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

Hilaire V. Kemami Wangun,[a] Albert Härtl,[a] Trinh Tam Kiet[b] and Christian Hertweck*[a,c]

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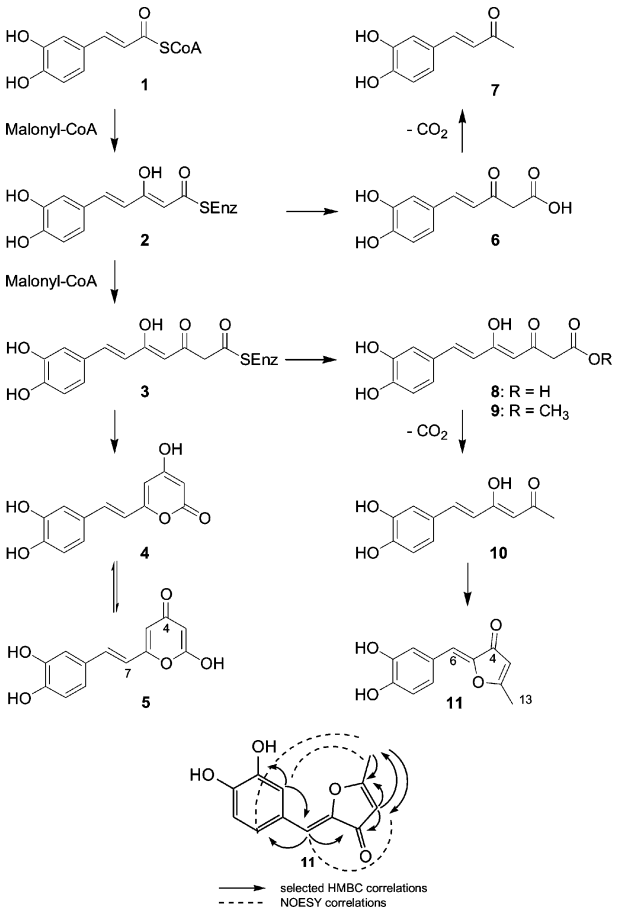
By bioassay-guided isolation, phenylpropanoid-derived polyketides, including an unusual 5-methyl-3(2*H*)-furanone derivative (inotilone) with potent cyclooxygenase (COX) and xanthone oxidase (XO) inhibitory activities were obtained from the fruiting body of the mushroom *Inonotus* sp.

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.[1] Many of these drugs target cyclooxygenases (COX), which catalyze the first two steps in the biosynthesis of the prostaglandins from the substrate arachidonic acid.[2,3] In this context, the selective inhibition of enzyme subtypes, COX-1 and COX-2, has become an important goal.[4] In contrast to rheumatoid arthritis, gouty arthritis is mediated by the crystallisation of uric acid (UA) in the joints.[5,6] 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.[7,8] 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.[9] 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: 3α-HSD, COX and xanthine oxidase. Bioassay-guided separation of the combined crude ethanolic and $CHCl_3$/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).



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.[10] In addition to **4**, another compound **5** with the same molecular formula ($C_{13}H_{10}O_5$) was isolated. Also the ^{1}H NMR spectrum of **5** showed signals similar to those of **4**.[10] However, the ^{13}C NMR spectrum, which showed a signal for a conjugated carbonyl at δ 179.1, clearly established **5** as the tautomeric γ-pyrone (*iso*-hispidin).

[a]*Dept. Biomolecular Chemistry, Leibniz-Institute for Natural Products Research and Infection Biology, Beutenbergstr. 11a, 07745, Jena, Germany. E-mail: christian.hertweck@hki-jena.de; Fax: INT+3641-656705; Tel: INT+3641-656700*
[b]*Centre of Biotechnology, Vietnam National University, 144 Xuan Thuy Street, Hanoi, Vietnam*
[c]*Friedrich-Schiller-University, Jena*

The molecular formula of the second main product (**9**) was determined as $C_{14}H_{14}O_6$ based on HR-EIMS and its ^{13}C NMR spectrum. Similar to **4** and **5**, the ^1H-NMR spectrum showed signals attributable to the ABX spin coupling system of a trisubstituted phenyl moiety at δ 6.77 (1H, d, J = 8.1 Hz, H-12), δ 7.02 (1H, dd, J = 8.2, 1.8 Hz, H-13), δ 7.07 (1H, d, J = 1.8 Hz H-9), a *trans* disubstituted double bond at δ 7.45 (1H, d, J = 15.8 Hz, H-7) and δ 6.50 (1H, d, J = 15.8 Hz, H-6), and two exchangeable phenolic hydroxyl protons at δ 9.15 and 9.65. In addition, a chelated proton at δ 15.20 was detected. Analyses of ^{13}C, DEPT 135 and HMQC NMR spectra of **9** showed 14 carbon signals including six sp^2 methines, four quaternary sp^2 carbons (three of which are oxygenated), one methylene carbon at δ 45.6, a methoxy carbon at δ 51.8, a carbonyl carbon at δ 191.8, and a carboxyl carbon at δ 167.9. HMBC NMR spectra proved to be very helpful in defining their connectivities. The correlation of the H-9 (δ 7.07) with C-7 (δ 141.0), C-8 (δ 126.2), C-10 (δ 145.6), and C-11 (δ 148.4), the correlation of H-12 (δ 6.77) with H-8, H-10, H-11, and H-13 and the correlation of H-13 (δ 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 (δ 167.9), C-3 (δ 191.8) and C-4 (δ 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^{-1}, a conjugated carbonyl (1632 cm^{-1}) a carboxyl group at 1733 cm^{-1}, and aromatic rings (1567, 1513 and 1435 cm^{-1}). Consequently, **9** represents the methyl ester of the open chain derivative of **4** or **5**, and was named inonotic acid methyl ester.

The molecular formula of compound **11** was determined as $C_{12}H_{10}O_4$ based on HR-EIMS and ^{13}C NMR data. Similar to **4**, **5** and **9**, the ^1H NMR spectrum of **11** showed signals attributable to the ABX spin coupling system of a trisubstituted phenyl moiety. Two olefinic protons at δ 6.49 (1H, s, H-6), δ 5.82 (1H, d, J = 0.6 Hz, H-4) and a methyl group at δ 2.39 (3H, s, H-13) were also observed. Two proton signals were attributable to the phenolic exchangeable hydroxyl protons. The ^{13}C NMR and DEPT 135 spectra of **11** showed 11 sp^2 carbon signals including five methines and five quaternary oxygenated carbons including one carbonyl. The occurrence of the carbonyl moiety was confirmed by the ^{13}C spectrum, which showed one signal at δ 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 δ 2.39 (3H, s, H-13) with C-2 (δ 180.4), and C-3 (δ 105.4), and the correlation of the olefinic proton H-3 (δ 5.82) with C-4 (carbonyl moiety) and C-5 (δ 144.3) unambiguously revealed a disubstituted dihydrofuranone moiety. The correlation of the olefinic proton H-6 (δ 6.49) with C-4 (δ 186.6), C-5 (δ 144.3), C-7 (δ 122.9), C-8 (δ 117.9) and C-12 (δ 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 (δ 6.49) and H-3 (δ 5.82) and the correlation between the protons H-8 (δ 7.35) and H-12 (δ 7.17) with the methyl protons H-13 (δ 2.39). Thus the structure was established as 2-(3,4-dihydroxybenzylidene)-5-methyfuran-3-one, named inotilone (**11**). Only recently, related 5-methyl-3(2*H*)-furanone metabolites have been reported from *Phellinus igniarius*.[11]

Table 1 Inhibitory activities of **4**, **5**, **7**, **9**, and **11** against 3-αHSD, COX-1, COX-2, and XO

	$IC_{50}/\mu M$				
Compound	3α-HSD	COX-1	COX-2	COX-2/COX-1	XO
4	8.1	0.01	8×10^{-4}	0.08	4.4
5	12.1	0.05	0.13	2.6	13.8
7	8.9	0.03	0.01	0.3	10.1
9	16.1	0.46	0.21	0.4	7.1
11	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 caffeyl-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**.[12] A related sequence could be involved in the formation of the tri- and tetrahydroxyaurone aglycones of sulfurein and cernuosides.[13,14]

All compounds were evaluated for their inhibitory activities in hydroxysteroid dehydrogenase (3α-HSD), COX-1, COX-2 and XO enzyme assays according to previously documented procedures. Their inhibitory potencies, expressed as IC_{50} 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.[3] In all cases, except for compound **11**, strong 3α-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 α-pyrone is more active than the γ-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 caffeyl 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 Spericord 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 ^1H and 75.475 MHz for ^{13}C

in DMSO-d6. 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_{18} (7 μm) column. TLC was carried out with silica gel 60 F_{254} plates. Spots were visualized by spraying with vanilline/H_2SO_4 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 $CHCl_3$–MeOH (9 : 1, 8 : 2, 1 : 1 *v*/*v*) as eluent. Final purification was achieved by preparative HPLC (Spherisorb ODS-2 RP_{18}, 5 μm (Promochem), 250 × 25 mm, acetonitrile–H_2O (83 : 17 *v*/*v*), at a flow rate of 10 ml min^{-1} 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 $CHCl_3$–MeOH 80 : 20 as eluent. Further purification was done by HPLC using gradient (water–acetonitrile 95 : 5 to 5 : 95; 30 min) R_t = 14 min; UV (MeOH) λ_{max} 248, 361 nm; IR (film) 3059, 1649, 1590, 1494, 1411, 1276, 1202, 1050, 1000 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) data see Table 2; ^{13}C NMR (DMSO-d_6, 75 MHz) data see Table 2; *m*/*z* 245 $[M - H]^-$; HR-EIMS (found $[M - H]^-$): 245.0464 calcd. for $C_{15}H_{15}O_6$: 245.0445).

Inonotic acid methyl ester (9). Was obtained as a yellow oil by open column chromatography on Sephadex LH 20 using $CHCl_3$–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_t = 20.5 min; UV (MeOH) λ_{max} 261, 380 nm; IR (film) 3094, 1733, 1632, 1567, 1513, 1435, 1282, 1022, 974 cm^{-1}; ^{1}HNMR (DMSO-d_6, 300 MHz) data see Table 2; ^{13}C NMR (DMSO-d_6, 75 MHz) data see Table 2; *m*/*z* 277 $[M - H]^-$; HR-EIMS (found $[M - H]^-$): 277.0682 calcd. for $C_{14}H_{13}O_6$: 277.0707).

Inotilone (11). Was obtained as a yellow oil by open column chromatography on Sephadex LH 20 using $CHCl_3$–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_t = 16 min; UV (MeOH) λ_{max} 264, 312, 378 nm; IR (film) 3184, 1682, 1588, 1435, 1287, 1014, 951 cm^{-1}; ^{1}HNMR (DMSO-d_6, 300 MHz) data see Table 2; ^{13}C NMR (DMSO-d_6, 75 MHz) data see Table 2; *m*/*z* 217 $[M - H]^-$; HR-EIMS (found $[M - H]^-$): 217.0495, calcd. for $C_{12}H_9O_4$: 217.0495).

Biological assays

The 3α-hydroxy steroid dehydrogenase assay (3-αHSD) was measured spectrophotometrically, and conducted according to the method described by Penning.[15] The inhibitory activities of the test compounds are indicated in terms of IC_{50}. 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.*[16] The inhibitory activities of the test compounds are given in terms of IC_{50}. 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.*[17] The inhibitory activities of the test compounds are indicated in terms of IC_{50}. Allopurinol was used as the reference.

Table 2 ^{1}H and ^{13}C NMR data[a] for compounds **5**, **9**, and **11**

	5		**9**		**11**	
N°	δ ^{1}H (*J*/Hz)	δ ^{13}C	δ ^{1}H (*J*/Hz)	δ ^{13}C	δ ^{1}H (*J*/Hz)	δ ^{13}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		

[a] Recorded in DMSO-d6.

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