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A new cyclooxygenase (COX) inhibitory pterocarpan from *Indigofera aspalathoides*: structure elucidation and determination of binding orientations in the active sites of the enzyme by molecular docking

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Abstract—A new compound indigocarpan (**1**) and the known compound mucronulatol (**2**) were isolated from chloroform extracts of *Indigofera aspalathoides*. Their structures were established by spectroscopic methods, including single-crystal X-ray analysis (in the case of **1**). The isolates were evaluated for cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inhibitory activities and antioxidant properties. The new compound **1** showed significant COX-1 inhibition (IC_{50} 30.5 μM) and its in vivo anti-inflammatory activity was found to be comparable to that of ibuprofen. Molecular docking studies revealed the binding orientations of **1** in the active sites of COX-1 and COX-2.
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Indigofera aspalathoides Vahl. belongs to the family Fabaceae and grows abundantly in Southern India. This plant has been used in traditional Indian medicine for the treatment of oedematous tumours, gastric hyperacidity and ulcers, toothache and abscesses.[1–3] Our preliminary activity screening showed that a crude chloroform extract of *I. aspalathoides* exhibited significant anti-inflammatory activity in carrageenan induced rat paw edema assay. The chloroform extract was subjected to repeated column chromatography on silica gel and Sephadex LH-20 to furnish compounds **1** and **2**.

Compound **1** was isolated as a white amorphous powder (mp 139–140 °C), $[\alpha]_D^{25}$ −185 (*c* 0.1, $CHCl_3$). The APCI-MS showed an ion peak at *m/z* 317 $[M+H]^+$. In the FT-IR spectrum, diagnostic peaks were observed for methyl groups (2937, 2842 cm^{-1}) and the aromatic ring (1607, 1501 cm^{-1}). The structure of **1** was deduced from detailed analysis of the ^1H and ^{13}C NMR data aided by 2D NMR experiments (^1H–^1H COSY, HMQC and HMBC) (Table 1) (Fig. 1).

The ^1H NMR spectrum of **1**, showed the characteristic multiplets associated with the CH–CH–CH_2 grouping of the pterocarpan skeleton,[4,5] the methylene protons at δ 3.66 (1H, m, Hβ-6), and 4.32 (1H, dd, $J = 10.4$, 4.6 Hz, Hα-6), two methine protons at δ 3.53 (1H, m, Hα-6a) and 5.53 (1H, d, $J = 6.4$ Hz, Hα-11a), two methoxy protons at δ 3.85 (3H, s), and 3.88 (3H, s), four aromatic protons at δ 6.44 (1H, d, $J = 8.0$ Hz, H-7), 6.66 (1H, d, $J = 8.5$ Hz, H-2), 6.73 (1H, d, $J = 8.0$ Hz, H-8), and 7.22 (1H, d, $J = 8.5$ Hz, H-1) and two phenolic OH at δ 5.46 (1H, s, OH-10) and 5.93 (1H, s, OH-3). The presence of two phenolic OH groups was confirmed by the formation of the corresponding diacetate **1a**.[6]

The multiplicity of signals in the ^{13}C NMR spectrum of **1**, as determined by DEPT experiments, indicated the presence of two methoxy, one methylene, six methine and eight quaternary carbons. The ^{13}C NMR values for all the carbons were assigned on the basis of the HMQC and HMBC spectra and were in good agreement with the proposed structure. Moreover, X-ray analysis[7] was performed to determine the relative configuration of the stereocentres (C-6a and C-11a) and compound **1** was unequivocally confirmed as (6a*R*,11a*S*)-4,9-dimethoxy-pterocarpan-3,10-diol. Figure 2 shows the ORTEP

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Table 1. ^{1}H and ^{13}C NMR data of indigocarpan (**1**)[a]

C. no	δ_C	DEPT	δ_H [mult., J (Hz)]	HMBC (H → C)
1	126.49	CH	7.72 d (8.5)	C-3, C-4a, C-11a
1a	112.99	C		
2	108.88	CH	6.66 d (8.5)	C-4, C-1a, C-3
3	149.65	C		
4	134.81	C		
4a	148.44	C		
6	66.43	CH_2	α: 4.32 dd (10.4, 4.6); β: 3.66 m	C-6a, C-11a, C-4a
6a	39.88	CH	3.53 m	C-11a
7	103.77	CH	6.44 d (8.0)	C-8, C-7a, C-9, C-10a
7a	121.37	C		
8	114.75	CH	6.73 d (8.0)	C-7, C-10, C-9
9	148.07	C		
10	146.06	C		
10a	130.63	C		
11a	79.20	CH	5.53 d (6.4)	C-1a, C-1, C-6, C-4a
4-OCH_3	61.15	CH_3	3.88 s	C-4
9-OCH_3	56.44	CH_3	3.85 s	C-9

[a] All spectra were recorded in $CDCl_3$ (^{1}H, 300 MHz; ^{13}C, 75 MHz).

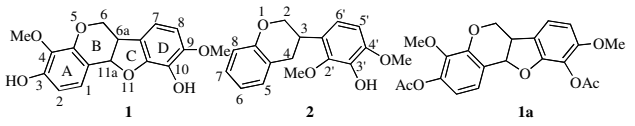

Figure 1. Chemical structures of indigocarpan (**1**), mucronulatol (**2**) and indigocarpan diacetate (**1a**).

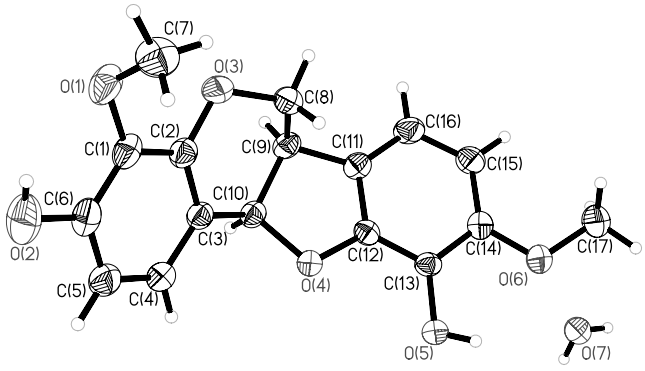

Figure 2. ORTEP drawing of the X-ray structure of **1**.

projection of compound **1**. The trivial name indigocarpan is suggested for **1**.

Compound **2** was recognized as an isoflavan by the characteristic complex ^{1}H NMR signals assignable to the CH_2–CH–CH_2 grouping of the 3-aryl chroman system.[8,9] The NMR spectral data and melting point of **2** were similar to those of mucronulatol, previously isolated from *Tephrosia strigosa*,[10] *Dalbergia variabilis* and *Machaerium mucronulatum*.[11]

The anti-inflammatory activity of the compounds isolated was evaluated employing the COX catalyzed prostaglandin biosynthesis assay in vitro.[12–14] COX is the enzyme catalyzing the rate-limiting step in prostaglandin biosynthesis, converting arachidonic acid into prostaglandin and is generally accepted to be the target of NSAIDs.[15–17] Indigocarpan and mucronulatol exhibited IC_{50} values of 30.5 and 51.9 μM, respectively, for COX-1 inhibitory activity. However, these compounds were found to have moderate potency (52.5% and 40.9% inhibition, respectively, at 100 μg/mL concentration) towards the COX-2 enzyme. The diacetyl derivative of indigocarpan **1a** was found to be the least active with 66.9% (COX-1) and 37.9% (COX-2) inhibition at 100 μg/mL concentration. Since indigocarpan was found to possess significant COX-1 inhibitory activity, we thought of performing molecular docking studies to understand the ligand–protein interactions and COX-1/COX-2 selectivity in detail. Thus the FlexX method[18,19] was used to dock these compounds into the active sites of COX-1 and COX-2.

All the calculations were performed using SYBYL 6.9[20] software installed on a SGI octane 2 workstation. The crystal structures of COX-1 and COX-2 enzymes complexed with indomethacin [1PGG.pdb, 4COX.pdb][21] were used for the docking. The active site of the enzyme was defined to include residues within a 6.5 Å radius to any of the inhibitor atoms. The energy minimization of **1** was carried out using the MMFF94[22] method and the partial charges were calculated using the same method. After the FlexX docking, the top scoring conformation of **1** was superimposed onto the active sites of the enzymes and hydrogen atoms were added. The cofactor heme and sugar molecules were removed. The whole complex was minimized in a stepwise manner until the gradient convergence 0.05 kcal/mol was reached. The distance dependent dielectric function ($\varepsilon = 4r$) was used. The calculated binding energies of indigocarpan–COX-1 and indigocarpan–COX-2 complexes are given in Table 2.

Since the X-ray analysis of **1** provided the relative configuration as (6a*R*,11a*S*), we planned to dock both enantiomers of **1** on the active sites of the enzymes. The FlexX program could dock **1** into the active sites of COX-1 and COX-2 successfully. The (6a*R*,11a*S*) enantiomer of **1** showed binding energies of −32.16 and −28.10 kcal/mol

Table 2. Binding energies of **1**, (*R*)-**2**, and (*S*)-**2** with COX-1 and COX-2

S. no.	Compound	Enzyme	Interaction energy[a] (kcal/mol)
1	**1** (6a*R*,11a*S*)	COX-1	−32.16
2	**1** (6a*R*,11a*S*)	COX-2	−28.10
3	**1** (6a*S*,11a*R*)	COX-1	−27.48
4	**1** (6a*S*,11a*R*)	COX-2	−26.12
5	**2** (*R*)	COX-1	−28.34
6	**2** (*R*)	COX-2	−31.22
7	**2** (S)	COX-1	−29.95
8	**2** (S)	COX-2	−27.28

[a] $E_{complex} - (E_{ligand} + E_{protein})$.

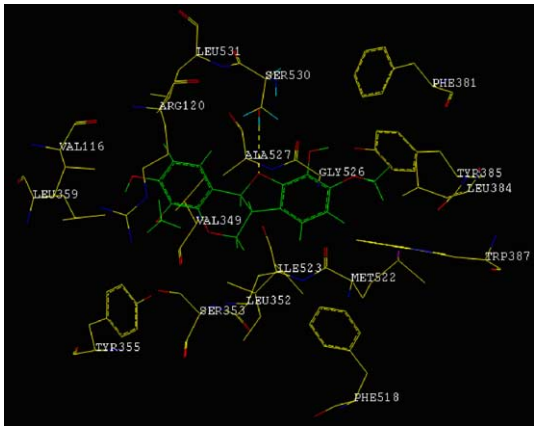

Figure 3. Binding of (6a*R*,11a*S*)-indigocarpan into the active site of COX-1. The hydrogen bonding interaction is shown as broken line.

for COX-1 and COX-2, respectively. The corresponding binding energies of the (6a*S*,11a*R*) enantiomer were found to be −27.48 and −26.12 kcal/mol, respectively. These docking results suggest that **1** is a preferred ligand for COX-1 rather than for COX-2 and provides a rationale for the selectivity of the enzyme inhibitory activity.

A hydrogen bonding interaction between the furan oxygen atom of **1** and the hydroxyl group of Ser530 of COX-1 (O···H–O–Ser530 = 2.574 Å) was observed (Fig. 3). The same residue of COX-2 was involved in a hydrogen bonding interaction with the C-10 hydroxyl group of **1** (O–H···O–Ser530 = 2.658 Å) (Fig. 4). That the interaction with the Ser530 hydroxyl group is important for enzyme inhibitory activity is well exemplified by the binding interaction of aspirin, a well known anti-inflammatory agent, with COX-1/COX-2.[23] A weak non bonded interaction between the furan oxygen in **1** and the side chain methyl group of Ala527 was observed in the indigocarpan–COX-2 complex. Significant favourable van der Waals interactions were observed between the active site amino acid residues of COX-1 and **1**. The oxygen atom of the C-9 methoxy group and the corresponding phenyl ring **D** were found to be involved in favourable nonbonded interactions with the phenyl ring of Tyr385 and Trp387, respectively. The C-6 methylene moiety of the pyran ring **B** also exhibited a favourable interaction with methyl group of Leu352. The methoxy and hydroxyl groups in ring **A** were not involved in any significant interaction with the

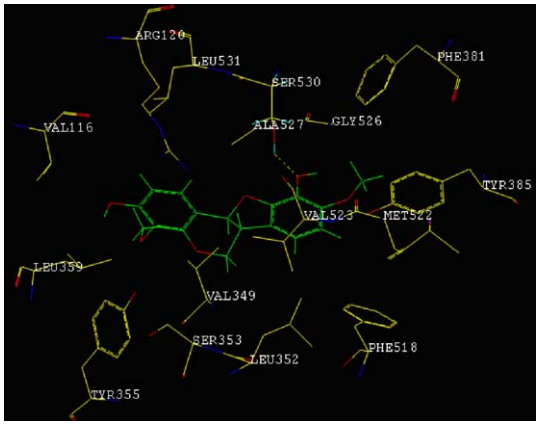

Figure 4. Binding of (6a*R*,11a*S*)-indigocarpan into the active site of COX-2. The hydrogen bonding interaction is shown as broken line.

active sites of the enzyme indicating that these groups do not contribute much to the enzyme inhibitory activity. However, in the case of indigocarpan–COX-2 complex formation, the van der Waals interactions were found to be inferior. Therefore, we anticipate that the favourable van der Waals interactions observed between indigocarpan and COX-1 may be the reason for the observed selectivity.

To understand the inferior enzyme inhibitory activity of mucronulatol (**2**) compared to that of **1**, the *R* and *S* enantiomers of **2** were docked into the active sites of COX-1 and COX-2. From the interaction energies we found that (*R*)-**2** and (*S*)-**2** were selective towards COX-2 and COX-1, respectively. However, the poor FlexX scores obtained during the docking of the diacetate **1a** in the active sites of COX-1 and COX-2 revealed that the binding of **1a** with the enzymes was not energetically favourable and accounted for the poor COX inhibitory property of **1a**. Thus, the comparison of the observed enzyme inhibitory activity of **1**, **1a** and **2** along with the docking studies suggest that the presence of the furan ring **C** and the free hydroxyl group at C-10 is essential for the desired biological activity.

The new isolate **1** was tested for in vivo anti-inflammatory activity using carrageenan induced rat paw oedema assay[24] at 125 mg/kg dose level which resulted in 51.77% (3 h) inhibition. Ibuprofen was used as positive control and exhibited 54.5% (3 h) inhibition at 100 mg/kg dose. It has been found that in the case of inflammatory disorders, excessive free radical generation takes place and several NSAIDs and phenolic compounds with anti-inflammatory activity are reported to act as radical scavengers.[25] The *o*-methoxy phenolic moiety has been found to be an essential structural feature for antioxidant properties.[26] Accordingly, the antioxidant properties of **1** and **2** were evaluated using DPPH radical scavenging in an in vitro assay.[27] It was found that **1** ($IC_{50} = 25.42\,\mu M$) exhibited better radical scavenging activity than **2** ($IC_{50} = 34.83\,\mu M$).

In conclusion, a new anti-inflammatory compound indigocarpan (**1**) was isolated from *I. aspalathoides*. The

relative stereochemistry was determined by X-ray analysis. This is the first report of mucronulatol and pterocarpan for COX inhibitory activity. The mixed functioning antioxidant and COX inhibitory properties may provide superior anti-inflammatory activity.[28] Thus, the pterocarpan nucleus can serve as a new pharmacophore for lead generation and optimization of novel anti-inflammatory agents. Further studies on the chemical and biological properties of indigocarpan analogues are currently underway.

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6. *Acetylation of* **1** (**1a**):[29] $InCl_3$ (0.06 mg, 1 mol%) was added to a magnetically stirred mixture of **1** (10 mg, 0.03 mmol) and Ac_2O (12.24 mg, 0.01 mL, 4 equiv) and the mixture was stirred at room temperature until the TLC showed complete consumption of **1** (40 min). The mixture was diluted with water (5 mL) and extracted with EtOAc (3×10 mL). The combined EtOAc extracts were washed with water (5 mL), dried (Na_2SO_4), and concentrated under reduced pressure to afford the diacetate **1a** (12 mg, 95%): ^{1}H NMR ($CDCl_3$) δ 2.32 (3H, s, $-CH_3$), 2.33 (3H, s, $-CH_3$), 3.57 (1H, m, H-6a), 3.65 (1H, m, H-6β), 3.81 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 4.38 (1H, dd, $J = 8.9$ and 2.9 Hz, H-6α), 5.56 (1H, d, $J = 6.0$ Hz, H-11a), 6.49 (1H, d, $J = 8.2$ Hz, H-7), 6.75 (1H, d, $J = 8.4$ Hz, H-2), 7.04 (1H, d, $J = 8.2$ Hz, H-8), 7.28 (1H, d, $J = 8.4$ Hz, H-1); ^{13}C NMR ($CDCl_3$) δ 20.46 ($COCH_3$), 20.73 ($COCH_3$), 39.70 (C-6a), 56.35 (OCH_3), 60.94 (OCH_3), 66.50 (C-6), 79.19 (C-11a), 104.52 (C-7), 115.79 (C-2), 118.85 (C-1a), 120.88 (C-7a), 121.51 (C-8), 124.40 (C-10a), 125.67 (C-1), 140.65 (C-4), 144.26 (C-3), 149.73 (C-4a), 151.70 (C-9), 152.77 (C-10), 168.33 (C=O), 169.09 (C=O); APCI-MS 400.7 $[M]^+$, 382.9, 370.9, 358.9, 340.9, 194.8, 152.9.
7. X-ray crystal structure analysis of compound **1**:[30] The X-ray measurements were made on a Siemens P4 Diffractometer with graphite-monochromated Mo Kα ($\lambda = 0.71073$ Å) radiation at 293 (2) K. The final unit cell parameters were based on all 2785 reflections measured, 2731 of which were independent R (int) = 0.0248. The space group was $P2_12_12_1$ with unit cell parameters $\alpha = 7.108$ (1) A°, $b = 16.810$ (1) A° and $c = 25.462$ (2) A°. The model was refined by full-matrix least-squares procedures on F^2 using SHELXL-97 to a value of $R_1 = 0.0329$ for 2414 reflections with $I > 2\sigma$ (I). ORTEP for Windows was used to prepare the molecular graphics.
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30. Crystallographic data for compound **1** have been deposited with the Cambridge Crystallographic Data Centre (CCDC 231472). Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 IEZ, UK. [fax: +44-0-1223-336033, e-mail: deposit@ccdc.cam.ac.uk].