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(−)-Dibromophakellin: An α_{2B} adrenoceptor agonist isolated from the Australian marine sponge, *Acanthella costata*

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ABSTRACT

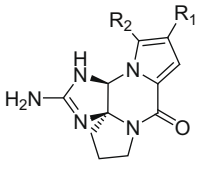
Bioassay-guided fractionation of the organic extract from the marine sponge *Acanthella costata* resulted in the isolation of the known natural product, (−)-dibromophakellin (**1**). Using a fluorescence imaging plate reader (FLIPR) based assay, compound **1** was identified as displaying agonist activity against the α_{2B} adrenoceptor, with an EC_{50} of 4.2 μM. Debromination and Suzuki–Miyaura coupling reactions were undertaken in order to provide structure activity data about the pyrrole ring of this marine metabolite. These synthetic studies generated the known natural product analogues, (−)-phakellin (**2**), and (−)-monobromophakellin (**3**), along with the new synthetic derivatives (−)-4-bromo-5-phenylphakellin (**5**) and (−)-4,5-diphenylphakellin (**6**). Substitution of the C-5 Br of **1** with H (**2** and **3**) or phenyl (**5** and **6**) resulted in loss of activity indicating that Br at C-5 is required for agonist activity.

1. Introduction

The α_2-adrenoceptors (α_2-ARs) are members of the G-protein coupled receptor (GPCR) superfamily and respond to the physiological agonists noradrenaline and adrenaline. The α_2-ARs are widely distributed throughout the body and are located in both the CNS and peripheral tissue. The receptors are located both presynaptically and postsynaptically.[1,2] Central effects of α_2-ARs include analgesia, sedation, hypotension, regulation of hypothermia, gastric protection and gastric motility. In the periphery they are present in smooth muscle and are involved in venous contraction.[1,2] Through receptor binding and molecular cloning studies, three subtypes have been identified, α_{2A}, α_{2B} and α_{2C}. A fourth subtype, the α_{2D} has been found in the rat and is thought to be the species orthologue of the human α_{2A}.[3]

A number of non-selective α_2 agonists have been used clinically for antihypertensive treatment, pain control and as anesthetic adjuncts.[4,5] The hypotensive and sedative effects caused by these agonists have been found to be mediated through the α_{2A} subtype. These are however unwanted side effects for potential new clinical applications of the other receptor subtypes. The discovery of a compound with selective agonist activity for these subtypes may provide a useful lead in the development of new drug therapies in areas such as gastric protection and post anesthetic care.[5]

From HTS of a library containing 61 674 crude biota extracts we discovered that the organic extract from the marine sponge *Acanthella costata* (Axinellidae) showed micromolar activity in an α_{2B} adrenoceptor FLIPR assay. Bioassay-guided fractionation of the crude organic extract resulted in the isolation of the previously reported natural product, (−)-dibromophakellin (**1**),[6] which was subsequently shown to account for the activity displayed in the crude organic extract (Fig. 1). Herein we report the isolation and α_{2B} adrenoceptor agonist activity of (−)-dibromophakellin (**1**), along with several synthetic studies aimed at providing structure activity about the pyrrole ring of this bioactive lead molecule.



R_1 = Br R_2 = Br (**1**)
R_1 = H R_2 = H (**2**)
R_1 = Br R_2 = H (**3**)

Figure 1. Chemical structures for natural products **1**–**3**.

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2. Results and discussion

A freeze-dried and ground sample of *A. costata* was extracted with DCM and MeOH. The resulting organic extracts were combined and chromatographed using C_{18} bonded silica HPLC to yield (−)-dibromophakellin (**1**, 57.6 mg, 0.576% dry wt).

The TFA salt of (−)-dibromophakellin (**1**) was isolated as an optically active stable gum. Compound **1** was identified as (−)-dibromophakellin following comparison of the NMR and $[\alpha]_D$ data with literature values.[6] Compound **1** was tested in an α_{2B} adrenoceptor FLIPR assay and displayed an EC_{50} value of 4.2 μM. Due to the potency of this marine metabolite, synthetic studies were undertaken in order to provide some structure activity data about the pyrrole ring of **1**. Dehalogenation of (−)-dibromophakellin was performed under catalytic hydrogen transfer conditions using HCO_2NH_4 and Pd/C in MeOH.[7] Purification of the reaction mixture by C_{18} bonded silica HPLC afforded the TFA salt of the previously reported natural product derivative, (−)-phakellin (**2,** 3.5 mg, 35%).[6] The structure of **2** was identified following comparison of spectroscopic data with literature values.[6] (−)-Phakellin (**2**) showed no agonist activity when screened at concentrations up to 83 μM in the α_{2B} adrenoceptor FLIPR assay. These data suggested that the lipophilic nature of either one or both bromine atoms in **1** was essential for α_{2B} adrenoceptor agonist activity; hence we decided to see whether we could replace either or both bromine atoms with a lipophilic aryl substituent. A review outlining palladium catalyzed cross-coupling reactions involving pyrroles has been published[8] and Suzuki–Miyaura couplings[9] were chosen for these synthetic manipulations due to the simplicity and ease of this reaction type. Regioselective cross-couplings between 4,5-di or 3,4,5-tribrominated pyrrole moieties and aryl boronic acids have been reported in the literature to occur preferentially at the C-5 position.[10,11] Also, monoarylation of 4,5-dibrominated pyrroles has been reported to take place adjacent to the ring nitrogen in Negishi-type cross-couplings with zincated species.[12] The Suzuki–Miyaura cross-coupling reaction was initially attempted with **1** and phenyl boronic acid, using Cs_2CO_3 in the presence of $Pd(OAc)_2$ and PPh_3, in xylene/EtOH/H_2O (5:1:1) at reflux overnight.[10] The reaction mixture was separated using C_{18} bonded silica HPLC to yield the TFA salt of (−)-monobromophakellin (**3**, 1.9 mg, 8%)[6] and unreacted starting material. Dehalogenation in Suzuki–Miyaura coupling reactions is not unexpected[13,14] and has been observed to occur significantly at C-4 for 4-bromo- and 4,5-dibromopyrrole-2-carboxylates.[15] This side reaction can be suppressed by the use of a Boc protecting group on the pyrrole nitrogen, which serves to reduce the electron density of the pyrrole.[15–17] These results prompted us to try the coupling reactions after Boc protection of the guanidine in **1**.[18] This protection was performed under standard conditions of Boc_2O in DCM (Scheme 1).[19] Following rapid silica flash chromatography of the reaction mixture, we obtained a mixture of the mono-, di- and tri-Boc protected products[18] (**4**), which was used immediately for the Suzuki–Miyaura coupling. The mixture **4** was treated with phenyl boronic acid, and Na_2CO_3 in the presence of $Pd(OAc)_2$ and PPh_3 in DME/H_2O at reflux overnight. MS analysis of the crude reaction mixture indicated the presence of both mono- (**5**) and diphenylated (**6**) products. Chromatography using C_{18} bonded silica HPLC yielded the TFA salt of the new natural product analogues, (−)-4-bromo-5-phenylphakellin (**5**, 6.0 mg, 6%) and (−)-4,5-diphenylphakellin (**6**, 2.1 mg, 2%). This reaction was not optimized. The structures of both of these compounds were elucidated following 1D/2D NMR and HRMS data analysis. Since C-5 of the pyrrole system in **1** is more electron deficient,[10,20] it was predicted that 5-Br would be the first position for the Suzuki–Miyaura coupling to take place and hence the formation of the monophenyl analogue (**5**) was not unexpected. A strong ROESY correlation between H-6 and H-17 and H-21 confirmed that the phenyl ring of **5** was indeed substituted at C-5. Diarylated products resulting from Suzuki–Miyaura cross-coupling reactions of 4,5-dibrominated pyrroles using similar reaction conditions have also been reported.[11,12]

Compounds **3**, **5** and **6** were subsequently tested in the α_{2B} adrenoceptor FLIPR assay. Surprisingly none of these analogues showed any activity when screened up to 83 μM. Isosteric replacement of the C-5 Br with a Cl, CF_3 or CH_3 substituent may have proven more successful, however due to lack of material other synthetic manipulations could not be pursued. In conclusion, substitution of the C-5 Br of **1** with H (**2** and **3**) or phenyl (**5** and **6**) resulted in loss of α_{2B} adrenoceptor agonist activity. These data indicated the importance of Br at C-5 for this receptor.

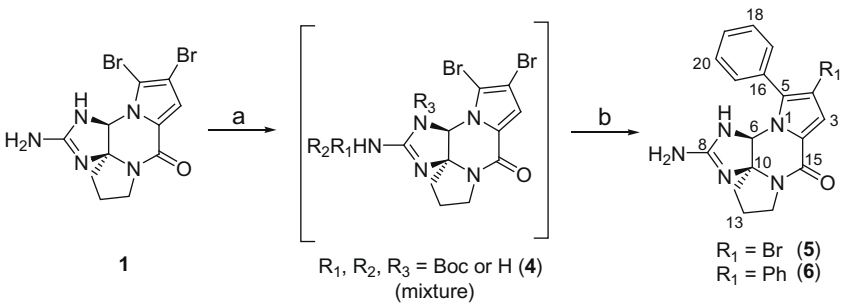
3. Conclusion

Bioassay-guided fractionation of the organic extract from the marine sponge *A. costata* identified (−)-dibromophakellin (**1**) as an α_{2B} adrenoceptor agonist, with an EC_{50} of 4.2 μM. A series of small-scale synthetic reactions were performed on this marine natural product in order to provide structure activity data about the pyrrole ring. These synthetic studies identified that the substitution of the C-5 Br of **1** with H or phenyl resulted in loss of activity, indicating that Br at C-5 is required for α_{2B} adrenoceptor agonist activity.

4. Experimental

4.1. General methods

Optical rotations were recorded on a Jasco P-1020 polarimeter. UV and IR spectra were recorded on a Camspec M501 spectrophotometer and a Bruker Tensor 27 spectrometer, respectively. NMR spectra were recorded at 30 °C on either a Varian 500 MHz Unity INOVA or 600 MHz NMR system spectrometer. The latter spectrometer was equipped with a triple resonance cold probe. The ^1H and ^{13}C chemical shifts were referenced to the proto-deutero solvent peaks for DMSO-d_6 at δ_H 2.49 and δ_C 39.5. LRESIMS were



Scheme 1. Synthesis of compounds **5** and **6**. Reagents and conditions: (a) Boc_2O, DCM, TEA, rt, 16 h; (b) $Pd(OAc)_2$, $Na_2CO_3{\cdot}10H_2O$, $PhB(OH)_2$, PPh_3, DME/H_2O, Ar, Δ, 18 h.

recorded on a Waters ZQ mass spectrometer. HRESIMS were recorded on a Bruker Daltonics Apex III 4.7e Fourier-transform mass spectrometer. A Waters 600 pump equipped with a Waters 996 PDA detector and a Waters 717 autosampler were used for HPLC. A ThermoElectron C_{18} Betasil 5 μm 143 Å column (21.2 × 150 mm) was used for semi-preparative HPLC separations. Sigma diatomaceous earth (95% SiO_2, acid-washed) packed into an SPE cartridge (10 × 20 mm) or sintered glass column (25 × 25 mm) was used for reaction purifications. Merck Silica Gel 60 (40–63 μm) packed into a glass column (25 × 200 mm) was used for normal phase silica flash chromatography. Alltech Davisil 40–60 μm 60 Å C_{18} bonded silica packed into a glass column (25 × 200 mm) was used for reversed phase silica flash chromatography. Alltech Davisil 40–60 μm 60 Å C_{18} bonded silica was used for preadsorption work. All solvents used for chromatography, UV, optical rotations and MS were Lab-Scan HPLC grade, and the H_2O was Millipore Milli-Q PF filtered. All synthetic reagents were purchased from Sigma–Aldrich.

4.2. Biological material

A. costata was collected by scuba diving (−25 m) at Sykes Reef (Capricorn-Bunker Group Great Barrier Reef) during August 1996, and kept frozen prior to freeze-drying and extraction. Voucher specimen G307233 has been deposited at the Queensland Museum, South Brisbane, Queensland, Australia.

4.3. Extraction and isolation

A freeze-dried and ground sample of *A. costata* (10 g) was exhaustively extracted with DCM (1 L) and MeOH (1 L). Both extracts were combined and evaporated to yield a brown gum (1.5 g) that was preadsorbed to C_{18} (5 g) then packed into a stainless steel cartridge (25 × 50 mm). The cartridge was attached to a C_{18} HPLC column and a linear gradient from H_2O (1% TFA) to MeOH (1% TFA) at a flowrate of 9 mL/min over 35 min was run. One min fractions were collected from time = 0 min; fraction 14 contained the TFA salt of (−)-dibromophakellin (**1**, 57.6 mg, 0.576% dry wt).

4.3.1. TFA salt of (−)-dibromophakellin (1)

Light brown gum; identified by comparison with literature data.[6]

4.4. Debromination of (−)-dibromophakellin (1)

To an Ar purged solution of **1** (16.0 mg, 43 μmol) in MeOH (2.0 mL) was added HCO_2NH_4 (38.0 mg, 603 μmol) and 10% Pd/C (30.0 mg), and the mixture was stirred under Ar at rt for 24 h.[7] The reaction solution was filtered through an SPE cartridge packed with diatomaceous earth and the column washed with 100% MeOH (10 mL). The MeOH eluent was preadsorbed to C_{18} (1 g) then packed into a stainless steel cartridge (10 × 30 mm). The cartridge was attached to a C_{18} HPLC column and a linear gradient from H_2O (1% TFA) to MeOH (1% TFA) at a flowrate of 9 mL/min over 59 min was run. One min fractions were collected from time = 0 min; fractions 18 and 19 contained the TFA salt of (−)-phakellin (**2**, 3.5 mg, 35%).

4.4.1. TFA salt of (−)-phakellin (2)

Colourless gum; identified by comparison with literature data.[6]

4.5. Suzuki–Miyaura coupling reaction 1 (no Boc protection of 1)

Using conditions similar to that described by Schröter and Bach,[10] to a suspension of (−)-dibromophakellin (**1**, 28 mg, 0.056 mmol), Cs_2CO_3 (94 mg, 0.29 mmol), phenyl boronic acid (35 mg, 0.29 mmol), PPh_3 (15 mg, 0.057 mmol) in xylene (2.5 mL), EtOH (0.5 mL) and H_2O (0.5 mL) was added $Pd(OAc)_2$ (3.6 mg, 0.016 mmol) under an atmosphere of Ar. The reaction mixture was heated under reflux for 18 h, allowed to cool to rt then the solvent evaporated. MeOH (10 mL) was added to the residue and the crude product was filtered through an SPE cartridge (10 × 20 mm) packed with diatomaceous earth and the column washed with 100% MeOH (10 mL). The solvent was evaporated and the residue was preadsorbed to C_{18} (1 g) then packed into a stainless steel cartridge (10 × 30 mm). The cartridge was attached to a C_{18} HPLC column and isocratic conditions of H_2O containing 1% TFA were employed for 10 min at a flowrate of 9 mL/min followed by a linear gradient to 50% H_2O (1% TFA)/50% MeOH (1% TFA) over 50 min. One min fractions were collected from time = 0 min; fractions 44 and 54 contained the TFA salts of (−)-monobromophakellin (**3**, 1.93 mg, 8%) and (−)-dibromophakellin (**1**, 4.00 mg), respectively.

4.5.1. TFA salt of (−)-monobromophakellin (3)

Colourless gum; identified by comparison with literature data.[6]

4.6. Boc protection of 1

Boc_2O (1.50 g, 6.87 mmol) was added to a stirred solution of (−)-dibromophakellin (**1**, 395 mg, 0.785 mmol) and TEA (1 mL, 726 mg, 7.17 mmol) in DCM (20 mL) and the mixture stirred overnight at rt. The solvent was evaporated and the residue subjected to purification by rapid silica flash chromatography using $CHCl_3$/MeOH (99:1) as the eluent. Ten fractions were collected then analyzed by MS. Fraction 6 contained a mixture of the mono-, di- and tri-Boc protected (−)-dibromophakellin (**4**, 111.6 mg), which was used in the next step without further purification. (+)-LRESIMS *m/z* (rel. int.) 488 (10), 490 (20), 492 (10), 588 (10), 590 (20), 592 (10), 688 (5), 690 (10), 692 (5).

4.7. Suzuki–Miyaura coupling reaction 2 (Boc protection of 1)

In a Wheaton vial (5 mL), $Pd(OAc)_2$ (4.2 mg, 0.019 mmol) was added to a suspension of Boc protected (−)-dibromophakellin [**4**, 110.4 mg, 0.187 mmol (M_W 589)], $Na_2CO_3{\cdot}10H_2O$ (188 mg, 0.656 mmol), phenyl boronic acid (46 mg, 0.375 mmol), PPh_3 (9.8 mg, 0.037 mmol) in DME (3 mL) and H_2O (390 μL) under an atmosphere of Ar. The reaction mixture was stirred and heated under reflux for 18 h. MS analysis of the crude reaction mixture indicated the presence of the mono- (**5**) and diphenylated (**6**) products. MeOH (10 mL) was added to the reaction mixture and the crude product was filtered through a sintered glass column packed with diatomaceous earth and washed with 100% MeOH (50 mL). The solvent was evaporated and the residue (100 mg) was preadsorbed to C_{18} (1 g) then packed into a stainless steel cartridge (10 × 30 mm). The cartridge was attached to a C_{18} HPLC column and isocratic conditions of 75% H_2O (1% TFA)/25% MeOH (1% TFA) were employed for 40 min at a flowrate of 9 mL/min followed by a linear gradient to 20% H_2O (1% TFA)/80% MeOH (1% TFA) over 20 min. Thirty seconds fractions were collected from time = 0 min; fraction 92 contained the TFA salt of (−)-dibromophakellin (**1**, 10.0 mg), and fractions 111 and 117 contained the TFA salts of (−)-4-bromo-5-phenylphakellin (**5**, 6.0 mg, 6%) and (−)-4,5-diphenylphakellin (**6**, 2.1 mg, 2%), respectively.

4.7.1. TFA salt of (−)-4-bromo-5-phenylphakellin (5)

Stable brown gum; $[\alpha]_D^{19}$ −121 (*c* 0.400, MeOH); UV (MeOH) λ_{max} (log ε) 226 (4.11), 296 (4.01) nm; IR ν_{max} (KBr) 1694, 1663, 1556, 1467. 1431, 1382, 1291, 1205, 1134, 1023 cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6) δ 2.06 (2H, m, H-13a, H-13b), 2.22 (1H, ddd, *J* = 12.0, 6.0, 1.8 Hz, H-14a), 2.44 (1H, ddd, *J* = 12.0, 12.0,

9.0 Hz, H-14b), 3.51 (1H, ddd, *J* = 10.8, 9.6, 8.4 Hz, H-12a), 3.70 (1H, ddd, *J* = 10.8, 9.0, 3.6 Hz, H-12b), 6.54 (1H, d, *J* = 0.6 Hz, H-6), 7.00 (1H, s, H-3), 7.53 (1H, m, H-19), 7.57 (4H, m, H-17, H-18, H-20, H-21), 7.77 (1H, br s, 8-NH_2), 8.19 (1H, s, H-7), 8.39 (1H, br s, 8-NH_2), 10.09 (1H, s, H-9); ^{13}C NMR (125 MHz, DMSO-d_6) δ 19.1 (C-13), 38.6 (C-14), 44.7 (C-12), 66.6 (C-6), 82.4 (C-10), 99.3 (C-4), 114.8 (C-3), 123.7 (C-2), 128.1 (C-16), 129.1[a] (2C, C-17, C-21), 129.2 (C-19), 129.7[a] (2C, C-18, C-20), 132.3 (C-5), 154.6 (C-15), 156.2 (C-8); (+)-LRESIMS *m/z* (rel. int.) 386 (100), 388 (100); (+)-HRESIMS *m/z* 386.0622 ($C_{17}H_{17}{}^{79}BrN_5O$ $[M+H]^+$ requires 386.0611). [a]Interchangeable signals.

4.7.2. TFA salt of (−)-4,5-diphenylphakellin (6)

Stable amorphous solid; $[\alpha]_D^{23}$ −86 (*c* 0.140, MeOH); UV (MeOH) λ_{max} (log ε) 240 (4.05), 297 (3.70) nm; IR ν_{max} (KBr) 1689, 1659, 1564, 1467, 1442, 1412, 1299, 1206, 1183, 1135, 1025 cm^{-1}; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.09 (2H, m, H-13a, H-13b), 2.23 (1H, ddd, *J* = 12.6, 4.2, 4.2 Hz, H-14a), 2.46 (1H, ddd, *J* = 12.6, 11.4, 10.8 Hz, H-14b), 3.54 (1H, ddd, *J* = 10.8, 9.0, 8.4 Hz, H-12a), 3.72 (1H, ddd, *J* = 10.8, 6.0, 5.4 Hz, H-12b), 6.50 (1H, br s, 8-NH_2), 6.51 (1H, s, H-6), 7.06 (2H, d, *J* = 7.2, H-23, H-27), 7.09 (1H, s, H-3), 7.14 (1H, t, *J* = 7.2, H-25), 7.20 (2H, dd, *J* = 7.2, 7.2, H-24, H-26), 7.42 (2H, m, H-17, H-21), 7.50 (3H, m, H-18, H-19, H-20), 7.70 (1H, s, H-7), 8.18 (1H, br s, 8-NH_2), 9.96 (1H, s, H-9); ^{13}C NMR (125 MHz, DMSO-d_6) δ 19.2 (C-13), 38.6 (C-14), 44.5 (C-12), 66.2 (C-6), 82.4 (C-10), 113.1 (C-3), 123.2 (C-2), 125.6 (C-4), 126.2 (C-25), 127.7 (2C, C-23, C-27), 128.3 (2C, C-24, C-26), 129.0 (C-19), 129.3 (2C, C-18, C-20), 129.6 (C-16), 130.2 (2C, C-17, C-21), 131.5 (C-5), 134.2 (C-22), 155.4 (C-15), 156.0 (C-8); (+)-LRESIMS *m/z* (rel. int.) 384 (100); (+)-HRESIMS *m/z* 384.1821 ($C_{23}H_{22}N_5O$ $[M+H]^+$ requires 384.1819).

4.8. α_{2B} Adrenoceptor FLIPR assay

HEK 293 cells stably expressing the human α_{2B} adrenoreceptor (hα_{2B}) and Gqi5 protein were obtained from AstraZeneca (Mölndal, Sweden). All cell culture reagents, Pluronic F-27 and Fluo-4 AM were purchased from Invitrogen (Carlsbad, CA). Cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS), 0.3 mg/mL hygromycin B and 1 mg/mL geneticin under a humidified 5% CO_2 atmosphere at 37 °C. The α_{2B} FLIPR assay was carried out in 384 well poly-D-lysine-coated, black/clear bottom microtitre plates (BD Bioscience, Bedford, MA) using the Calcium-3 dye FLIPR™ kit from Molecular Devices (Sunnyvale, CA). To each well 20 μL of 2.5 × 10^5 cells/mL in DMEM containing 10% FBS was added and the plates incubated overnight under a humidified 5% CO_2 atmosphere at 37 °C. The following day, media was removed and cells loaded with dye by adding to each well 20 μL of Calcium-3 non wash dye (Molecular Devices) diluted in dye loading buffer (Hanks balanced salt solution containing 20 mM HEPES, 0.25 M probenicid, pH 7.4, 0.00176 mM Fluo-4, 0.0176% Pluronic F-27, 1.7% DMSO). Plates were incubated for 1 h at rt. A baseline fluorescence reading for 10 s (Emission 488 nm/Excitation 520 nm) was then performed on the FLIPR™ (FLuorometric Imaging Plate Reader, Molecular Devices). Each compound/extract was solubilized in 100% DMSO and 20 μL of each solution prediluted (1:60) in assay buffer (Hanks balanced salt solution containing 20 mM HEPES, pH 7.4, final DMSO concentration 0.83%) then dispensed into the 384-well plate (total assay volume = 40 μL). Fluorescence was measured every 1 s for 60 s on the FLIPR to determine agonist response. Noradrenaline (Sigma–Aldrich) in assay buffer containing 2 mM ascorbic acid was used as the positive control and gave an EC_{50} of 0.5–2.0 nM. EC_{50} values for all compounds were obtained from at least 2 independent experiments with each dose for the concentration response curve performed in triplicate.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.01.065.

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