH	7.19, d (15.4)	147.8, CH	7.24, d (15.2)
14	132.8, C		132.8, C		130.7, C	
15	148.4, CH	5.64, d (9.5)	148.4, CH	5.64, d (9.5)	148.6, CH	5.67, d (9.8)
16	34.3, CH	2.56, m	34.3, CH	2.56, m	33.1, CH	2.50, m
17	38.4, CH_2	1.39, m; 1.27, m	38.4, CH_2	1.39, m; 1.27, m	37.0, CH_2	1.35, m; 1.25, m
18	28.6, CH_2	1.26, m	28.6, CH_2	1.26, m	27.1, CH_2	1.21, m
19	30.5, CH_2	1.27, m	30.5, CH_2	1.27, m	29.4, CH_2	1.25, m
20	33.0, CH_2	1.27, m	33.0, CH_2	1.27, m	31.6, CH_2	1.23, m
21	23.7, CH_2	1.29, m	23.7, CH_2	1.29, m	22.4, CH_2	1.27, m
22	14.4, CH_3	0.89, t (7.0)	14.4, CH_3	0.89, t (7.0)	13.9, CH_3	0.87, t (7.0)
23	12.7, CH_3	1.81, s	12.7, CH_3	1.81, s	12.3, CH_3	1.75, s
24	20.9, CH_3	0.99, d (6.7)	20.9, CH_3	0.99, d (6.7)	20.3, CH_3	0.97, d (6.7)

[a] Measured in methanol-d_4. [b] Measured in $CDCl_3$.

possessed similar $^{3}J_{1,2}$ value (almost zero) and NOE correlations as **5**, suggesting that both shared the same relative configuration, whereas the larger coupling constant between H-1 and H-2 (4.3 Hz) and NOE correlations between H-1 and H-2 observed for **6b** suggested an opposite configuration at C-1.

Compound **7** had the molecular formula $C_{23}H_{35}Br_2NO_5$ as indicated by HRESIMS. Comparison of its NMR spectroscopic data (Table 3) with those of gymnastatins T–Y (**1–6**) suggested that it featured a different bicyclic ring system but shared the same aliphatic side chain. Further analysis of 2D NMR spectra of **7** suggested the presence of the same ring system as reported earlier for dankastatins A–C.[15,17] This was confirmed by the COSY correlations between H-1α/H-2, H-1β/H-2, H-2/H-3α, H-2/H-3β, H-5/H-6, and H-8/H-9 as well as by the HMBC correlations from H-1β and H-3α to C-9, from H-3β to C-4 and C-5, and from H-6 and H-9 to C-7 (Fig. 7). The location of the two bromine atoms was deduced by comparing the carbon signals with those of dankastatins A–C. The coupling constants between H-1β and H-2 ($J_{1\beta,2}$ = 11.0 Hz), between H-2 and H-3β ($J_{2,3\beta}$ = 12.4 Hz), and between H-5 and H-6 ($J_{5,6}$ = 9.9 Hz) indicated *trans*-axial arrangement of these protons. Combined with the NOE correlations between H-1β/H-3β, H-1β/H-9, H-3β/H-9, H-9/H-8, H-8/H-6, and between H-5/H-3α, the relative configuration of the ring system was determined as shown in Fig. 8. The stereochemistry of the side chain was assigned as 12*E* and 14*E* due to the large coupling constants ($^{3}J_{12,13}$ = 15.2 Hz) and the NOE correlations between H-12/H_3-23, and H-13/H-15. Therefore, the structure of **7** was elucidated and the compound was named dankastatin D.

The three known analogues were identified as gymnastatins I–K (**8–10**) by comparison of their NMR and mass spectroscopic data with the literature.[16]

Cytotoxicity of all isolated alkaloids (**1–10**) was evaluated against the mouse lymphoma cell line (L5178Y) (Table 4). Among them, compounds **1**, **4**, **8** and **9** exhibited significant activity with IC_{50} values of 1.3, 0.99, 0.55 and 0.078 μM, respectively, even stronger than that of positive control kahalalide F. Comparison of the cytotoxicity of **4** *vs.* **6** and **9** *vs.* **10** suggested the conjugated ketone system to be important for the activity of those compounds. Gymnastatin I (**8**) showed similar activity compared to its chlorinated analogue gymnastatin A, whereas gymnastatin J (**9**) exhibited much stronger activity than its chlorinated analogue gymnastatin B.

When comparing the structures of the brominated alkaloids isolated in our study with those of chlorinated analogues reported previously,[12–17] it became evident that addition of NaBr to the medium caused an increase in the structural diversity of the fungal metabolites. Gymnastatins T (**1**), V (**3**), Y (**6**), I (**8**), J (**9**), and K (**10**) can be considered to be derivatives of gymnastatins G,[14] aranochlor A,[18] gymnastatin D, A, B, and C,[13] that are formed by a mere replacement of chlorine atoms *vs.* bromine atoms, whereas the structures of gymnastatins U (**2**), V (**4**), Y (**5**),

Table 4 Cytotoxicity of isolated brominated alkaloids

Compound	IC_{50} (μM)
1	1.3
2	3.0
3	3.6
4	0.99
5	14.1
6	6.2
7	3.0
8	0.55
9	0.078
10	9.6
Gymnastatin A	0.64[b]
Gymnastatin B	5.8[b]
Kahalalide F[a]	4.3

[a] Positive control. [b] Data from ref. 12.

and K (**7**) differ also with regard to other substituents and/or their stereochemistry, compared to their chlorinated analogues.

The brominated tyrosine-derived alkaloids isolated in this study (**1–10**) can be divided into three different subtypes including bicyclo[3.3.1]nonane derivatives (**1** and **2**), 1-oxa-spiro [4.5]decane derivatives (**3–6** and **8–10**), and 2-oxa-bicyclo[4.4.0] decane derivative (**7**). A plausible biosynthetic pathway modified after Hammerschmidt *et al.*[12] and Amagata *et al.*[14] is proposed. Gymnastatin N is suggested to be an important intermediate, which is corroborated by the co-isolation of its oxidative product 12′-hydroxygymnastatin N (**11**) and aldehyde product (**12**) originating from reduction of the carboxyl group of gymnastatin N (Fig. 9). The present study provides further evidence regarding the biosynthetic relationships of *Gymnascella* derived alkaloids, and proved the plasticity of the fungal secondary metabolism, which can be useful for biosynthetic research and the quest for new bioactive compounds.

Experimental section

General procedures

Optical rotations were measured with a Jasco P-2000 polarimeter. NMR spectra were recorded on a Bruker ARX 600 NMR spectrometer. Chemical shifts were referenced to the solvent residual peaks. Mass spectra (ESI) were recorded with a LC-MS HP1100 Agilent Finnigan LCQ Deca XP Thermoquest and HRESIMS were recorded with a UHR-QTOF maXis 4G (Bruker Daltonics) mass spectrometer. HPLC analysis was performed with a Dionex UltiMate-3400SD system coupled with an LPG-3400SD pump and a photodiode array detector (DAD 300RS). The analytical column (125 × 4 mm) was prefilled with Eurosphere-10 C_{18} (Knauer, Germany), and the following gradient was used (MeOH, 0.1% formic acid in H_2O): 0 min (10% MeOH); 5 min (10% MeOH); 35 min (100% MeOH); 45 min (100% MeOH). Semi-preparative HPLC was performed using a Merck Hitachi HPLC System (UV detector L-7400; pump L-7100; Eurosphere-100 C_{18}, 300 × 8 mm, Knauer, Germany), with mixtures of MeOH–H_2O as mobile phase. Column chromatography included Merck MN silica gel 60 M (0.04–0.063 mm). TLC plates precoated with silica gel F_{254} (Merck) were used to monitor and collect fractions; detection was under 254 and 366 nm or by spraying the plates with anisaldehyde reagent. Distilled and spectral grade solvents were used for column chromatography and spectroscopic measurements, respectively.

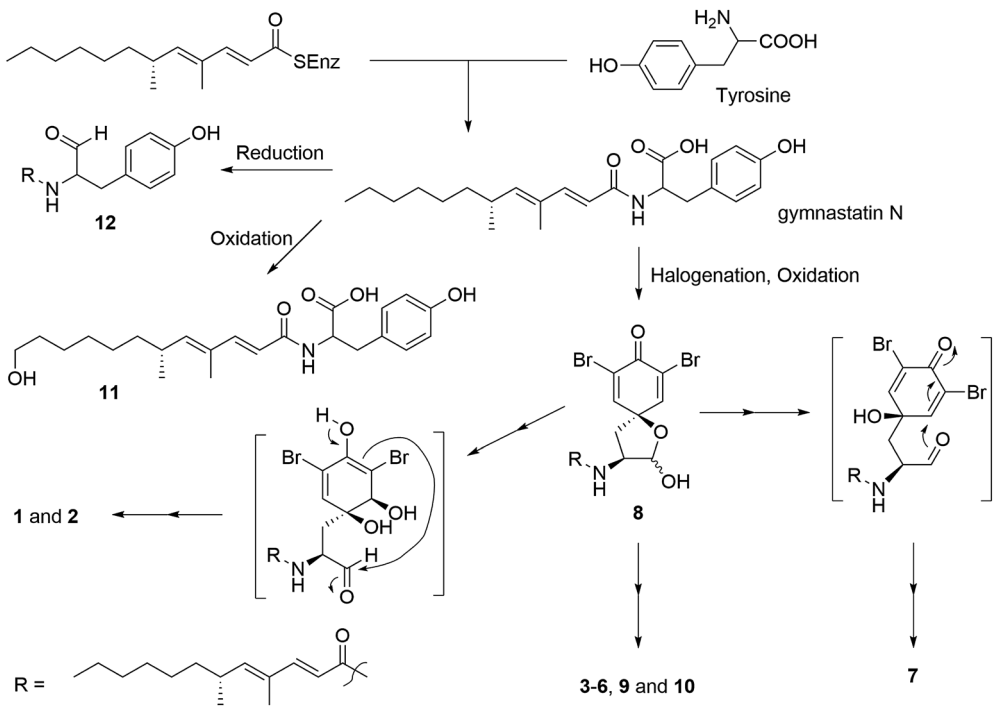

Fig. 9 Plausible biosynthesis of brominated alkaloids from *G. dankaliensis*.

Fungal material and cultivation

The fungus was isolated from a soil sample close to the Giza pyramids, Egypt, and identified by DNA amplification, sequencing of the ITS region and comparison with GenBank data (GenBank accession No. HM991265.1) as *G. dankaliensis* as previously described.[19] The fungal strain was grown on solid rice medium containing 3.5% NaBr (3.50 g NaBr, 100 mL distilled water was added to 100 g commercially available rice and then autoclaved) in two Erlenmeyer flasks (1 L each) at 27 °C under static conditions for 28 days.[20]

Extraction and isolation

Fungal culture was extracted twice with 500 mL ethyl acetate. The crude extract (9.35 g) was chromatographed on a silica column using *n*-hexane, *n*-hexane–ethyl acetate (60 : 1), CH_2Cl_2–MeOH gradient to give five fractions [A (1.87 g), B (1.32 g), C (2.13 g), D (3.15 g), E (0.87 g)]. Fraction B was further separated on a silica gel column with a CH_2Cl_2–MeOH gradient (100 : 0 to 150 : 1), affording twelve subfractions (B1–12). Fraction B9 (43.1 mg) was purified by semi-preparative HPLC using MeOH–H_2O (78 : 22) to yield **8** (4.1 mg) and **9** (4.7 mg), while fraction B10 (21.2 mg) was purified by semi-preparative HPLC using MeOH–H_2O (76 : 24) to give **7** (1.6 mg). Fraction C was fractionated by silica gel column chromatography with a gradient of CH_2Cl_2–MeOH (500 : 1 to 25 : 1) as solvent system, to give eight subfractions (C1–8). Fraction C2 (185.2 mg) was further purified by semi-preparative HPLC using MeOH–H_2O (from 77 : 23 to 80 : 20 in 25 minutes) to afford **3** (14.2 mg), **4** (7.5 mg), and **10** (17.9 mg). Fraction D was separated by silica gel column chromatography eluting with CH_2Cl_2–MeOH (200 : 1 to 15 : 1), to give eight subfractions (FrD1–8). **1** (7.2 mg) and **2** (3.5 mg) were obtained from FrD3 (285.1 mg) and FrD4 (252.3 mg) by semi-preparative HPLC using MeOH–H_2O (72 : 28), respectively. FrD5 (301.4 mg) was further purified by semi-preparative HPLC using MeOH–H_2O (70 : 30) to give **5** (1.7 mg) and **6** (2.1 mg).

Gymnastatin T (1). Colorless needle crystal; $[\alpha]_D^{20}$ −47.3 (*c* 0.50, MeOH); UV (MeOH): λ_{max} 271.2 (4.02) nm; HRESIMS *m*/*z* 500.1644 $[M + H]^+$ (calcd 500.1622 for $C_{23}H_{35}BrNO_6$); ^1H and ^{13}C NMR data, Table 1.

Gymnastatin U (2). White powder; $[\alpha]_D^{20}$ −37.5 (*c* 0.65, MeOH); UV (MeOH): λ_{max} 271.2 (3.83) nm; HRESIMS *m*/*z* 500.1641 $[M + H]^+$ (calcd 500.1622 for $C_{23}H_{35}BrNO_6$); ^1H and ^{13}C NMR data, Table 1.

Gymnastatin V (3). Colorless oil; $[\alpha]_D^{20}$ −1.8 (*c* 0.56, MeOH); UV (MeOH): λ_{max} 271.2 (3.73) nm; HRESIMS *m*/*z* 482.1534 $[M + H]^+$ (calcd 482.1537 for $C_{23}H_{33}BrNO_5$); ^1H and ^{13}C NMR data, Table 2.

Gymnastatin W (4). White powder; $[\alpha]_D^{20}$ −27.0 (*c* 0.88, MeOH); UV (MeOH): λ_{max}271.9 (3.98) nm; HRESIMS *m*/*z* 576.0952 $[M + H]^+$ (calcd 576.0955 for $C_{24}H_{36}Br_2NO_5$); ^1H and ^{13}C NMR data, Table 2.

Gymnastatin X (5). Colorless oil; $[\alpha]_D^{20}$ −10.2 (*c* 0.22, MeOH); UV (MeOH): λ_{max} 270.4 (3.94) nm; HRESIMS *m*/*z* 498.1845 $[M + H]^+$ (calcd 498.1850 for $C_{24}H_{37}BrNO_5$); ^1H and ^{13}C NMR data, Table 2.

Gymnastatin Y (6). White powder; $[\alpha]_D^{20}$ −3.9 (*c* 1.04, MeOH); UV (MeOH): λ_{max} 271.9 (3.94) nm; HRESIMS *m*/*z* 484.1689 $[M + H]^+$ (calcd 484.1693 for $C_{23}H_{35}BrNO_5$); ^1H and ^{13}C NMR data, Table 3.

Dankastatin D (7). Colorless oil; $[\alpha]_D^{20}$ −29.4 (*c* 0.25, MeOH); UV (MeOH): λ_{max} 270.4 (3.73) nm; HRESIMS *m*/*z* 564.0959 $[M + H]^+$ (calcd 564.0955 for $C_{23}H_{36}Br_2NO_5$); ^1H and ^{13}C NMR data, Table 3.

X-ray crystallographic analysis of gymnastatin T (1)

Crystallization conditions. X-ray quality crystal of **1** was obtained by slow evaporation from MeOH solution. A suitable single crystal was carefully selected under a polarizing microscope.

Data collection. Bruker Kappa APEX2 CCD diffractometer, Mo-Kα radiation (λ = 0.71073 Å), multilayer mirror, ω- and ϕ-scan; data collection with APEX2, cell refinement and data reduction with SAINT,[21] experimental absorption correction with SADABS.[22]

Structure analysis and refinement. The structure was solved by direct methods using SHELXS-97; refinement was done by full-matrix least squares on F^2 using the SHELXL-97 program suite.[23] All non-hydrogen positions were refined with anisotropic displacement parameters. Hydrogen atoms were positioned geometrically and refined using riding models. Graphics were drawn with DIAMOND,[24] analyses of the inter- and intramolecular hydrogen bonding interactions were done with PLATON for *Windows*.[25]

Crystal data of 1. $C_{23}H_{34}BrNO_6 \cdot CH_4O$, M = 532.46, orthorhombic system, space group $P2_12_12_1$, a = 7.0251(3) Å, b = 11.3852(5) Å, c = 32.3080(15) Å, V = 2584.1(2) Å^3, Z = 4, D_{calc} = 1.369 g cm^{-3}, crystal size 0.40 × 0.05 × 0.01 mm^3, μ(Mo-Kα) = 1.633 mm^{-1}, 2.5° < θ < 25.3°, N_t = 39 194, N = 6086 (R_{int} = 0.0445), R_1 = 0.03626, wR_2 = 0.1637, S = 1.092, Flack parameter[26] 0.043(4).

Cytotoxicity assay

Cytotoxicity against the mouse lymphoma cell line L5178Y (European Collection of Authenticated Cell Cultures; Catalogue No. 87111908) was tested using the MTT method with kahalalide F as positive control and media with 0.1% DMSO as negative control as described previously.[27] The cells were grown in RPMI medium and 10% FCS (fetal calf serum) (Biochrom/Merck). Thiazolyl blue tetrazolium bromide (MTT; # M2128 Sigma) was used as an indicator for cell viability. The viability assay reaction is based upon the oxidation of the yellowish MTT solution to solid formazan *via* the mitochondrial dehydrogenases in living cells.[28] The crystals formed were solubilized with acidified isopropanol and the intensity was determined colorimetrically at 570 nm.[29]

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