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Orthidines A–E, tubastrine, 3,4-dimethoxyphenethyl-β-guanidine, and 1,14-sperminedihomovanillamide: potential anti-inflammatory alkaloids isolated from the New Zealand ascidian *Aplidium orthium* that act as inhibitors of neutrophil respiratory burst

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ABSTRACT

In addition to the known dihydroxystyrylguanidine alkaloid tubastrine (**1**), five new dimers, orthidines A–E (**2**–**6**) and the biosynthetically unrelated 1,14-sperminedihomovanillamide (orthidine F, **7**) were isolated from the New Zealand ascidian *Aplidium orthium*. The structures of the new compounds, elucidated by interpretation of spectroscopic data, encompass benzodioxane neolignan-type scaffolds (**2**–**5**) and a 1,2,3,4-tetrasubstituted cyclobutane (**6**), the latter likely having arisen via $[\pi 2_s + \pi 2_s]$ dimerization of tubastrine. The subunit head-to-tail orientation of dimer **6** was established unambiguously by interpretation of data from a $^2J,^3J$-HMBC NMR experiment. The structure of **7** was also confirmed by facile synthesis. Compounds **1**–**4**, **6**, and **7** inhibited the in vitro production of superoxide by PMA-stimulated human neutrophils in a dose-dependent manner with IC_{50}s of 10–36 μM and this was associated with inhibition of superoxide production by neutrophils in vivo in a murine model of gouty inflammation.

1. Introduction

As part of our ongoing screening campaign of New Zealand biota to discover new anti-inflammatory natural products[1–4] we found that an extract of the marine organism *Aplidium orthium* (Ascidiacea), collected from Northeast Island of the Three Kings Islands, strongly inhibited superoxide production by stimulated human neutrophils.[5] Bioassay-guided fractionation of this extract using combinations of C_8 and C_{18} reversed phase and Sephadex LH-20 flash column chromatography led to the isolation of the known guanidinostyrene marine natural product tubastrine (**1**),[6,7] four new benzodioxanes (orthidines A–D, **2**–**5**), a cyclobutane dimer of tubastrine (orthidine E, **6**), and a biosynthetically unrelated dihomovanillamide derivative of spermine (orthidine F, **7**). Investigation of the chemical components of non-biologically active chromatography fractions yielded guanidine **8**, previously known as a synthetic compound.[8] The structures of **1**–**8** were determined by spectroscopic methods with confirmation of **7** also being achieved by synthesis. In vitro and in vivo anti-inflammatory activities are reported for the natural products **1**–**4** and **6**–**8**.

2. Results and discussion

2.1. Chemistry

The biologically active crude MeOH extract of the organism was subjected to reversed phase C_{18} column chromatography with a steep H_2O (0.05% TFA) to MeOH gradient. Biological activity was detected in the H_2O (TFA) and 10–20% MeOH/H_2O (0.05% TFA) fractions, further purification of which using reversed phase C_8 and Sephadex LH-20 gel permeation chromatography afforded the previously reported alkaloid tubastrine (**1**) and five new compounds (**2**, **3**, **4**, **6**, **7**). A second collection of the organism from the same location yielded the same series of compounds as well as the additional stereoisomer **5**. Investigation of the biologically inactive 30% MeOH/H_2O (0.05% TFA) C_{18} column fraction afforded

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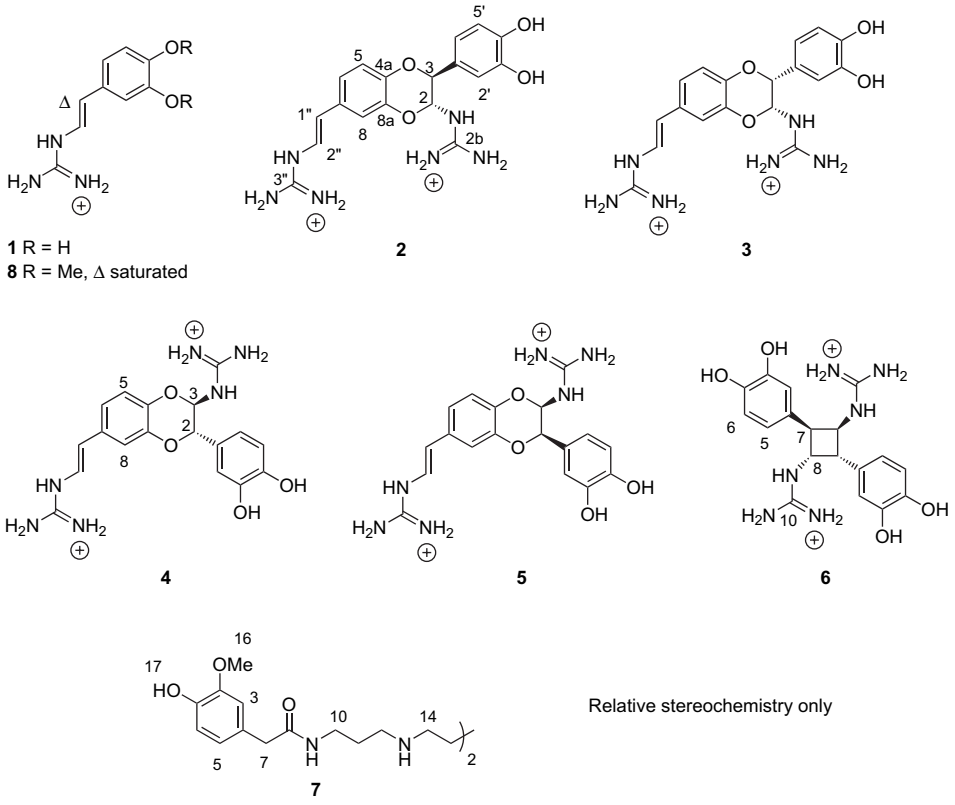

3,4-dimethoxyphenethyl-β-guanidine (**8**), which is reported for the first time as a natural product.[8]

The first eluting bioactive compound was identified as tubastrine (**1**), previously isolated from the hard coral *Tubastrea aurea*[6] and the ascidian *Dendrodoa grossularia*,[7] through combined use of HRESIMS and ^{1}H and ^{13}C, and two dimensional NMR spectroscopy. Its identity was confirmed by direct comparison of ^{1}H and ^{13}C NMR data with that previously reported.[7]

Orthidine A (**2**) was assigned a molecular formula of $C_{18}H_{20}N_6O_4$ on the basis of HRESIMS and NMR data. In addition to the pseudomolecular ion at *m*/*z* 385, a doubly ionized ion at *m*/*z* 193 attributable to $[M+2H]^{2+}$ was also observed. The UV–vis spectrum of **2** exhibited absorption maxima at 207, 226, and 284 nm with a shoulder at 311 nm indicative of an extended aromatic chromophore. Interpretation of the ^{1}H (Table 1) and ^{13}C (Table 2) NMR spectra including HSQC and HMBC data established the presence of a tubastrine-like substructure accounting for $C_9H_9N_3O_2$ of the required formula. In addition, a partial fragment consisting of a 3,4-dihydroxyphenyl group, two oxymethine carbons (δ_C 77.5, δ_H 4.91, d, J=5.6 Hz; δ_C 79.8, δ_H 5.63, d, J=5.6 Hz), and a guanidine group (δ_C 158.7) was established by interpretation of HSQC and HMBC NMR spectra, accounting for the remaining composition. HMBC correlations observed between H-8 (δ 7.00, d, J=2.0 Hz) and H-6 (δ 6.97, dd, J=8.4, 2.0 Hz) to δ 143.4 (C-4a) and from H-5 (δ 6.92, d, J=8.4 Hz) to δ 142.7 (C-8a) unequivocally assigned carbons C-4a and C-8a, respectively. Interfragment connectivity was established by observing HMBC correlations between H-2 (δ 5.63) and C-8a and between H-3 (δ 4.91) and C-4a leading to the construction of a 2,3-dihydrobenzodioxane framework for orthidine A (**2**). The relative stereochemistry and conformation of **2** was investigated by analysis of NOESY NMR correlations, and $^{3}J_{HH}$ and $^{3}J_{CH}$ coupling constants. NOESY correlations were observed between H-2 and H-3/H-2′/H-6′ and between H-3 and H-2/H-2′/H-6′. While the magnitude of the vicinal coupling between H-2 and H-3 (5.6 Hz) suggested a trans stereochemical relationship, an H-2 axial, H-3 axial arrangement was discounted due to the observation of strong NOESY correlations between H-2 and H-3. This inferred an H-2 equatorial, H-3 equatorial arrangement for **2** (Fig. 1). Further support for this relative stereochemistry and conformation was obtained from analysis of a *J*-HMBC NMR experiment,[9] which furnished coupling constants of 8.0 Hz for both $^{3}J_{C\text{-}8a\text{–}H\text{-}2}$ and $^{3}J_{C\text{-}4a\text{–}H\text{-}3}$. Such a coupling constant is consistent with C-8a/H-2 and C-4a/H-3 being in

Table 1
^{1}H NMR (600 MHz, CD_3OD) data for **2**–**5**

Position	**2**	**3**	**4**	**5**
	δ_H (mult., J in Hz)	δ_H (mult., J in Hz)	δ_H (mult., J in Hz)	δ_H (mult., J in Hz)
2	5.63 (d, 5.6)	5.87 (d, 1.9)	4.91 (d, 5.7)	5.23 (d, 2.0)
3	4.91 (d, 5.6)	5.23 (d, 1.9)	5.63 (d, 5.7)	5.86 (d, 2.0)
5	6.92 (d, 8.4)	7.03 (m)	6.88 (d, 8.4)	6.94 (d, 8.4)
6	6.97 (dd, 8.4, 2.0)	7.03 (m)	6.96 (dd, 8.4, 2.0)	7.03 (m)
8	7.00 (d, 2.0)	7.05 (m)	7.05 (d, 2.0)	7.13 (d, 2.0)
2′	6.86 (d, 1.8)	6.96 (d, 1.4)	6.90 (d, 1.7)	6.96 (m)
5′	6.78 (m)	6.84 (m)	6.79 (m)	6.84 (m)
6′	6.76 (m)	6.85 (m)	6.80 (m)	6.86 (m)
1″	6.22 (d, 14.0)	6.23 (d, 14.0)	6.23 (d, 13.9)	6.24 (d, 14.0)
2″	7.08 (d, 14.0)	7.12 (d, 14.0)	7.12 (d, 13.9)	7.12 (d, 14.0)

Table 2
^{13}C NMR[a] and HMBC[b] data for **2**–**5** (CD_3OD)

Position	**2**	**3**	**4**	**5**
	δ_C	δ_C	δ_C	δ_C
2	79.8 (3)	78.8 (3)	77.4 (2′, 6′)	76.6 (2′, 6′)
3	77.5 (2, 2′, 6′)	76.6 (2′, 6′)	79.8	78.8
4a	143.4 (3, 5, 6, 8)	143.7 (8)	141.9 (3, 5, 6, 8)	140.6 (3, 5, 6, 8)
5	118.6 (6)	119.1	118.7	119.3
6	121.2 (8, 1″)	121.4 (8, 1″)	121.2 (8, 1″)	121.6 (8, 1″)
7	131.5 (5, 2″)	132.2 (5, 2″)	131.5 (5, 2″)	131.9 (5, 2″)
8	115.5 (6, 1″)	116.1 (6, 1″)	115.3 (6, 1″)	115.9 (6, 1″)
8a	142.7 (2, 5, 8)	141.5 (2, 5, 8)	144.2 (2, 5)	144.6 (2, 5, 8)
1′	128.0 (2, 3, 2′, 5′)	126.9 (3, 5′)	128.0 (3, 5′)	126.9 (2, 2′, 5′)
2′	115.4 (3, 6′)	114.2 (3, 6′)	115.5 (2, 6′)	114.3 (2, 6′)
3′	146.8 (2′, 5′)	146.9 (5′)	146.7 (2′, 5′)	146.9 (5′)
4′	147.5 (2′, 6′)	147.1 (2′, 6′)	147.4 (2′, 6′)	147.1 (2′, 6′)
5′	116.4	116.6	116.5	116.5
6′	120.3 (3, 2′)	118.5 (3, 2′)	120.4 (2, 2′)	118.6 (2, 2′)
1″	116.8 (6, 2″)	116.7 (6, 8, 2″)	116.9 (6)	116.6 (6, 8)
2″	122.0 (1″)	122.3 (1″)	121.9	122.2 (1″)
4″	156.2 (2″)	156.2 (2″)	156.0 (2″)	156.2 (2″)
2b	158.7 (2)	159.1 (2)	158.6 (3)	159.1 (3)

[a] Obtained at 150 MHz.
[b] Numbers in parentheses are protons to which the carbon correlated to in ^{1}H–^{13}C HMBC NMR experiments.

antiperiplanar relationships (Fig. 1a). Thus it was concluded that orthidine A (**2**) bears the 2-guanidyl and 3-(3,4-dihydroxyphenyl) groups in a 2,3-trans diaxial (H-2 equatorial, H-3 equatorial) configuration. The lack of specific rotation and absence of CD maxima implied that **2** was a racemate.

1,4-Benzodioxane derivatives resulting from dimerization have been reported from a number of sources including neolignans americanol A and isoamericanol A isolated from the seeds of the plant *Phytolacca americana*,[10] ascidian metamorphosis-inducing sulfated 3,4-dihydroxystyrene dimers isolated from the marine sponge *Jaspis* sp.,[11] and *N*-acetyldopamine dimers isolated from the sclerotization of insect cuticles.[12–14] Facile racemization of the latter dimers was noted[13] and more recently such dimers have been reported to exhibit antioxidant and anti-inflammatory biological activities.[14] Tubastrine and related compounds[15,16] have been reported from a number of divergent phyla (Chordata, Porifera, and Cnidaria)—this is the first report of tubastrine dimers.

Orthidine B (**3**) had the same molecular formula as **2** and exhibited very similar ^{1}H and ^{13}C NMR resonances (Tables 1 and 2). Major differences were observed, however, for resonances associated with the oxymethines C-2 and C-3 (δ_C 78.8, δ_H 5.87; δ_C 76.6, δ_H 5.23). Analysis of HSQC and HMBC NMR data led to the same planar structure as orthidine A (**2**) with an HMBC correlation observed between H-2 and C-8a (δ 141.5) being important in establishing regiochemistry. The observation of a $^3J_{H\text{-}2\text{–}H\text{-}3}$ vicinal coupling constant of 1.9 Hz indicated a cis relative configuration between H-2 and H-3. As with **2**, NOESY correlations were also observed for orthidine B (**3**) from H-2 to H-3, H-2′, and H-6′ and from H-3 to H-2, H-2′, and H-6′. A *J*-HMBC NMR experiment determined the $^3J_{C\text{-}8a\text{–}H\text{-}2}$ coupling constant to be 10.3 Hz while the lack of an observed correlation attributed a coupling constant for $^3J_{C\text{-}4a\text{–}H\text{-}3}$ of less than 1 Hz. These results are consistent with H-2 being antiperiplanar to C-8a (i.e., guanidyl axial) and H-3 being orthogonal to C-4a (i.e., dihydroxylphenyl equatorial) (Fig. 1b). A lack of detectable specific rotation or CD maxima indicated that **3** was also isolated as a racemate.

Spectroscopic and spectrometric data observed for orthidine C (**4**) were very similar to those observed for orthidine A (**2**). Inspection of ^{1}H and ^{13}C NMR data (Tables 1 and 2) and analysis of HSQC and HMBC spectra indicated the presence of the same substructural fragments, i.e., a 3,4-dioxa-phenyl-(*E*)-ethenyl guanidine fragment similar to tubastrine and a second fragment comprised of a 3,4-dihydroxyphenyl, two oxymethines (δ_C 77.4, δ_H 4.91, d, J=5.7 Hz; δ_C 79.8, δ_H 5.63, d, J=5.7 Hz), and a guanidine group (δ 158.6). Interfragment HMBC correlations between H-2 and H-5 to C-8a (δ 144.2) and from H-3/H-6/H-8 to C-4a (δ 141.9) indicated that the 3,4-dihydroxyphenyl and guanidine groups were substituted on C-2 and C-3, respectively; the reversed attachment order to that observed for orthidine A (**2**). The magnitude of the $^3J_{H\text{-}2\text{–}H\text{-}3}$ vicinal coupling constant (5.7 Hz) supported a trans relative configuration and as with **2**, *J*-HMBC NMR data indicated that both $^3J_{C\text{-}8a\text{–}H\text{-}2}$ and $^3J_{C\text{-}4a\text{–}H\text{-}3}$ coupling constants were 7.6 Hz, inferring that both H-2 and H-3 were equatorial. Lack of chiroptical properties indicated **4** was also a racemate.

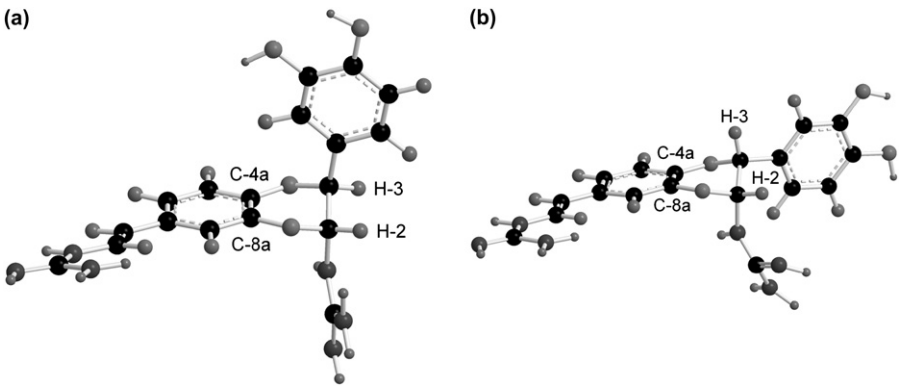

Figure 1. Relative stereochemistry of (a) orthidine A (**2**) and (b) orthidine B (**3**) as determined by interpretation of NOESY, $^3J_{HH}$ and $^3J_{CH}$ NMR data.

Table 3
^{1}H (600 MHz), ^{13}C (150 MHz), and HMBC[a] data for **6** (CD_3OD)

Position	δ_H (mult., J in Hz)	δ_C	^{1}H–^{13}C HMBC	NOESY
1, 1′		146.2	3/3′, 5/5′	
2, 2′		146.7	6/6′	
3, 3′	6.84 (d, 1.9)	116.9	5/5′, 7/7′	7/7′, 8/8′
4, 4′		128.0	6/6′, 7/7′, 8/8′	
5, 5′	6.75 (dd, 8.1, 1.9)	121.0	3/3′, 7/7′	7/7′, 8/8′
6, 6′	6.80 (d, 8.1)	116.6		
7, 7′	3.88 (dd, 8.0, 6.7)	50.1	3/3′, 5/5′, 7/7′, 8/8′	8/8′, 3/3′, 5/5′
8, 8′	4.66 (dd, 8.0, 6.7)	53.0	7/7′, 8/8′	7/7′, 3/3′, 5/5′
NH-9, 9′	7.94 (br d)[b]			
10, 10′		158.2	8/8′	

[a] Protons to which the carbon correlated to in ^{1}H–^{13}C HMBC NMR experiments.
[b] Obtained in DMSO-d_6 (300 MHz).

Compound **6** (orthidine E) was isolated as an optically inactive pale yellow gum and exhibited a pseudo-molecular ion at m/z 387 ($[M+H]^+$ $C_{18}H_{23}N_6O_4$) under electrospray ionization. As seen for orthidines A–C, a doubly ionized ion, this time at m/z 194 attributable to $[M+2H]^{2+}$, was also detected. Analysis of ^{1}H, ^{13}C, HSQC-DEPT, and HMBC NMR data (Table 3) established the presence of the now familiar 1,2,4-trisubstituted dihydroxylated phenyl ring, two coupled methines (H-7 (δ_H 3.88, dd, J=8.0, 6.7 Hz, δ_C 50.1); H-8 (δ_H 4.66, dd, J=8.0, 6.7 Hz, δ_C 53.0)), and guanidine (δ 158.2) fragments. These fragments accounted for exactly half of the required molecular formula, indicating that **6** was a dimer. Long-range HMBC correlations were observed from H-7 (δ 3.88) to C-3 (δ 116.9) and C-5 (δ 121.0) of the catechol ring and C-8 (δ 53.0) and from H-8 (δ 4.66) to C-7 (δ 50.1) and to the guanidine carbon, C-10 (δ 158.2), consistent with the presence of a dihydrotubastrine substructure. Inter-substructure HMBC correlations observed from H-7 to C-7 and from H-8 to C-8 suggested that **6** was a 1,2,3,4-tetrasubstituted cyclobutane related to tubastrine by a $[\pi 2_s+\pi 2_s]$ cycloaddition reaction. Such photochemically allowed reaction products can be described as deriving from head-to-head or head-to-tail cycloaddition. Examples of natural products resulting from head-to-head, e.g., sceptrin,[17] sagerinic acid,[18] monochaetin,[19] moslolignans,[20] and mooniines[21] or head-to-tail, e.g., *p*-coumaric and ferulic acid dimers,[22] 4,4′-dihydroxytruxillic acid,[23] achyrodimers A–C,[24] and piperarborenines A and B[25] dimerization have been reported.

To discern between the possible regioisomeric structures of **6** resulting from head-to-head (**A**) or head-to-tail (**B**) cycloaddition (Fig. 2a), a $^{2}J,^{3}J$-HMBC long-range NMR experiment was used.[26] This experiment, which introduces a STAR operator into proton-detected long-range heteronuclear correlation pulse sequences, allows differentiation between two-bond and three-bond long-range correlations to protonated carbons. By use of a J_{scale} parameter, an F_1 dimension skew is apparent in $^{2}J_{CH}$ correlations while no modulation of $^{3}J_{CH}$ correlations occurs. As shown in Figure 2b and c, correlations observed in the $^{2}J,^{3}J$-HMBC experiment of **6** carried out in both CD_3OD and D_2O solvents clearly show that H-7/7′ correlates to C-7/7′ with a $^{3}J_{CH}$-type signal, as does H-8/8′ with C-8/8′, while H-7/7′ exhibits a staggered F_1 skewed $^{2}J_{CH}$ correlation to C-8/8′ as also observed for H-8/8′ to C-7/7′. Thus orthidine E (**6**) represents a relatively rare example of a naturally occurring head-to-tail cycloaddition product.

Data obtained in an NOESY NMR experiment was used to determine the relative configuration of **6**. There are three relative stereochemical geometries possible for **6**, being cis–trans–cis (**C**), cis–cis–cis (**D**) or trans–trans–trans (**E**) as shown in Figure 3.[27–29] NOESY correlations observed between the cyclobutane protons

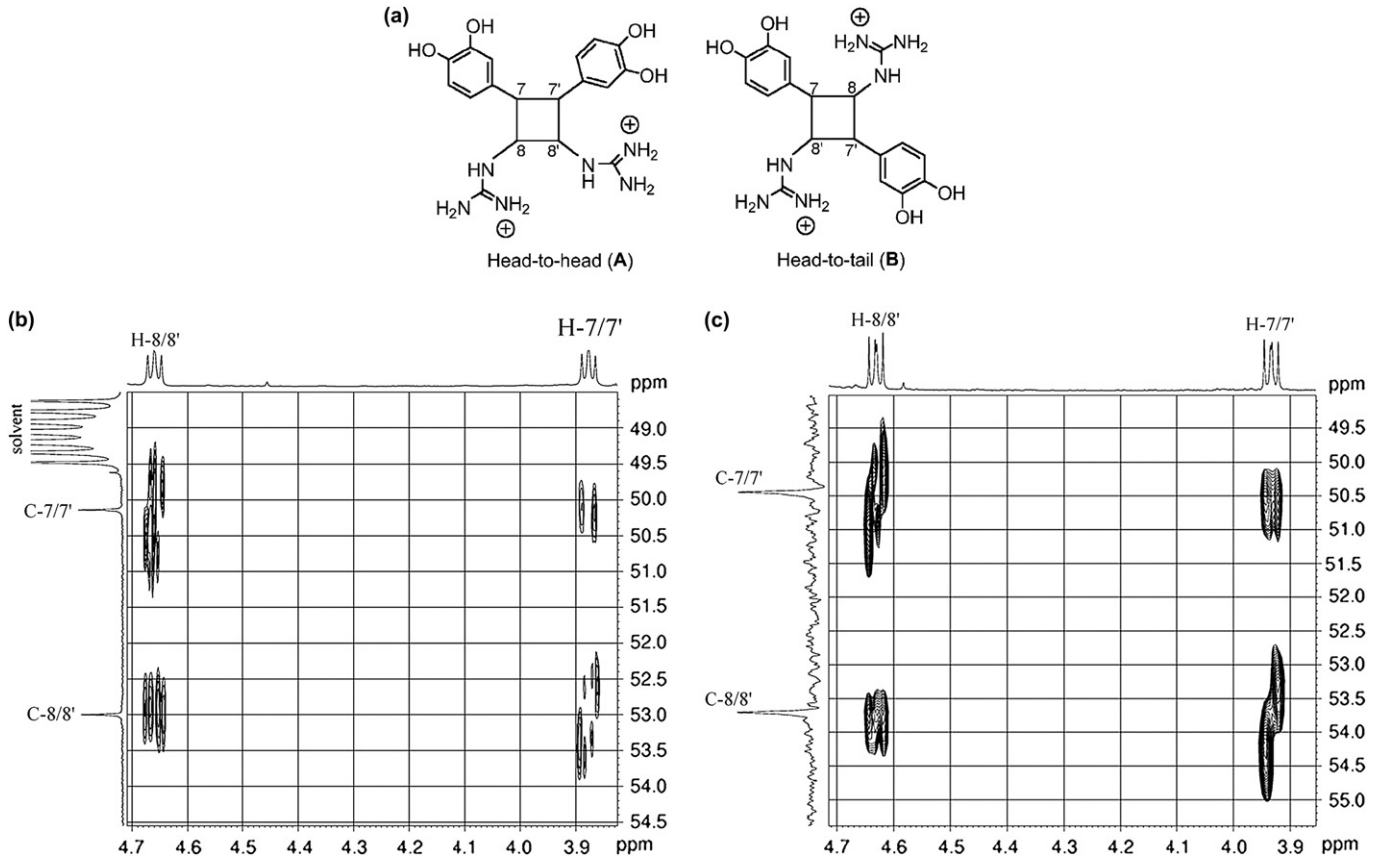

Figure 2. (a) Alternative head-to-head (**A**) or head-to-tail (**B**) cycloaddition structures of orthidine E (**6**) and relevant regions of $^{2}J,^{3}J$-HMBC NMR spectra (J_{scale} 16) of **6** obtained in (b) CD_3OD and (c) D_2O highlighting 'normal' shaped correlations between H-7/7′/C-7/7′ and H-8/8′/C-8/8′ and 'F_1 skew' shaped correlations between H-7/7′/C-8/8′ and H-8/8′/C-7/7′ establishing the $^{3}J_{CH}$ bonding arrangement between H-7/7′ and C-7/7′ and H-8/8′ and C-8/8′ and hence defining orthidine E as being head-to-tail (i.e., **B**).

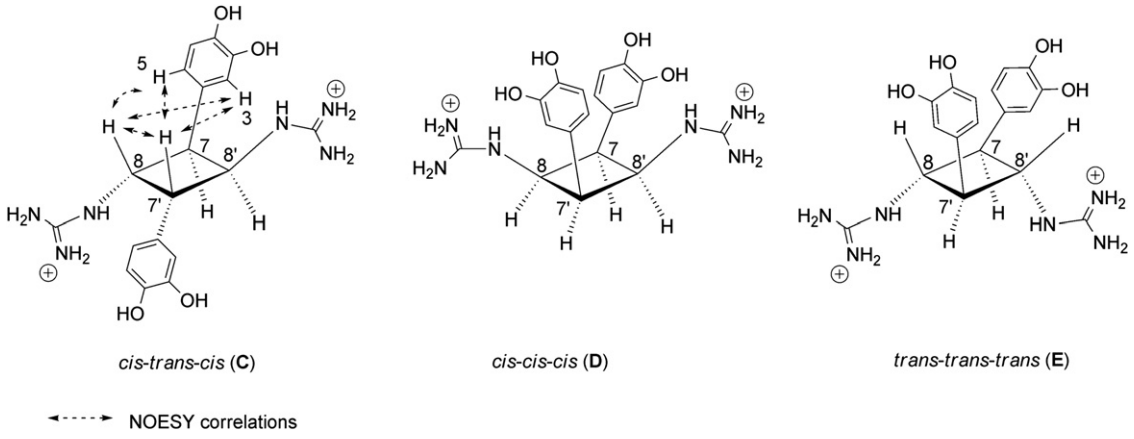

Figure 3. Alternative relative configurations (**C**–**E**) of orthidine E (**6**) with observed NOESY correlations indicated on **C**.

H-7/7′ and H-8/8′ are not consistent with the all-trans configuration **E**. NOESY correlations were also observed between H-7/7′ and H-8/8′ to H-3/3′ and H-5/5′, which is possible for either of configurations **C** and **D**. While all-cis configurations such as **D** have been discounted on the grounds of steric congestion,[28] previous studies indicating cyclobutane ^{1}H–^{1}H-coupling constants of $^{3}J_{cis}$ 11 Hz and $^{3}J_{trans}$ 6 Hz [27,29] provide stronger evidence that the coupling constants observed for **6** (H-7/H-8, dd, J=8.0, 6.7 Hz) arise from a cis–trans–cis (i.e., **C**), rather than the all-cis (**D**), relative configuration.

Orthidine F (**7**) had a molecular formula of $C_{28}H_{42}N_4O_6$ as determined by HRFABMS. Inspection of the ^{1}H and ^{13}C NMR spectra (DMSO-d_6) (Table 4) accounted for 14 carbons and 21 protons, including 3 exchangeables, indicating that **7** was another symmetrical dimer. The presence of a homovanilloyl fragment was suggested by inspection of ^{1}H NMR [δ 8.83, 1H, br s, OH-17; 6.85, 1H, d, J=1.8 Hz, H-3; 6.70, 1H, d, J=8.0 Hz, H-6; 6.64, 1H, dd, J=8.0, 1.8 Hz, H-5; 3.74, 3H, s, OCH_3; 3.29, 2H, s, H_2-7] and ^{13}C NMR [δ 147.2 (s), 145.0 (s), 127.0 (s), 121.1 (d), 115.2 (d), 113.2 (d), 55.5 (q), 41.9 (t)] data. Intra-fragment HSQC and long-range ^{1}H–^{13}C HMBC NMR correlations confirmed this. An isolated 1,5-diazaheptanyl 12-proton spin system, -$NHCH_2CH_2CH_2NHCH_2CH_2$-, was also identified by interpretation of COSY, selective TOCSY, HSQC, and HMBC NMR data. Connection to the homovanilloyl fragment via an amide bond was established by observation of HMBC correlations from NH-9 (δ 8.23) and CH_2-10 (δ_H 3.11) to C-8 (δ_C 170.9). This combined substructure accounted for all observed ^{1}H and ^{13}C NMR resonances and amounted to exactly half of the molecular formula, implying dimerization through C-15. This was supported by the observation of HMBC correlations from CH_2-15 (δ_H 1.65) to C-15 (δ_C 22.4) leading to the conclusion that orthidine F was the 1,14-dihomovanillamide derivative of spermine (**7**). Reaction of spermine with homovanillic acid in the presence of coupling agent PyBOP yielded **7** (39%) that was identical to the natural product in all respects. Structurally, orthidine F (**7**) is closely related to the plant derived dihydrocaffeamide spermine alkaloid kukoamine A,[30] which exhibits hypotensive activity and also inhibits the trypanosomatid parasite enzyme trypanothione reductase.[31]

Table 4
^{1}H (400 MHz), ^{13}C (100 MHz), and HMBC[a] data for **7** (DMSO-d_6)

Position	δ_H (mult., J in Hz)	δ_C	^{1}H–^{13}C HMBC
1, 1′		145.0	OH-17/17′, 3/3′, 5/5′
2, 2′		147.2	OH-17/17′, 6/6′, 16/16′
3, 3′	6.85 (d, 1.8)	113.2	5/5′, 7/7′
4, 4′		127.0	6/6′, 7/7′
5, 5′	6.64 (dd, 8.0, 1.8)	121.1	3/3′, 7/7′
6, 6′	6.70 (d, 8.0)	115.2	OH-17/17′
7, 7′	3.29 (s)	41.9	3/3′, 5/5′
8, 8′		170.9	7/7′, NH-9/9′, 10/10′
NH-9, 9′	8.23 (br t)		
10, 10′	3.11 (m)	35.7	11/11′
11, 11′	1.77 (m)	25.7	12/12′
12, 12′	2.82 (m)	44.3	10/10′, 11/11′
NH-13, 13′	9.05 (br m)		
14, 14′	2.83 (m)	45.8	
15, 15′	1.65 (m)	22.4	14/14′, 15/15′
16, 16′	3.74 (s)	55.5	
OH-17, 17′	8.83 (br s)		

[a] Protons to which the carbon correlated to in ^{1}H–^{13}C HMBC NMR experiments.

Inspection of non-biologically active column fractions by HPLC-DAD and NMR for related metabolites that could aid in structure–activity relationship studies yielded compound **8**. Inspection of ^{1}H, ^{13}C, COSY, HSQC, and HMBC NMR data established **8** as being 3,4-dimethoxyphenethyl-β-guanidine, previously reported as a synthetic product.[8]

Investigation of a second collection of *A. orthium* yielded **5** (orthidine D), which proved very difficult to purify and so was characterized as a 2:1 mixture with **4**. Whilst signal overlap hampered analysis of the ^{1}H NMR spectrum, adequate ^{13}C resonance dispersion allowed for complete assignment of the structure of the major component of the mixture (Tables 1 and 2). The planar structure of **5** was determined to be the same as orthidine C (**4**) (i.e., 2-(3,4-dihydroxyphenyl) and 3-guanidyl substituted) by interpretation of HSQC and HMBC NMR data. A $^{3}J_{H\text{-}2\text{–}H\text{-}3}$ vicinal coupling constant of 2.0 Hz indicated a C-2/C-3 cis relative configuration, which was supported by NOESY NMR correlations (data not shown). A J-HMBC NMR experiment determined the $^{3}J_{C\text{-}4a\text{–}H\text{-}3}$ coupling constant to be 10.2 Hz while the lack of an observed correlation attributed a coupling constant for $^{3}J_{C\text{-}8a\text{–}H\text{-}2}$ of less than 1 Hz. These results are consistent with H-3 being antiperiplanar to C-4a (i.e., guanidyl axial) and H-2 being orthogonal to C-8a (i.e., dihydroxylphenyl equatorial). The mixture of **4** and **5** was optically inactive suggesting that **5** was a racemate.

The biogenesis of orthidines A–D is likely to be similar to that proposed for neolignans or lignoids, which proceeds via phenol oxidation, followed by *O*-β radical coupling and ring closure of resultant quinone methides (Fig. 4).[32] If not enzymatically controlled, the coupling and ring closure steps would be expected to yield regio- and stereoisomers as observed in the current study.

2.2. Biological evaluation

Tubastrine (**1**) and orthidines A, B, C, E, and F (**2**, **3**, **4**, **6**, **7**) inhibited in vitro superoxide production by PMA-stimulated human neutrophils in a dose-dependent manner (Table 5).[5] The compounds exhibited good selectivity, with low or non-detectable

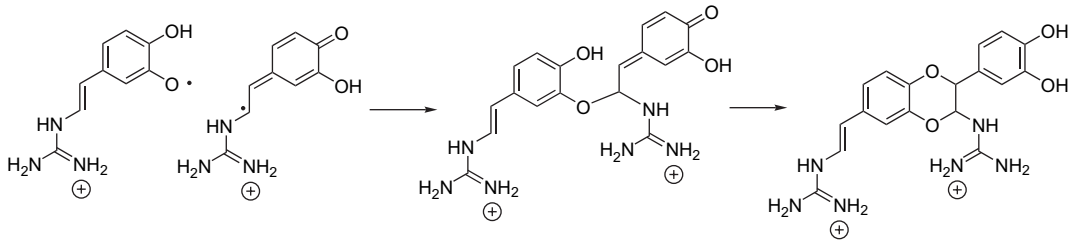

Figure 4. Proposed biogenesis of orthidines A and B from tubastrine.[32]

Table 5
Summary of biological activities observed for compounds **1**–**4**, **6**–**8**[a]

Compound	AI_{50}[b]	AP_{50}[c]	X/XO[d]
1	27.21±0.03 (*n*=2)	>258 (*n*=2)	156.0±6.38 (*n*=2)
2	15.06±0.23 (*n*=2)	118.0 (*n*=1)	133.4 (*n*=1)
3	11.80±1.34 (*n*=2)	>1000 (*n*=2)	118.4±2.64 (*n*=2)
4	14.13±1.13 (*n*=2)	>165 (*n*=2)	67.47±2.78 (*n*=2)
6	10.67±1.57 (*n*=2)	>129 (*n*=2)	67.96±6.91 (*n*=2)
7	36.25±6.18 (*n*=2)	101.2±13.69 (*n*=2)	215.8±13.46 (*n*=2)
8	262.8±48.77 (*n*=2)	>893 (*n*=2)	>893 (*n*=2)
SOD	0.65±0.06		
Methotrexate		6.92 nM±0.84 nM	
Allopurinol			42.24±5.86

[a] Values are IC_{50} values (units of μM unless stated otherwise) representing the mean of at least two determinations±standard error.
[b] AI_{50}: anti-inflammatory activity. Concentration of compound required to inhibit in vitro superoxide production by phorbol-12-myristate 13-acetate (PMA)-stimulated human neutrophils by 50%.
[c] AP_{50}: antiproliferative activity. Concentration of compound required to inhibit HL60 cell proliferation by 50%.
[d] X/XO: xanthine/xanthine oxidase inhibitory activity. Concentration of compound required to inhibit superoxide production in xanthine/xanthine oxidase assay by 50%.

antiproliferative activity (AP_{50}) against human leukemic HL60 cells. Significantly weaker suppression of superoxide production was observed for 3,4-dimethoxyphenethyl-β-guanidine (**8**), presumably due to the lack of phenolic functionality. In an effort to determine the influence of non-specific antioxidant effects, all compounds were tested in a xanthine/xanthine oxidase enzyme-based superoxide scavenging assay.[4] As also shown in Table 5, all of the compounds were considerably less effective as superoxide scavengers than as suppressors of superoxide production by neutrophils. The compounds were also tested for inhibition of neutrophil infiltration and superoxide production in an acute model of gouty arthritis as previously described.[4] At a dose of 25 μmol/kg, administered orally, all of the compounds inhibited superoxide production by neutrophils in vivo (30–70%) with no indication of acute liver toxicity compared to controls (data not shown). Interestingly, tubastrine (**1**) and orthidine E (**6**) also inhibited neutrophil infiltration in vivo. In contrast, compound **8** failed to inhibit in vivo neutrophil infiltration, providing further evidence that free phenolic groups play a key role in the bioactivity of the orthidine alkaloids.

3. Conclusion

Our search for new classes of anti-inflammatory agents has led to the isolation of a series of guanidine-containing alkaloids[33–36] from the New Zealand ascidian *A. orthium*. The known dihydroxystyrylguanidine tubastrine (**1**) was isolated in addition to five new alkaloids that contained benzodioxane (orthidines A–D) and cyclobutane (orthidine E) skeletons arising from the dimerization of tubastrine. In the case of orthidines A–D, relative stereochemistry assignment relied upon the acquisition and interpretation of $^3J_{CH}$ coupling constants that can be more readily obtained via recently reported indirect methods than the more classical carbon-detection methods. The use of a $^2J,^3J$-HMBC experiment was pivotal in determining the head-to-tail dimerization substructure of the cyclobutane analogue orthidine E. While structure elucidation of the numerous examples of naturally occurring cyclobutane dimers to date has relied upon mass spectrometric fragmentation patterns, X-ray crystallography or chemical shift comparisons with previously reported compounds, the ability to differentiate between $^2J_{CH}$ and $^3J_{CH}$ HMBC correlations provides a rapid and robust alternative method. A sixth new compound, the dihomovanillamide derivative of spermine was characterized by spectroscopic techniques and confirmed by synthesis. Several of the compounds were found to inhibit the in vitro production of superoxide by PMA-stimulated human neutrophils and were also active in an in vivo acute model of gouty arthritis providing further evidence that intervention of neutrophil-mediated processes can led to the discovery of potential anti-inflammatory agents. These results further highlight the tremendous diversity of natural product architecture biosynthesized by ascidians of the genus *Aplidium*.[37]

4. Experimental section

4.1. General methods (chemistry)

Flash column chromatography was performed using reversed phase Merck Lichroprep RP-8 or RP-18 40–63 μm, and size exclusion chromatography on Pharmacia Biotech Sephadex® LH-20. UV–vis spectra were run as MeOH solutions on a UV-2102 PC Shimadzu UV–vis scanning spectrophotometer. IR spectra were acquired as either dry films or Nujol mulls on a Spectrum One FTIR spectrometer with the 1603 cm^{-1} absorption band of polystyrene being used as reference. NMR spectra were recorded on either a Bruker Avance DRX-600 spectrometer at 600 MHz for ^1H and 150 MHz for ^{13}C, or a Bruker Avance DRX-400 spectrometer at 400 MHz for ^1H and 100 MHz for ^{13}C. Proto-deutero solvent signals were used as reference (DMSO-d_6: δ_H, 2.50; δ_C, 39.4. CD_3OD: δ_H, 3.30; δ_C, 49.0. $CDCl_3$: δ_H, 7.25; δ_C, 77.0 ppm), or in the case of ^1H and ^{13}C NMR data for **6** acquired in D_2O, external reference to DSS (δ_H 0.00; δ_C 0.0 ppm) was used. MS were recorded on either a Bruker Daltonics MicrOTOF Spectrophotometer or a VG-7070 mass spectrometer. Compound purity was determined by reversed phase HPLC (Waters 600 HPLC photodiode array system, Alltech Econosil C18, 3 μm, 33×7 mm, H_2O (0.05% TFA) to MeCN over 13.5 min at 2.0 mL/min and monitoring at 280 nm).

4.2. Biological material

The ascidian *A. orthium*[38] was collected (1263 g) by SCUBA at a depth of −30 m from Northeast Island, Three Kings Island group, New Zealand in November 2002 and a second collection (1022 g) was made in the same location in December 2003. Both collections were kept frozen until used. Specimens were identified by one of us (M.P.) and a voucher specimen is held at the National Institute for Water and Atmospheric Research, Private Bag 14-901, Kilbirnie, Wellington, New Zealand as MNP7030.

4.3. Isolation and purification

The frozen specimens from the first collection were freeze-dried (82.4 g) and extracted with MeOH (6×100 mL) followed by CH_2Cl_2 (2×100 mL). The combined extracts were filtered and dried in vacuo to produce a crude extract (26.2 g) that was subjected to reversed phase C_{18} flash column chromatography with a steep gradient from H_2O (0.05% trifluoroacetic acid (TFA)) to MeOH (0.05% TFA). The anti-inflammatory assay showed the activity to be concentrated in the water and 10–20% MeOH/H_2O (0.05% TFA) fractions. Repeated C_{18} and C_8 flash column chromatography (H_2O (0.05% TFA) to MeOH (0.05%TFA)) followed by size exclusion column chromatography on Sephadex LH-20 yielded **1** (12.5 mg, 0.02% dry wt), **2** (8.9 mg, 0.01% dry wt), **3** (15.3 mg, 0.02% dry wt), **4** (20.2 mg, 0.02% dry wt), **6** (16.1 mg, 0.02% dry wt), **7** (4.3 mg, 0.005% dry wt), and **8** (17.2 mg, 0.02% dry wt). A second collection of the same species (51.1 g dry wt, 15.4 g crude extract) was processed in the same manner yielding **1** (5.6 mg, 0.01% dry wt) and **5** in a 3:2 ratio with **3** (6.6 mg, 0.01% dry wt). Compounds **2**, **4**, **6**, **7**, and **8** were detected by analytical HPLC but not isolated.

4.3.1. Tubastrine (**1**)[6,7]

Pale yellow gum; UV (MeOH) λ_{max} (log ε) 205 (3.79), 221 (3.79), 287 (3.79), 306 sh (3.68) nm; IR ν_{max} (smear) 3326, 3176, 1677, 1607, 1522, 1445, 1280, 1195, 1113, 930 cm^{-1}; ESIMS *m/z* $[M+H]^+$ 194, HRESIMS *m/z* 194.0909, $C_9H_{12}N_3O_2$ requires 194.0924; ^1H NMR (DMSO-d_6, 400 MHz) δ 10.06 (1H, d, *J*=10.0 Hz, NH-9), 9.05 (1H, s, OH-1), 8.80 (1H, s, OH-2), 7.60 (4H, br s, NH_2×2), 7.10 (1H, dd, *J*=13.9, 10.2 Hz, H-8), 6.81 (1H, d, *J*=1.4 Hz, H-3), 6.68 (2H, m, H-5,6), 6.02 (1H, d, *J*=13.9 Hz, H-7); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 153.9 (C-10), 145.1 (C-2), 144.6 (C-1), 126.9 (C-4), 119.8 (C-8), 117.3 (C-5), 115.6 (C-6), 114.7 (C-7), 112.7 (C-3); purity 99% t_R=0.90 min.

4.3.2. Orthidine A (**2**)

Pale yellow gum; ^1H, ^{13}C, and HMBC NMR data are reported in Tables 1 and 2. UV (MeOH) λ_{max} (log ε) 207 (4.56), 226 (4.17), 284 (4.02), 311 sh (3.73) nm; IR ν_{max} (smear) 3333, 3189, 1676, 1606, 1507, 1438, 1267, 1201, 1139, 810 cm^{-1}; ESIMS *m/z* (rel int %) 385 (7), 326 (23), 193 (100); HRESIMS *m/z* $[M+H]^+$ 385.1606, $C_{18}H_{21}N_6O_4$ requires 385.1619; $[M+2H]^{2+}$ 193.0858, $C_9H_{11}N_3O_2$ requires 193.0846; purity 99% t_R=5.30 min.

4.3.3. Orthidine B (**3**)

Pale yellow gum; ^1H, ^{13}C, and HMBC NMR data are reported in Tables 1 and 2. UV (MeOH) λ_{max} (log ε) 205 (4.55), 226 (4.16), 284 (4.08), 308 sh (3.83) nm; IR ν_{max} (smear) 3344, 3187, 1677, 1606, 1507, 1439, 1267, 1202, 1141, 932 cm^{-1}; ESIMS *m/z* (rel int %) 385 (35), 326 (10), 193 (100); HRESIMS *m/z* $[M+H]^+$ 385.1612, $C_{18}H_{21}N_6O_4$ requires 385.1619; $[M+2H]^{2+}$ 193.0861, $C_9H_{11}N_3O_2$ requires 193.0846; purity 95% t_R=4.65 min.

4.3.4. Orthidine C (**4**)

Pale yellow gum; ^1H, ^{13}C, and HMBC NMR data are reported in Tables 1 and 2. UV (MeOH) λ_{max} (log ε) 205 (4.59), 224 (4.32), 284 (4.23), 311 sh (3.97) nm; IR ν_{max} (smear) 3326, 3173, 1677, 1606, 1507, 1442, 1263, 1197, 1118, 933 cm^{-1}; ESIMS *m/z* (rel int %) 385 (3), 326 (16), 193 (100); HRESIMS *m/z* $[M+H]^+$ 385.1634, $C_{18}H_{21}N_6O_4$ requires 385.1619; $[M+2H]^{2+}$ 193.0899, $C_9H_{11}N_3O_2$ requires 193.0846; purity 98% t_R=5.14 min.

4.3.5. Orthidine D (**5**)

Pale yellow gum; ^1H, ^{13}C, and HMBC NMR data are reported in Tables 1 and 2. UV (MeOH) λ_{max} (log ε) 206 (4.59), 227 (4.23), 287 (4.25), 310 sh (4.01) nm; ESIMS *m/z* (rel int %) 385 (7), 193 (100); HRESIMS *m/z* $[M+H]^+$ 385.1622, $C_{18}H_{21}N_6O_4$ requires 385.1619; $[M+2H]^{2+}$ 193.0848, $C_9H_{11}N_3O_2$ requires 193.0846; purity 99% t_R=4.84 min.

4.3.6. Orthidine E (**6**)

Pale yellow gum; ^1H, ^{13}C, and HMBC NMR data are reported in Table 3. ^1H NMR (600 MHz, D_2O) δ 6.96 (2H, d, *J*=8.2 Hz, H-6/6′), 6.90 (2H, d, *J*=2.2 Hz, H-3/3′), 6.85 (2H, dd, *J*=8.2, 2.2 Hz, H-5/5′), 4.63 (2H, dd, *J*=8.2, 6.5 Hz, H-8/8′), 3.93 (2H, dd, *J*=8.2, 6.5 Hz, H-7/7′); ^{13}C NMR (100 MHz, D_2O) δ 158.8 (C-10/10′), 146.7 (C-2/2′), 146.2 (C-1/1′), 130.1 (C-4/4′), 123.3 (C-5/5′), 118.9 (C-3/3′, 6/6′), 53.7 (C-8/8′), 50.5 (C-7/7′); UV (MeOH) λ_{max} (log ε) 206 (4.45), 230 (3.83), 285 (3.45) nm; IR ν_{max} (smear) 3335, 3173, 1671, 1610, 1521, 1444, 1369, 1287, 1201, 1119, 1022, 990 cm^{-1}; ESIMS *m/z* (rel int %) 387 (30), 194 (100); HRESIMS *m/z* $[M+H]^+$ 387.1768, $C_{18}H_{23}N_6O_4$ requires 387.1775; $[M+2H]^{2+}$ 194.0918, $C_9H_{12}N_3O_2$ requires 194.0924; purity 99% t_R=1.14 min.

4.3.7. Orthidine F (**7**)

Pale yellow gum; ^1H, ^{13}C, and HMBC NMR data reported in Table 4. UV (MeOH) λ_{max} (log ε) 206 (4.10). 230 (3.46), 285 (3.10) nm; IR ν_{max} (smear) 3328, 3188, 1677, 1514, 1433, 1270, 1202, 1132, 800 cm^{-1}; FABMS *m/z* $[M+H]^+$ 531, HRFABMS 531.3188, $C_{28}H_{43}N_4O_6$ requires 531.3183; purity 97% t_R=5.66 min.

4.3.8. Synthesis of orthidine F (**7**)

Homovanillic acid (98 mg, 0.54 mmol), spermine (54 mg, 0.27 mmol), and PyBOP (281 mg, 0.54 mmol) were stirred together in DMF (1 mL). Triethylamine (225 μL, 1.62 mmol) was added and the reaction mixture was stirred at ambient temperature under N_2 for 22 h. The crude product was subjected to reversed phase C_8 flash column chromatography followed by reversed phase phenyl-bonded column chromatography (H_2O to MeOH), with the product eluting in the 100% MeOH fraction yielding **7** (55.6 mg 39%) as a pale yellow gum. FABMS *m/z* $[M+H]^+$ 531, HRFABMS 531.3184, $C_{28}H_{43}N_4O_6$ requires 531.3183; UV, IR, and ^1H and ^{13}C NMR data were identical to those observed for the isolated natural product. Purity 98% t_R=5.64 min, and co-eluted with isolated natural product.

4.3.9. 3,4-Dimethoxyphenethyl-β-guanidine (**8**)[8]

Pale yellow gum; UV (MeOH) λ_{max} (log ε) 205 (4.07), 229 (3.52), 281 (3.11) nm; IR ν_{max} (smear) 3350, 3184, 1671, 1517, 1466, 1262, 1235, 1202, 1139, 1024, 801 cm^{-1}; FABMS *m/z* $[M+H]^+$ 224, HRFABMS 224.1394, $C_{11}H_{18}N_3O_2$ requires 224.1399; ^1H NMR (CD_3OD, 400 MHz) δ 6.89 (1H, d, *J*=8.2 Hz, H-6), 6.85 (1H, d, *J*=2.0 Hz, H-3), 6.78 (1H, dd, *J*=8.2, 2.0 Hz, H-5), 3.82 (3H, s, H_3-14), 3.79 (3H, s, H_3-13), 3.42 (2H, t, *J*=7.1 Hz, H_2-8), 2.81 (2H, t, *J*=7.1 Hz, H_2-7); ^{13}C NMR (CD_3OD, 100 MHz) δ 158.7 (C-10), 150.6 (C-2), 149.4 (C-1), 132.2 (C-4), 122.3 (C-5), 113.8 (C-3), 113.3 (C-6), 56.6 (C-14), 56.5 (C-13), 43.9 (C-8), 35.6 (C-7); purity 99% t_R=4.89 min.

4.4. Biological assays

Details of general procedures for the in vitro and in vivo biological assays undertaken in the present study have been reported previously.[4]

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References and notes

1. Pearce, A. N.; Berridge, M. V.; Harper, J. L.; Maas, E. W.; Perry, N. B.; Webb, V. A.; Copp, B. R. *Chem. N.Z.* **2007**, *71*, 47–49.
2. McNamara, C. E.; Larsen, L.; Perry, N. B.; Harper, J. L.; Berridge, M. V.; Chia, E. W.; Kelly, M.; Webb, V. L. *J. Nat. Prod.* **2005**, *68*, 1431–1433.
3. Pearce, A. N.; Chia, E. W.; Berridge, M. V.; Maas, E. W.; Page, M. J.; Webb, V. L.; Harper, J. L.; Copp, B. R. *J. Nat. Prod.* **2007**, *70*, 111–113.
4. Pearce, A. N.; Chia, E. W.; Berridge, M. V.; Clark, G. R.; Harper, J. L.; Larsen, L.; Maas, E. W.; Page, M. J.; Perry, N. B.; Webb, V. L.; Copp, B. R. *J. Nat. Prod.* **2007**, *70*, 936–940.
5. Tan, A. S.; Berridge, M. V. *J. Immunol. Methods* **2000**, *238*, 59–68.
6. Sakai, R.; Higa, T. *Chem. Lett.* **1987**, 127–128.
7. Barenbrock, J. S.; Köck, M. *J. Biotechnol.* **2005**, *117*, 225–232.
8. Short, J. H.; Biermacher, U.; Dunnigan, D. A.; Leth, T. D. *J. Med. Chem.* **1963**, *6*, 275–283.
9. Meissner, A.; Sørensen, O. W. *Magn. Reson. Chem.* **2001**, *39*, 49–52.
10. Fukuyama, Y.; Hasegawa, T.; Toda, M.; Kodama, M.; Okazaki, H. *Chem. Pharm. Bull.* **1992**, *40*, 252–254.
11. Tsukamoto, S.; Kato, H.; Hirota, H.; Fusetani, N. *Tetrahedron* **1994**, *50*, 13583–13592.
12. Andersen, S. O.; Jacobsen, J. P.; Roepstorff, P. *Tetrahedron* **1980**, *36*, 3249–3252.
13. Noda, N.; Kubota, S.; Miyata, Y.; Miyahara, K. *Chem. Pharm. Bull.* **2000**, *48*, 1749–1752.
14. Xu, M.-Z.; Lee, W. S.; Han, J.-M.; Oh, H.-W.; Park, D.-S.; Tian, G.-R.; Jeong, T.-S.; Park, H.-Y. *Bioorg. Med. Chem.* **2006**, *14*, 7826–7834.
15. Urban, S.; Capon, R. J.; Hooper, J. N. A. *Aust. J. Chem.* **1994**, *47*, 2279–2282.
16. Sperry, S.; Crews, P. *J. Nat. Prod.* **1998**, *61*, 859–861.
17. Walker, R. P.; Faulkner, D. J.; Van Engen, D.; Clardy, J. *J. Am. Chem. Soc.* **1981**, *103*, 6772–6773.
18. Lu, Y.; Foo, L. Y. *Phytochemistry* **1999**, *51*, 91–94.
19. Isaza, J. H.; Ito, H.; Yoshida, T. *Phytochemistry* **2001**, *58*, 321–327.
20. Wang, Q.; Terreaux, C.; Marston, A.; Tan, R.-X.; Hostettmann, K. *Phytochemistry* **2000**, *54*, 909–912.
21. Rahman, A.-U.; Khattak, K. F.; Nighat, F.; Shabbir, M.; Hemalal, K. D.; Tillekeratne, L. M. *Phytochemistry* **1998**, *48*, 377–383.
22. Ford, C. W.; Hartley, R. D. *J. Sci. Food Agric.* **1990**, *50*, 29–43.
23. Tachibana, S.; Ohkubo, K.; Towers, G. H. N. *Phytochemistry* **1992**, *31*, 81–83.
24. Sagawa, T.; Takaishi, Y.; Fujimoto, Y.; Duque, C.; Osorio, C.; Ramos, F.; Garzon, C.; Sato, M.; Okamoto, M.; Oshikawa, T.; Ahmed, S. U. *J. Nat. Prod.* **2005**, *68*, 502–505.
25. Lee, F.-P.; Chen, Y.-C.; Chen, J.-J.; Tsai, I.-L.; Chen, I.-S. *Helv. Chim. Acta* **2004**, *87*, 463–468.
26. Krishnamurthy, V. V.; Russell, D. J.; Hadden, C. E.; Martin, G. E. *J. Magn. Reson.* **2000**, *146*, 232–239.
27. Ulrich, H.; Rao, D. V.; Stuber, F. A.; Sayigh, A. A. R. *J. Org. Chem.* **1970**, *35*, 1121–1125.
28. Wei, K.; Li, W.; Koike, K.; Chen, Y.; Nikaido, T. *J. Org. Chem.* **2005**, *70*, 1164–1176.
29. Montaudo, G.; Caccamese, S. *J. Org. Chem.* **1973**, *38*, 710–716.
30. Funayama, S.; Yoshida, K.; Konno, C.; Hikino, H. *Tetrahedron Lett.* **1980**, *21*, 1355–1356.
31. Ponasik, J. A.; Strickland, C.; Faerman, C.; Savvides, S.; Karplus, P. A.; Ganem, B. *Biochem. J.* **1995**, *311*, 371–375.
32. Arnoldi, A.; Merlini, L. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2555–2557.
33. Berlinck, R. G. S.; Kossuga, M. H. *Nat. Prod. Rep.* **2005**, *22*, 516–550.
34. Berlinck, R. G. S. *Nat. Prod. Rep.* **2002**, *19*, 617–649.
35. Berlinck, R. G. S. *Nat. Prod. Rep.* **1999**, *16*, 339–365.
36. Berlinck, R. G. S. *Nat. Prod. Rep.* **1996**, *13*, 377–409.
37. Zubía, E.; Ortega, M. J.; Salvá, J. *Mini-Rev. Org. Chem.* **2005**, *2*, 389–399.
38. Millar, R. H. *N.Z. Oceanogr. Inst. Mem.* **1982**, *85*, 5–117.