

First total synthesis of antrocamphin A and its analogs as anti-inflammatory and anti-platelet aggregation agents†

Chia-Lin Lee,^{a,h} Chi-Huan Huang,^{‡a} Hui-Chun Wang,^a Da-Wei Chuang,^a Ming-Jung Wu,^b Sheng-Yang Wang,^c Tsong-Long Hwang,^d Chin-Chung Wu,^a Yeh-Long Chen,^e Fang-Rong Chang^{*a,f} and Yang-Chang Wu^{*a,g,h}

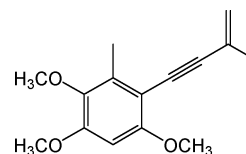
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Naturally occurring antrocamphin A (**1**) is a potent anti-inflammatory compound from the edible fungus *Antrodia camphorata* (*Taiwanofungus camphoratus*), whose wild fruiting body is used as a valuable folk medicine in Taiwan. This study is the first total synthesis of antrocamphin A (**1**) and its analogs. Their inhibition ability on NO release, superoxide anion generation, elastase release and platelet aggregation are reported herein.

Introduction

The endemic fungus *Antrodia camphorata* (*Taiwanofungus camphoratus*), also known as Niu-Chang-Chih (Jang-Jy), is used as a folk medicine and a dietary supplement in Taiwan. Its wild fruiting body is very valuable.^{1–3} The chemical composition of this fungus can be classified into three categories: 1). Polysaccharides, 2). Triterpenoids and 3). Enynyl-benzenoids. The polysaccharides are regarded as immunomodulation agents, such as functional foods derived from other fungi, e.g., *Ganoderma lucidum* and *Agaricus blazei*.¹ Triterpenoids are considered to have anti-cancer and anti-inflammatory effects. However, the major chemical component, antrocamphin A (**1**) (Fig. 1), which belongs to the third class, is the key component for anti-inflammatory activity. Evidence is

Fig. 1 Antrocamphin A (**1**).

shown below through a complete bioassay-guided fractionation study.^{3,4}

Antrocamphin A (**1**), a potent anti-inflammatory compound naturally found in *A. camphorata* (*T. camphoratus*) and was first isolated by Chen *et al.* in 2007.⁴ Wang *et al.* demonstrated its mechanism in suppressing pro-inflammatory molecules (NO and PGE₂), from being released *via* the down-regulation of iNOS and COX-2 expression through the NF-κB pathway.³ Previous studies indicate that compound **1** may serve as a promising lead drug for the treatment of various diseases that are induced by inflammation. Therefore, antrocamphin A was chosen to be a candidate of further investigation.

Currently, there are only two literature^{3,4} reports on the subject of antrocamphin A (**1**). To understand the diverse biological properties and the structure–activity relationship (SAR) of the lead compound **1**, the first total synthesis of compound **1** and its analogs were achieved in the current investigation. All compounds were evaluated for anti-inflammatory and anti-platelet aggregation activities.

Results and discussion

The retrosynthetic analysis of compound **1** is illustrated in Scheme 1. The key step was the Sonogashira reaction. Compound **2** (2-iodo-3,5,6-trimethoxytoluene) was coupled with 2-methyl-1-buten-3-yne (**3**), which led to the target compound. Compound **2** was synthesized from 2,3,5-trimethoxytoluene (**8**), obtained from *o*-vanillin (**5**), in four steps described by Singh and co-workers.⁵ We prepared 2-hydroxy-3-methoxytoluene (**4**) by hydrogenating of *o*-vanillin (**5**) over 10% palladium-on-carbon. Compound **4** was then converted to quinone **6** by treatment with the oxidant, potassium nitrosodisulfonate (Fremy's salt). Quinone **6** was reduced to hydroquinone **7** using TiCl₃ and then methylated with Me₂SO₄ in the presence of K₂CO₃ resulting in the desired compound **8**. Compound (**2**), 2-iodo-3,5,6-trimethoxytoluene, a key synthon,

^aGraduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung 807, Taiwan. E-mail: aaronfrc@kmu.edu.tw; Fax: +886 7 311 4773; Tel: +886 7 312 1101 ext. 2162

^bDepartment of Chemistry, National Sun Yat-sen University, Kaohsiung 804, Taiwan

^cDepartment of Forestry, National Chung-Hsing University, Taichung 402, Taiwan

^dGraduate Institute of Natural Products, Chang Gung University, Tao-Yuan 333, Taiwan

^eDepartment of Medicinal and Applied Chemistry, Kaohsiung Medical University, Kaohsiung 807, Taiwan

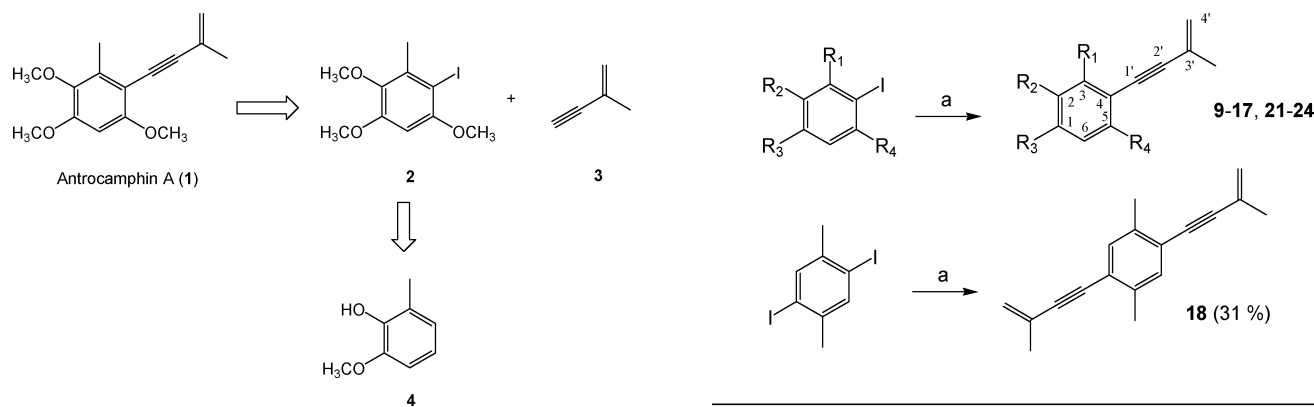
^fDepartment of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung 804, Taiwan

^gGraduate Institute of Integrated Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan. E-mail: yachwu@mail.cmu.edu.tw; Fax: +886 4 220 60248; Tel: +886 4 220 57153

^hNatural Medicinal Products Research Center, China Medical University Hospital, Taichung 40402, Taiwan

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‡ Equal contributions as first author.



Scheme 1 Retrosynthetic analysis of antrocamphin A.

was prepared *via* iodination of **8** using I_2 and CF_3COOAg . The Sonogashira reaction was utilized to couple iodo **2** with 2-methyl-1-buten-3-yne (**3**), using the catalysts $Pd(PPh_3)_4$ and CuI . This key reaction led to the desired compound, antrocamphin A (**1**) (Scheme 2).⁶ The Sonogashira coupling reaction was also applied to afford a series of designed analogues (**9–22**) from commercially available iodobenzene products or further iodination benzenoid compounds (Scheme 3).

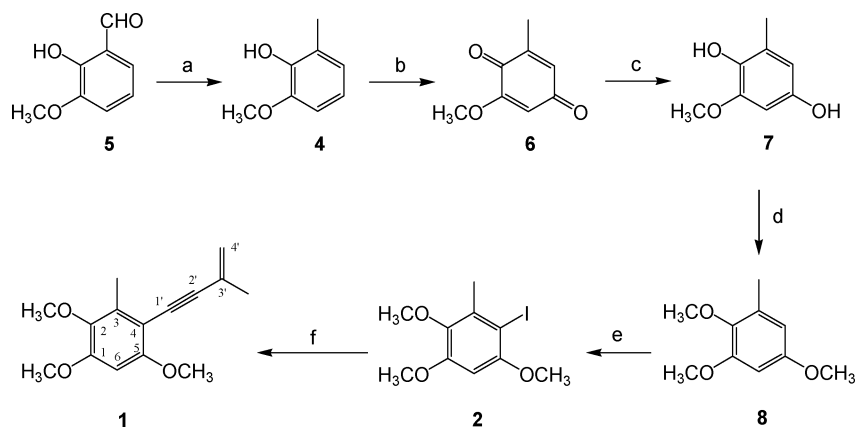
Synthesized compounds **1** and **9–22** were screened using a nitric oxide (NO) inhibitory assay, where curcumin was used as the positive control. In this study, all analogs were tested on their cytotoxicity toward RAW 264.7 macrophages. Cell viability rates for each test compound showed $> 90\%$ at the dosage of $20 \mu g mL^{-1}$. All compounds were evaluated against lipopolysaccharide (LPS)-induced NO production in RAW 264.7 macrophage cell line at the dosage of $20 \mu g mL^{-1}$. As shown in Table 1, compounds **1**, **20**, **21** and **22** showed potent anti-inflammatory activity with NO inhibition rates of 98.1%, 97%, 77%, 84%, respectively.

Compounds **1**, **9**, **15**, **17**, **20** and **21** were synthesized to study the SAR of the CH_3 substitution at C-3 presented in the natural product **1**; however, C3- CH_3 did not influence the ability of NO inhibition. To evaluate the SAR of the OCH_3 functions on the 3'-methyl-but-3'-en-1-ynyl-benzene skeleton, analogs **10–12**, **15** and

Compound	Substituent group				Yield (%)
	R ₁	R ₂	R ₃	R ₄	
9	H	H	H	H	93
10	H	H	OCH ₃	H	87
11	OCH ₃	H	H	H	73
12	H	OCH ₃	H	H	55
13	H	H	CH ₃	H	89
14	H	H	CN	H	83
15	H	OCH ₃	OCH ₃	OCH ₃	81
16	H	H	NO ₂	H	83
17	CH ₃	H	H	H	77
19	CH ₃	OCH ₃	OCH ₃	H	73
20	H	H	OCH ₃	OCH ₃	63
21	CH ₃	H	OCH ₃	OCH ₃	17
22	CH ₃	I	OCH ₃	OCH ₃	13

Scheme 3 Synthesis of **9–22**. Reagents and conditions: (a) 2-methyl-1-buten-3-yne (**3**), $Pd(PPh_3)_4$, CuI , Et_3N/THF , N_2 , rt.

20 were designed, which did not have the C3- CH_3 substitution. Compounds **10–12** possess only one OCH_3 group at C-1, C-3 and C-2, respectively. Moreover, **20** and **15** have two and three OCH_3 groups at C-1 and C-5, and C-1, C-2 and C-5, respectively. Among the five compounds, we speculated that the position is more important than the number of OCH_3 substitutions. A similar result was also observed in the test of compounds **19** and **21**, which both have a CH_3 group at C-3 and the same number of OCH_3 substitutions in different positions. Furthermore, a comparison



Scheme 2 Synthetic scheme of antrocamphin A (**1**). Reagents and conditions: (a) 10% Pd/C , H_2 , rt, 75%; (b) KH_2PO_4 , $(KSO_3)_2NO$, rt, 78%; (c) $TiCl_3$, rt, 90%; (d) $(CH_3O)_2SO_2$, K_2CO_3 , acetone, reflux, 88%; (e) I_2 , CF_3COOAg , CH_2Cl_2 , 0 °C, 74%; (f) 2-methyl-1-buten-3-yne (**3**), $Pd(PPh_3)_4$, CuI , Et_3N/THF , N_2 , rt, 10%.

Table 1 Effects of all compounds on NO in LPS-challenged RAW 264.7 cells^a

Compound ^b	NO inhibition (%)	Cell viability (%)
1	98.10	99.71
9	17.23	101.55
10	33.56	101.98
11	-4.43	90.44
12	27.27	105.80
13	23.11	100.95
14	-0.55	91.77
15	24.98	101.91
16	15.16	94.97
17	6.23	99.73
18	16.61	92.23
19	28.51	99.07
20	97.00	117.08
21	77.00	114.00
22	84.00	102.00
Curcumin ^c	99.00	103.00

^a All compounds were administrated 1 h before inflammation induction by adding LPS (1 $\mu\text{g mL}^{-1}$). ^b Dosage of test compound was 20 $\mu\text{g mL}^{-1}$. ^c Positive control.

with the structures of **1** and **20–22** concluded that the simultaneous existence of OCH₃ groups at C-1 and C-5 may be necessary for the potential ability of NO inhibition.

LPS-challenged RAW 264.7 macrophages are an *in vitro* model of murine cell lines for studying anti-inflammatory effects. To further explore the anti-inflammatory activities of these target compounds in human cell systems with different modes of action, all analogs were evaluated against superoxide anion generation and elastase release by human neutrophils in response to *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)/cytochalasin B (CB). Antrocamphin A has been reported as a new anti-inflammatory drug lead against superoxide anion generation in FMLP-activated human neutrophils.⁴ Activated neutrophils produced high concentrations of the superoxide anion and elastase known to be involved in airway mucus hypersecretion, which is a neutrophilic inflammatory disease like asthma.⁷ Therefore, all analogues were tested for their ability to inhibit superoxide anion generation and elastase release. Diphenyleneiodonium (DPI) and phenylmethylsulfonyl fluoride (PMSF) were used as positive controls in this model assay, respectively.

Interestingly, the analogs showed a broad-spectrum of activity in this model (Table 2). Most of the compounds could inhibit both inflammatory mediators and showed more potent effects than the natural product **1**. Compound **18** with a dienynyl functionality exhibited the best activity, which indicated the enynyl substitution played an important role for this model assay. On the whole, we found dimethoxy (**19–22**) and monomethoxy (**10–12**) derivatives were more favorable than trimethoxy and non-methoxy ones. Some of the compounds showed superior activities to the reference drug, PMSF, against elastase release by neutrophils.

Furthermore, all synthesized derivatives were also subjected to an anti-platelet aggregation assay with collagen and thrombin as inducers; aspirin was used as a positive control. As shown in Table 3, compounds **9**, **10**, **11** and **19** showed potent inhibition against collagen-induced platelet aggregation with IC₅₀ values of 3.36, 1.16, 7.55 and 7.22 $\mu\text{g mL}^{-1}$, respectively; their activities were better than aspirin's activity (IC₅₀ 13.58 $\mu\text{g mL}^{-1}$). Comparing the

Table 2 Effects of all compounds on superoxide anion generation and elastase release by human neutrophils in response to FMLP/CB

Compound	Superoxide		Elastase	
	IC ₅₀ / $\mu\text{g mL}^{-1a}$	or (Inh %)	IC ₅₀ / $\mu\text{g mL}^{-1a}$	or (Inh %)
1	(46.30 \pm 7.24)	***	(25.55 \pm 3.99)	**
9	4.82 \pm 0.41		(37.22 \pm 3.12)	***
10	5.63 \pm 0.71		4.14 \pm 0.35	
11	6.69 \pm 0.84		7.94 \pm 0.51	
12	4.05 \pm 0.41		3.31 \pm 0.06	
13	4.66 \pm 0.41		44.28 \pm 1.67	***
14	(7.95 \pm 6.06)		(17.89 \pm 1.42)	***
15	3.40 \pm 1.49		(26.74 \pm 5.88)	*
16	(-10.03 \pm 4.84)		22.78 \pm 1.01	***
17	(25.55 \pm 6.70)	*	(35.55 \pm 6.12)	**
18	0.45 \pm 0.03		1.32 \pm 0.67	
19	1.96 \pm 0.28		3.47 \pm 0.23	
20	4.69 \pm 0.99		8.72 \pm 1.15	
21	1.60 \pm 0.28		5.33 \pm 1.21	
22	(12.13 \pm 2.24)	**	(81.36 \pm 8.82)	
DPI ^b	0.22 \pm 0.13			
PMSF ^b			22.80 \pm 5.07	

Per cent of inhibition (Inh%) at a 10- $\mu\text{g mL}^{-1}$ concentration. Results are presented as mean \pm S.E.M. (n = 3). **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared with the control value.^a Concentration necessary for 50% inhibition (IC₅₀). ^b Diphenyleneiodonium (DPI) and phenylmethylsulfonyl fluoride (PMSF) were used as positive controls for superoxide anion generation and elastase release, respectively.

Table 3 Anti-platelet aggregation data of all compounds

Compound	IC ₅₀ / $\mu\text{g mL}^{-1}$	
	Collagen (10 $\mu\text{g mL}^{-1}$)	Thrombin (0.05 U/mL)
1	> 50	> 50
9	3.36	40.83
10	1.16	44.06
11	7.55	> 50
12	> 50	> 50
13	> 50	> 50
14	12.14	36.09
15	15.43	> 50
16	> 50	> 50
17	> 50	> 50
18	14.50	12.92
19	7.22	> 50
20	> 50	> 50
21	> 50	> 50
22	> 50	> 50
Aspirin ^a	13.58	> 200

^a Positive control.

bioassay results, most of the compounds did not possess good inhibition toward the platelet aggregation induced by thrombin. No clear SAR can be concluded from the anti-platelet aggregation effect. Interestingly, compounds **1** and **20–22**, which possess the powerful ability of NO inhibition, did not have anti-platelet aggregation activity.

Conclusions

In summary, we have achieved the first total synthesis of the active natural product antrocamphin A (**1**), in a total yield

of 3.7% in six steps. Moreover, 14 analogs, including new compounds, **15**, **16** and **18–22**, were synthesized. Most of these compounds possess OMe groups in the benzene ring. We tried to synthesize derivatives with different substitutions around the ring, for example, 2-iodo-3,6-diacetoxy-5-methoxytoluene or 4-iodo-3,6-dihydroxy-5-methoxytoluene coupled with 2-methyl-1-buten-3-yne using different combinations of catalyst [Pd(PPh₃)₄ or PdCl₂(PPh₃)₂], solvent (THF or DMF or ether) and base (Et₃N). Unfortunately, this resulted in no reaction or reaction without any target compound. Accordingly, only the reagents with OMe groups, which were easily obtained, were chosen to evaluate the amount and position of OMe groups related to the activity in this investigation.

Comparing anti-inflammatory activity, compounds **1**, **20**, **21** and **22** were identified to be potent NO inhibition agents; the dienynyl compound **18** was a new drug lead against superoxide anion generation and elastase release. Moreover, analog **10** is the most potential compound against platelet aggregation induced by collagen. Overall, our data demonstrate that enynyl-benzenoids may have a chance to be developed as safe and potential anti-inflammatory or a cardiovascular protecting agent in the future.

Acknowledgements

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