

# Inotilone and related phenylpropanoid polyketides from *Inonotus* sp. and their identification as potent COX and XO inhibitors

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By bioassay-guided isolation, phenylpropanoid-derived polyketides, including an unusual 5-methyl-3(2*H*)-furanone derivative (inotilone) with potent cyclooxygenase (COX) and xanthone oxidase (XO) inhibitory activities were obtained from the fruiting body of the mushroom *Inonotus* sp.

## Introduction

Arthritis is a general term for severe inflammatory processes in joints or joint tissue. Nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac and indomethacin, have emerged as the most commonly used anti-inflammatory agents for the therapy of rheumatoid arthritis.<sup>1</sup> Many of these drugs target cyclooxygenases (COX), which catalyze the first two steps in the biosynthesis of the prostaglandins from the substrate arachidonic acid.<sup>2,3</sup> In this context, the selective inhibition of enzyme subtypes, COX-1 and COX-2, has become an important goal.<sup>4</sup> In contrast to rheumatoid arthritis, gouty arthritis is mediated by the crystallisation of uric acid (UA) in the joints.<sup>5,6</sup> Gout can be treated with drugs that either increase the urinary excretion of UA, or with xanthine oxidase (XO) inhibitors that block the terminal step of UA biosynthesis.<sup>7,8</sup> The purine analogue allopurinol is currently the only XO inhibitor in clinical use. Unfortunately, it seems to be associated with an infrequent but severe hypersensitivity.<sup>9</sup> Thus, the search for new potent inhibitors of these enzymes, which could be useful as lead structures for new anti-inflammatory and anti-arthritic therapeutics, plays a pivotal role. Here we report on the isolation, structural elucidation and biological evaluation of natural anti-inflammatory COX and XO inhibitors from the mushroom *Inonotus* sp.

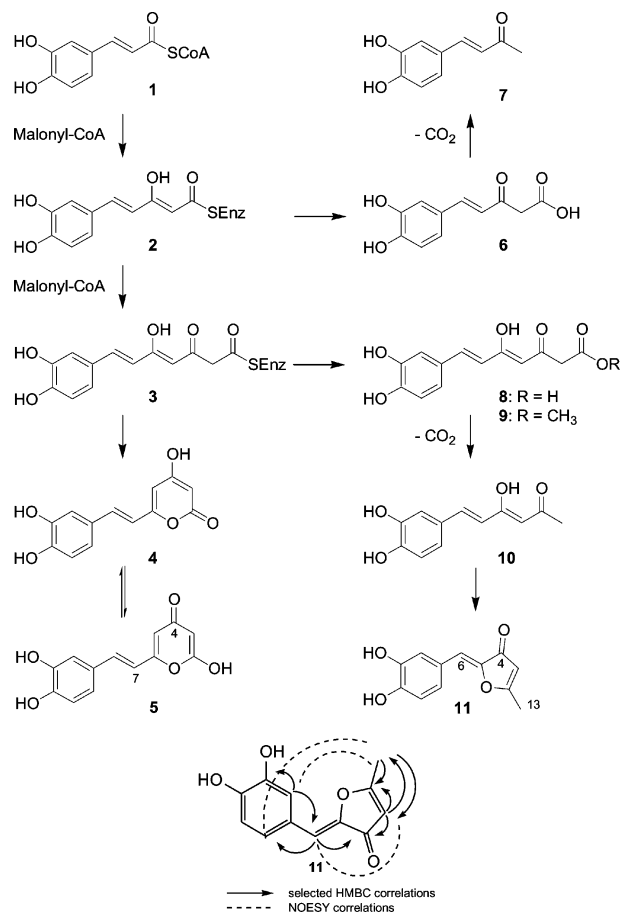
## Results and discussion

Extracts from the fruiting body *Inonotus* sp. exhibited significant inhibitory activities against key enzymes involved in inflammatory processes: 3 $\alpha$ -HSD, COX and xanthine oxidase. Bioassay-guided separation of the combined crude ethanolic and CHCl<sub>3</sub>/MeOH extracts of the fruiting body using open column and preparative HPLC yielded several phenolic compounds **11** (4 mg), **9** (20 mg), **5** (4 mg) together with the known compounds **4** (500 mg) and **7** (6 mg) (Scheme 1).

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**Scheme 1** Structures of *Inonotus* sp. metabolites and model for their biosynthesis. Key HMBC and NOESY correlations of **11**.

The main product from *Inonotus* sp. was identified as the known metabolite hispidin (**4**) by comparison of MS, IR and NMR data.<sup>10</sup> In addition to **4**, another compound **5** with the same molecular formula (C<sub>13</sub>H<sub>10</sub>O<sub>5</sub>) was isolated. Also the <sup>1</sup>H NMR spectrum of **5** showed signals similar to those of **4**.<sup>10</sup> However, the <sup>13</sup>C NMR spectrum, which showed a signal for a conjugated carbonyl at  $\delta$  179.1, clearly established **5** as the tautomeric  $\gamma$ -pyrone (*iso*-hispidin).

The molecular formula of the second main product (**9**) was determined as C<sub>14</sub>H<sub>14</sub>O<sub>6</sub> based on HR-EIMS and its <sup>13</sup>C NMR spectrum. Similar to **4** and **5**, the <sup>1</sup>H-NMR spectrum showed signals attributable to the ABX spin coupling system of a trisubstituted phenyl moiety at  $\delta$  6.77 (1H, d,  $J$  = 8.1 Hz, H-12),  $\delta$  7.02 (1H, dd,  $J$  = 8.2, 1.8 Hz, H-13),  $\delta$  7.07 (1H, d,  $J$  = 1.8 Hz H-9), a *trans* disubstituted double bond at  $\delta$  7.45 (1H, d,  $J$  = 15.8 Hz, H-7) and  $\delta$  6.50 (1H, d,  $J$  = 15.8 Hz, H-6), and two exchangeable phenolic hydroxyl protons at  $\delta$  9.15 and 9.65. In addition, a chelated proton at  $\delta$  15.20 was detected. Analyses of <sup>13</sup>C, DEPT 135 and HMQC NMR spectra of **9** showed 14 carbon signals including six sp<sup>2</sup> methines, four quaternary sp<sup>2</sup> carbons (three of which are oxygenated), one methylene carbon at  $\delta$  45.6, a methoxy carbon at  $\delta$  51.8, a carbonyl carbon at  $\delta$  191.8, and a carboxyl carbon at  $\delta$  167.9. HMBC NMR spectra proved to be very helpful in defining their connectivities. The correlation of the H-9 ( $\delta$  7.07) with C-7 ( $\delta$  141.0), C-8 ( $\delta$  126.2), C-10 ( $\delta$  145.6), and C-11 ( $\delta$  148.4), the correlation of H-12 ( $\delta$  6.77) with H-8, H-10, H-11, and H-13 and the correlation of H-13 ( $\delta$  7.02) with C-7, C-8, C-9, C-11 and C-12, revealed an *ortho* substitution of the phenolic hydroxyl protons. Other important information was obtained from the observed correlation of the methylene protons (H-2) with C-1 ( $\delta$  167.9), C-3 ( $\delta$  191.8) and C-4 ( $\delta$  100.3). Structural deductions from NMR data were supported by the IR spectrum of **9**, which showed absorption bands for hydroxyl groups at 3183 cm<sup>-1</sup>, a conjugated carbonyl (1632 cm<sup>-1</sup>) a carboxyl group at 1733 cm<sup>-1</sup>, and aromatic rings (1567, 1513 and 1435 cm<sup>-1</sup>). Consequently, **9** represents the methyl ester of the open chain derivative of **4** or **5**, and was named inonic acid methyl ester.

The molecular formula of compound **11** was determined as C<sub>12</sub>H<sub>10</sub>O<sub>4</sub> based on HR-EIMS and <sup>13</sup>C NMR data. Similar to **4**, **5** and **9**, the <sup>1</sup>H NMR spectrum of **11** showed signals attributable to the ABX spin coupling system of a trisubstituted phenyl moiety. Two olefinic protons at  $\delta$  6.49 (1H, s, H-6),  $\delta$  5.82 (1H, d,  $J$  = 0.6 Hz, H-4) and a methyl group at  $\delta$  2.39 (3H, s, H-13) were also observed. Two proton signals were attributable to the phenolic exchangeable hydroxyl protons. The <sup>13</sup>C NMR and DEPT 135 spectra of **11** showed 11 sp<sup>2</sup> carbon signals including five methines and five quaternary oxygenated carbons including one carbonyl. The occurrence of the carbonyl moiety was confirmed by the <sup>13</sup>C spectrum, which showed one signal at  $\delta$  186.6. The protonated carbons and their corresponding protons and the full connection of compound **11** were established using HMQC and HMBC experiments, respectively. The correlation of the methyl proton  $\delta$  2.39 (3H, s, H-13) with C-2 ( $\delta$  180.4), and C-3 ( $\delta$  105.4), and the correlation of the olefinic proton H-3 ( $\delta$  5.82) with C-4 (carbonyl moiety) and C-5 ( $\delta$  144.3) unambiguously revealed a disubstituted dihydrofuranone moiety. The correlation of the olefinic proton H-6 ( $\delta$  6.49) with C-4 ( $\delta$  186.6), C-5 ( $\delta$  144.3), C-7 ( $\delta$  122.9), C-8 ( $\delta$  117.9) and C-12 ( $\delta$  124.7) enabled us to connect the dihydrofuranone moiety with the rest of the molecule. The configuration of the C-5 double bond was established based on molecular modeling and NOESY, which showed a correlation between H-6 ( $\delta$  6.49) and H-3 ( $\delta$  5.82) and the correlation between the protons H-8 ( $\delta$  7.35) and H-12 ( $\delta$  7.17) with the methyl protons H-13 ( $\delta$  2.39). Thus the structure was established as 2-(3,4-dihydroxybenzylidene)-5-methylfuran-3-one, named inotilone (**11**). Only recently, related 5-methyl-3(2H)-furanone metabolites have been reported from *Phellinus igniarius*.<sup>11</sup>

**Table 1** Inhibitory activities of **4**, **5**, **7**, **9**, and **11** against 3- $\alpha$ HSD, COX-1, COX-2, and XO

Compound	IC <sub>50</sub> /μM				
	3 $\alpha$ -HSD	COX-1	COX-2	COX-2/COX-1	XO
<b>4</b>	8.1	0.01	8 × 10 <sup>-4</sup>	0.08	4.4
<b>5</b>	12.1	0.05	0.13	2.6	13.8
<b>7</b>	8.9	0.03	0.01	0.3	10.1
<b>9</b>	16.1	0.46	0.21	0.4	7.1
<b>11</b>	50.4	0.36	0.03	0.08	9.1
Indomethacin	15.4	0.10	6.00	60	n.a.
Allopurinol	n.a.	n.a.	n.a.	n.a.	4.4

The structures of compounds **5**, **9** and **11**, as well as the isolation of the known **4** and **7** suggest that all metabolites share the same biosynthetic origin. All compounds represent linear or cyclized polyketides derived from caffeoyl-CoA (**1**). While **7** appears to be a shunt product resulting from a premature release from the polyketide synthase, **4**, **5**, **9** and **11** are the result of two rounds of elongation. The structurally unusual **11** could be the product of a decarboxylation-radical ring closure sequence *via* the known metabolite hispolon **10**.<sup>12</sup> A related sequence could be involved in the formation of the tri- and tetrahydroxyaurone aglycones of sulfurein and cernuosides.<sup>13,14</sup>

All compounds were evaluated for their inhibitory activities in hydroxysteroid dehydrogenase (3 $\alpha$ -HSD), COX-1, COX-2 and XO enzyme assays according to previously documented procedures. Their inhibitory potencies, expressed as IC<sub>50</sub> values, are shown in Table 1 and are compared with those of the references, indomethacin and allopurinol. The results in the present study demonstrated that the phenolic compounds exhibit strong COX inhibitory effects with a prevalence for COX-2 in the case of the compounds **4**, **7**, **9** and **11**. It should be highlighted that hispidin (**4**) and the novel inotilone (**11**) selectively inhibit COX-2 at concentrations as low as those of the marketed selective inhibitors meloxicam and nimesulide.<sup>3</sup> In all cases, except for compound **11**, strong 3 $\alpha$ -HSD inhibitory effects were noted, as well as moderate inhibitory effects toward XO, except hispidin (**4**), which exhibited an inhibitory activity at a level comparable with that of the standard allopurinol. As far as the tautomeric compounds **4** and **5** are concerned, it seems that the  $\alpha$ -pyrone is more active than the  $\gamma$ -pyrone.

In summary, we have isolated and characterized three new phenylpropanoid polyketides with potent COX and XO inhibitory activities from the mushroom *Inonotus* sp. Apart from their potent anti-arthritic activities, these metabolites represent new members of caffeoyl derived polyketides, out of which the structure of inotilone is most notable.

## Experimental

### General experimental procedures

IR spectra (film) were recorded on a JASCO FT/IR-4100 spectrometer equipped with an ATR device. UV spectra were measured with a Spicord 200 Carl Zeiss spectrometer. High-resolution electron impact mass spectra (HR-EIMS) were recorded on an AMD 402 double-focussing mass spectrometer with BE geometry. NMR spectra were recorded on a Bruker Avance 500 DRX spectrometer at 300.133 MHz for <sup>1</sup>H and 75.475 MHz for <sup>13</sup>C

in DMSO-d<sub>6</sub>. Chemical shifts are given in ppm relative to TMS as internal standard. HSQC and NOESY (mixing time 0.7 s) data were obtained in the phase-sensitive mode TPPI. Column chromatography was performed using silica gel (60, Merck; 0.063–0.2 μm) and Sephadex LH-20. HPLC was performed using a Gilson binary gradient HPLC system equipped with a UV detector (UV/VIS-151)(370 nm) using a preparative reverse phase C<sub>18</sub> (7 μm) column. TLC was carried out with silica gel 60 F<sub>254</sub> plates. Spots were visualized by spraying with vanilline/H<sub>2</sub>SO<sub>4</sub> followed by heating. All solvents used were spectral grade or distilled prior to use.

### Strains

The fruiting body of *Inonotus* sp. was collected in Vietnam. Its identity was verified by Prof. Trinh Tam Kiet from the Mycological Research Center, Hanoi State University, Vietnam, where a specimen was deposited.

### Extraction and isolation

The fruiting body of *Inonotus* sp. (25 g dry weight) was cut into small species, dried and crushed. The resulting powder was extracted three times with ethanol (2 L) and chloroform–methanol (1 : 1) (3 × 2 L, 3 days each). The extracts were subjected to silica gel chromatography (silica gel 60, Merck, 0.063~0.1 mm, column 4 × 60 cm), using stepwise CHCl<sub>3</sub>–MeOH (9 : 1, 8 : 2, 1 : 1 v/v) as eluent. Final purification was achieved by preparative HPLC (Spherisorb ODS-2 RP<sub>18</sub>, 5 μm (Promochem), 250 × 25 mm, acetonitrile–H<sub>2</sub>O (83 : 17 v/v), at a flow rate of 10 ml min<sup>-1</sup> and UV detection at 372 nm). Yields: 500 mg of **4**, 4 mg of **5**, 6 mg of **7**, 20 mg of **9**, and 4 mg of **11**.

**iso-Hispidin (5).** Was obtained as a red oil by open column chromatography on Sephadex LH20 using CHCl<sub>3</sub>–MeOH 80 : 20 as eluent. Further purification was done by HPLC using gradient (water–acetonitrile 95 : 5 to 5 : 95; 30 min) *R*<sub>t</sub> = 14 min; UV (MeOH) λ<sub>max</sub> 248, 361 nm; IR (film) 3059, 1649, 1590, 1494, 1411, 1276, 1202, 1050, 1000 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz) data see Table 2; <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 75 MHz) data see Table 2; *m/z*

245 [M – H]<sup>-</sup>; HR-EIMS (found [M – H]<sup>-</sup>): 245.0464 calcd. for C<sub>15</sub>H<sub>15</sub>O<sub>6</sub>: 245.0445).

**Inonotic acid methyl ester (9).** Was obtained as a yellow oil by open column chromatography on Sephadex LH 20 using CHCl<sub>3</sub>–MeOH (v/v = 90 : 10) as eluent. Further purification was achieved by HPLC using a water–acetonitrile gradient (95 : 5 to 5 : 95; 30 min) *R*<sub>t</sub> = 20.5 min; UV (MeOH) λ<sub>max</sub> 261, 380 nm; IR (film) 3094, 1733, 1632, 1567, 1513, 1435, 1282, 1022, 974 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz) data see Table 2; <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 75 MHz) data see Table 2; *m/z* 277 [M – H]<sup>-</sup>; HR-EIMS (found [M – H]<sup>-</sup>): 277.0682 calcd. for C<sub>14</sub>H<sub>13</sub>O<sub>6</sub>: 277.0707).

**Inotilone (11).** Was obtained as a yellow oil by open column chromatography on Sephadex LH 20 using CHCl<sub>3</sub>–MeOH (v/v = 85 : 15) as eluent. Further purification was achieved by HPLC using a water–acetonitrile gradient (95 : 5 to 5 : 95; 30 min); *R*<sub>t</sub> = 16 min; UV (MeOH) λ<sub>max</sub> 264, 312, 378 nm; IR (film) 3184, 1682, 1588, 1435, 1287, 1014, 951 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz) data see Table 2; <sup>13</sup>C NMR (DMSO-d<sub>6</sub>, 75 MHz) data see Table 2; *m/z* 217 [M – H]<sup>-</sup>; HR-EIMS (found [M – H]<sup>-</sup>): 217.0495, calcd. for C<sub>12</sub>H<sub>9</sub>O<sub>4</sub>: 217.0495).

### Biological assays

The 3α-hydroxy steroid dehydrogenase assay (3-αHSD) was measured spectrophotometrically, and conducted according to the method described by Penning.<sup>15</sup> The inhibitory activities of the test compounds are indicated in terms of IC<sub>50</sub>. Indomethacin was used as reference.

The peroxidative activity of cyclooxygenases I and II was measured using luminol as a specific chemiluminescent substrate according to the method described by Forghani *et al.*<sup>16</sup> The inhibitory activities of the test compounds are given in terms of IC<sub>50</sub>. Indomethacin was used as reference.

The xanthine oxidase activity was measured using lucigenin as the chemiluminescence substrate, and conducted according to the method described by Pierce *et al.*<sup>17</sup> The inhibitory activities of the test compounds are indicated in terms of IC<sub>50</sub>. Allopurinol was used as the reference.

**Table 2** <sup>1</sup>H and <sup>13</sup>C NMR data<sup>a</sup> for compounds **5**, **9**, and **11**

N <sup>o</sup>	<b>5</b>		<b>9</b>		<b>11</b>	
	δ <sup>1</sup> H (J/Hz)	δ <sup>13</sup> C	δ <sup>1</sup> H (J/Hz)	δ <sup>13</sup> C	δ <sup>1</sup> H (J/Hz)	δ <sup>13</sup> C
1				167.9		
2		165.4	3.55 s	45.6		180.4
3	4.42 d (1.2)	86.5		191.8	5.82 q	105.5
4		179.1	5.91 s	100.3		186.6
5	5.59 d (1.2)	109.0		178.3		144.3
6		156.1	6.50 d (15.8)	118.6	6.49 s	111.9
7	6.12 d (15.8)	118.5	7.45 d (15.8)	141.0		122.9
8	6.87 d (15.8)	130.8		126.2	7.35 d (2.0)	117.9
9		127.4	7.07 d (1.8)	114.7		145.4
10	6.94 d (1.5)	113.5		145.6		148.1
11		145.6		148.4	6.80 d (8.2)	115.9
12		146.5	6.77 d (8.1)	115.7	7.17 dd (8.2, 2.0)	124.7
13	6.70 d (8.1)	115.7	7.02 dd (8.1, 1.8)	121.5	2.39 s	15.67
14	6.82 dd (8.1, 1.5)	119.2				
1'			3.65 s	51.8		

<sup>a</sup> Recorded in DMSO-d<sub>6</sub>.

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## References

- 1 S. I. Rennard, *Proc. Am. Thorac. Soc.*, 2004, **1**, 282.
- 2 J. R. Vane and R. M. Botting, *Inflammation Res.*, 1998, **47**(Suppl 2), 78.
- 3 J. R. Vane, Y. S. Bakhle and R. M. Botting, *Annu. Rev. Pharmacol. Toxicol.*, 1998, **38**, 97.
- 4 D. L. Simmons, R. M. Botting and T. Hla, *Pharmacol. Rev.*, 2004, **56**, 387.
- 5 N. Dalbeth and D. O. Haskard, *Rheumatology (Oxford, U. K.)*, 2005, **44**, 1090.
- 6 H. K. Choi, D. B. Mount and A. M. Reginato, *Ann. Intern. Med.*, 2005, **143**, 499.
- 7 G. Rastelli, L. Costantino and A. Albasini, *J. Am. Chem. Soc.*, 1997, **119**, 3007.
- 8 S. Ishibuchi, H. Morimoto, T. Oe, T. Ikebe, H. Inoue, A. Fukunari, M. Kamezawa, I. Yamada and Y. Naka, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 879.
- 9 K. R. Hande, R. M. Noone and W. J. Stone, *Am. J. Med.*, 1984, **76**, 47.
- 10 L. R. Brady and R. G. Benedict, *J. Pharm. Sci.*, 1972, **61**, 318.
- 11 S. Mo, S. Wang, G. Zhou, Y. Yang, Y. Li, X. Chen and J. Shi, *J. Nat. Prod.*, 2004, **67**, 823.
- 12 A. A. N. Ali, R. Jansen, H. Pilgrim, K. Liberra and U. Lindequist, *Phytochemistry*, 1996, **41**, 927.
- 13 M. Shimokoriyama and S. Hattori, *J. Am. Chem. Soc.*, 1953, **75**, 1900.
- 14 M. K. Seikel and T. A. Geissman, *J. Am. Chem. Soc.*, 1950, **72**, 5725.
- 15 T. M. Penning, *J. Pharm. Sci.*, 1985, **74**, 651.
- 16 F. Forghani, M. Ouellet, S. Keen, M. D. Percival and P. Tagari, *Anal. Biochem.*, 1998, **264**, 216.
- 17 L. A. Pierce, W. O. Tarnow-Mordi and I. A. Cree, *Int. J. Clin. Lab. Res.*, 1995, **25**, 93.