Acridone Alkaloids from **Glycosmis chlorosperma** as DYRK1A Inhibitors

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**ABSTRACT:** Two new acridone alkaloids, chlorospermines A and B (1 and 2), were isolated from the stem bark of *Glycosmis chlorosperma*, together with the known atalaphyllidine (3) and acrifoline (4), by means of bioguided isolation using an in vitro enzyme assay against DYRK1A. Acrifoline (4) and to a lesser extent chlorospermine B (2) and atalaphyllidine (3) showed significant inhibiting activity on DYRK1A with IC₅₀’s of 0.075, 5.7, and 2.2 μM, respectively. Their selectivity profile was evaluated against a panel of various kinases, and molecular docking calculations provided structural details for the interaction between these compounds and DYRK1A.

**RESULTS AND DISCUSSION**

From the 720 organic extracts tested on DYRK1A, only 11 (eight EtOAc and three alkaloid extracts) showed a significant inhibiting activity (IC₅₀ ≤ 10 μg/mL), of which the EtOAc bark extract of *G. chlorosperma* exhibited the most potent activity, with an IC₅₀ value of 0.66 μg/mL (Figure 1). This extract was subjected to C₁₈ flash chromatography to afford 12 fractions. The biologically active fraction F6 (IC₅₀ 0.23 μg/mL) was further purified using silica gel and C₁₈ column chromatographies, leading to the isolation of two new acridone alkaloids, chlorospermines A and B (1 and 2), along with the known atalaphyllidine (3) and acrifoline (4). The HRESIMS spectrum of chlorospermine A (1) indicated an [M + H]⁺ ion at m/z 406.1656, which, in conjunction with the ¹³C NMR spectroscopic data, is consistent with a molecular...
formula of C_{24}H_{24}NO_{5} (calcd 406.1654), indicating 14 indices of hydrogen deficiency. The UV spectrum exhibited characteristic absorption bands at 275 and 303 nm for an acridone core. The IR spectrum of 1 showed strong absorption bands at 3210 cm\(^{-1}\) for hydroxy groups and 1595 cm\(^{-1}\) for a carbonyl function. The \(^1\)H NMR data of 1 (Table 1) revealed the presence of two 2,2-dimethylpyran moieties. The first ring showed a set of doublets at \(\delta_H 6.95\) and 5.52 for H-1\(^{\prime}\) and H-2\(^{\prime}\), respectively, each with a 9.8 Hz coupling constant. The second 2,2-dimethylpyran ring was slightly different, and its \(^1\)H NMR showed the presence of only one singlet at \(\delta_H 6.60\) as well as a C-2\(^{\prime}\) hydroxy group. The four methyl groups of the two pyran rings resonated as two singlets (six protons each) at \(\delta_H 1.52\) and 1.70. In addition, the \(^1\)H NMR spectrum showed aromatic resonances for a 1,2,3-trisubstituted benzene ring as well as an N-methyl resonance at \(\delta_H 3.75\). The \(^13\)C and DEPT \(^13\)C NMR spectra confirmed the presence of 24 carbons, consisting of five methyl, six sp\(^2\) methine, two sp\(^3\) quaternary, and 11 sp\(^2\) quaternary carbons. The location of the first pyran ring was revealed by the NOESY correlation between H-1\(^{\prime}\) and the N-methyl protons and was confirmed by the HMBC correlations from H-1\(^{\prime}\) and H-2\(^{\prime}\) to C-3\(^{\prime}\) and C-4. The HMBC correlations from H-1\(^{\prime}\) to C-2\(^{\prime}\), C-1, and C-2 established the location of the second pyran moiety (Figure 2). Analysis of COSY, HSQC, and HMBC data confirmed the assignment of the entire structure, and the compound was given the trivial name chlorospermine A (1).
Compound 2 was obtained as an amorphous powder. The HRESIMS showed an [M + H]+ ion at m/z 348.1238, which in conjunction with the 13C NMR spectroscopic data established the molecular formula of 2 as C21H18NO4 (calc. 348.1236). The UV spectrum also revealed the existence of an acridone skeleton with absorption bands at 273 and 399 nm. The 1H NMR data revealed the presence of a 2,2-dimethylpyran moiety, consisting of a set of doublets at δ 6.77 and 5.54, each with a 9.7 Hz coupling constant. These resonances were assigned to H-1′ and H-2′, respectively (Table 1). The gem-dimethyl group of the pyran ring resonated as a six-proton singlet at δH 1.53. The 1H NMR spectrum also showed a set of doublets at δH 6.83 and 7.68 (H-1″ and H-2″, respectively), each with a coupling constant of 1.9 Hz, suggesting the presence of a furan ring. In addition, the 1H NMR spectrum showed aromatic resonances for a 1,2,3-trisubstituted benzene ring as well as a resonance for an aromatic methyl group at δH 2.12 (Table 1).

The HMBC correlations from H-1″ to C-2 and from H-2″ to C-2 established the location of the furan ring (Figure 2). The HMBC correlations from H-1″ to C-1 and from H-2″ to C-2 revealed the aromatic substituents on the furan ring. The HMBC correlations from H-1″ to C-3, C-4, and C-11 and from H-2″ to C-4 suggested that the 2,2-dimethylpyran moiety was attached to C-4 and C-3 (Figure 2). The HMBC correlations from H-1″ to C-3, C-4, and C-11 and from H-2″ to C-2 established the location of the furan ring (Figure 2).

Table 2. IC50’s (in μM) of Compounds 1–4 against Various Kinases

<table>
<thead>
<tr>
<th>comp</th>
<th>CDK1</th>
<th>CDK5</th>
<th>CLK1</th>
<th>DYRK1A</th>
<th>GSK3</th>
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<tr>
<td>1</td>
<td>&gt;10</td>
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<td>&gt;10</td>
</tr>
<tr>
<td>4</td>
<td>5.3</td>
<td>0.17</td>
<td>0.075</td>
<td>2</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>refa</td>
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<td>0.085</td>
<td>2.1</td>
<td>0.052</td>
<td>0.005</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*aAssays were carried out in duplicate. Specific activities for CDK1, CDK5, CLK1, CK1, DYRK1A, GSK-3 were respectively 60.1, 16.4, 28.9, 6.4, 29.4, and 35.4 pmol phosphate incorporated/μL kinase preparation/30 min. 6-Bromoindirubin-3-monoxime.

(4) showed potent DYRK1A and CLK1 inhibition activities with IC50 values of 0.075 and 0.17 μM, respectively, whereas compounds 2 and 3 were less active, and chlorospermine A (1) was inactive. Despite the fact that no relevant structure–activity relationships could be established within this chemical series, it could be postulated that free hydroxy groups at C-1 and C-6 are critical for achieving inhibition of DYRK1A and CLK1. We next evaluated the selectivity of acrifoline (4) by determining its IC50 values on a panel of 14 kinases (Table 3 and Figure S3). Results revealed a modest selectivity with a preference toward DYRK1A. Acrifoline (4) and analogues thereof are currently being investigated in more detail in terms of its molecular, cellular, and animal model effects.

In order to better understand the influence of the substitution pattern of the acridone tricyclic system on their specific binding to DYRK1A, we performed a molecular docking study of the compounds isolated into the DYRK1A ATP binding domain and compared the results obtained with the β-carboline alkaloid harmine. Molecular docking calculations (Figure 3A–D) provided more insight into the proposed structural details of the interaction between compounds 1–4 and the DYRK1A binding site. On the basis of molecular modeling, acrifoline (4) is proposed to interact with Glu203 and the conserved Lys188 through hydrogen bonds involving the C-6 hydroxy group and with the hinge region (backbone atoms of Glu239 and Leu241) through hydrogen bonds involving the C-1 hydroxy group. These interactions are similar to those observed in the crystal structure of the complex between harmine and DYRK1A (Figure 3E). Atalaphyllidine (3) is proposed to be positioned in a different way and forms hydrogen bonds between oxygen atoms at C-9, C-1, and C-5 and backbone atoms of Glu239, Leu241, and Ile165, respectively. Chlorospermine B (2) possesses yet another conformation, with hydrogen bonds between the C-5 hydroxy group and the side chains of Glu203 and Lys188. Finally, chlorospermine A (1) shows hydrogen bonds between oxygen atoms in positions C-5, C-9, and C-2 and backbone NH of Leu241 and side chains of Asn244 and Asp307 (from the DFG motif), respectively. In addition, a possible π–π stacking interaction between the "gatekeeper" residue Phe238 and compounds 2 and 4 might also contribute to their overall binding energy.

The predicted binding conformations of compounds 1–4 are strongly influenced by the substitution pattern of the acridone tricyclic system. For example, although no steric clashes prevent atalaphyllidine (3) from adopting the same conformation as acrifoline (4), its interaction with the DYRK1A binding site is driven by the presence of the C-5 hydroxy substituent, whereas the positioning of 4 is driven by the C-6 hydroxy substituent. Alternatively, chlorospermines A (1) and B (2) cannot adopt the same orientation as 4 because of steric clashes between the cyclic substituent at C-1/C-2 and the NCH3 group and the side chains of Leu241 and Val173, respectively (Figure S2, Supporting Information).

The analysis of these molecular modeling results in light of the biological data (Table 2) suggests that strong, stabilizing interactions of the ligand with both conserved Lys188 and backbone atoms in the hinge region (Glu239 and/or Leu241)
are required for good biological activity (e.g., compound 4 and harmine, Figure 3D,E). When only one of these interactions is present, the strength of the protein–ligand interaction is predicted to be reduced (e.g., compounds 2 and 3, Figure 3B,C). Compound 1 is predicted to interact mainly with the DFG motif, with the Asn244 residue, and to a lesser extent with the hinge region, which could explain the lack of biological activity determined experimentally for this compound (Table 2).

In conclusion, this is the first report of a natural acridone showing a potent DYRK1A-inhibiting activity. Molecular docking calculations provided structural details for the interaction between acrifoline (4), atalaphyllidine (3), chlorospermines A and B (1 and 2), and DYRK1A, which are in agreement with the biological data. Docking studies predict a binding mode for acrifoline (4), which showed the most potent DYRK1A-inhibiting activity, similar to that of harmine, a β-carboline alkaloid, and leucettines, currently considered the most potent bioavailable inhibitors of this enzyme.14 Acridone could therefore represent a novel molecular scaffold in the search for new DYRK1A inhibitors.

**EXPERIMENTAL SECTION**

**General Experimental Procedures.** UV spectra were recorded on a PerkinElmer Lambda 5 spectrophotometer. IR spectra were recorded with a Nicolet FTIR 205 spectrophotometer. The NMR spectra were recorded on a Bruker 500 MHz instrument (Avance 500) for 1H and 125 MHz for 13C using CDCl3 as solvent. HRESIMS was run on a Thermoquest TLM LCQ Deca ion-trap spectrometer.
Kromasol analytical and preparative C18 columns (250 × 4.6 mm and 250 × 21.2 mm; id: 5 μm, Thermo) were used for preparative HPLC separations using a Waters autopurification system equipped with a binary pump (Waters 2525), a UV–vis diode array detector (190–600 nm, Waters 2996), and a PL-ELS 1000 ELSD Polymer Laboratory detector. Silica gel 60 (6–35 μm) and analytical and preparative TLC plates (Si gel 60 F254) were purchased from SDS (France). A C18 140 g Versapack cartridge was used for flash chromatography using a Combiblack-Companion apparatus (Srlab). All other chemicals and solvents were purchased from SDS (France).

**Plant Material.** *Glycosmis chloropera* was collected in Keluang, Johor Province, Malaysia, in July 2006. The plant was identified by T. Leong Eng, Botanist, University of Malaya. A voucher specimen (KL-S280) has been deposited at the Herbarium of the Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

**Extraction and Isolation.** The air-dried and powdered bark of *G. chloropera* (1 kg) was extracted with EtOAc (3 × 1 L, 1 h each, 38 °C, 100 bar), using a Zipertex static high-pressure, high-temperature extractor, developed in the ICSN Pilot Unit. The EtOAc crude extract was concentrated in a vacuum at 40 °C to yield 9.4 g of residue. This residue (8.9 g) was subjected to flash chromatography using a C18 140 g Versapack cartridge with a gradient of MeCN–H2O (20:80 to 100:0) at 20 mL/min to afford 12 fractions, F1–F12, according to their TLC profiles. Fraction F6 (788 mg, Ig′c 0.23 μg/mL) was subjected to silica gel column chromatography (CC) using a gradient of CH2Cl2–MeOH (100:0–80:20) at 20 mL/min to increasing polarity, leading to 18 fractions (F1–F18) on the basis of TLC. Fraction F6′ (259 mg) was subjected to silica gel CC using a gradient of CH3Cl–MeOH (100:0–80:20) to afford atalaphyllidine (3, 18.9 mg) and acrifoline (4, 6.7 mg). Fraction F10′ (16 mg) was subjected to preparative TLC using the system EtOAc–CH2Cl2 (90:10) to afford chlorospermine B (2, 2.5 mg). The purification of fraction F15′ (51 mg) using preparative TLC with the solvent system EtOAc–MeOH (98:2) afforded chlorospermine A (1, 3.5 mg).

**Chlorospermine A (1):** red, amorphous powder; UV (MeOH) λmax (log ε) 273 (4.4) nm; IR νmax 3210, 1595, 1665, 1178, 833 cm−1; 1H and 13C NMR data, see Table 1; HRESIMS m/z [M + H]+ 406.1656 (calcd for C21H18NO4, 406.1236). Molecular Modeling. Sequence alignments were generated using ClustalX, version 2.0.21 Molecular docking calculations were carried out using Gold 5.2,21 with the Goldscore scoring function and the human DYRK1A protein structure (PDB code 3ANR25). Sequence alignment between the rat DYRK1A (used for the protein kinase assay) and the human DYRK1A (used for molecular docking) showed that there were only three mutations between these two proteins, in positions 32, 404, and 543 (Figure S1, see Supporting Information). Residue 404 is distant from the binding site (more than 43 Å), and the other two are toward the beginning and the end of the sequence, not present in the protein structure used for the docking, implying that the docking results can be reliably compared with the protein kinase assay data. The binding site was defined as a sphere with 15 Å radius around the CB atom of the “gatekeeper” Phe238 residue. Three-dimensional coordinates of the ligands were obtained using Corina, version 3.44 (http://www.molecular-networks.com/). Images were generated using PyMol version 1.6 (http://www.schrodinger.com/).

**ASSOCIATED CONTENT**

3 Supporting Information

NMR spectra for compounds 1 and 2, sequence alignment for rat and human DYRK1A, kinase activity data (4), and superimposition of compounds 1–4 in the DYRK1A binding site are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on May 5, 2014, with incorrect graphics in Figures 1 and 3. The corrected version reposted on May 6, 2014.