Prenylflavonoids and Phloroglucinol Derivatives from Hops (*Humulus lupulus*)

Feng Zhao,[†,‡] Yuki Watanabe,[‡] Hajime Nozawa,[†] Akihiro Daikonnya,[‡] Keiji Kondo,[†] and Susumu Kitanaka*[,‡]

Central Laboratories for Key Technology, Kirin Brewery Co., Ltd., 1-13-5 Fukuura, Kanazawa-ku, Yokohama-shi, Kanagawa 236-0004, Japan, and College of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi, Chiba 274-8555, Japan

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The ethyl acetate soluble fraction of hops (*Humulus lupulus*) showed potent inhibitory activity on the production of nitric oxide (NO) induced by a combination of LPS and IFN-γ. Four known prenylflavonoids (**1**−**4**) and a new prenylflavonoid (**5**), hulupinic acid (**6**), lupulone (**7**), and its six new derivatives (**8**−**13**) were isolated from the active fraction. The structures were determined on the basis of physiochemical properties and spectroscopic analysis. Their inhibitory activities on the production of NO in macrophage RAW 264.7 cells were examined.

The hop plant (*Humulus lupulus* L., Cannabinaceae) is used in folk medicine as a tranquilizer or bitter stomachic. The female inflorescences, hop cones, are widely used in the brewing industry to add bitterness and aroma to beer. Recently, some flavanones and chalcones with prenyl or geranyl groups have been identified in hops and beers,[1−5] and their biological activities such as the inhibition of bone resorption,[6] inhibition of diacylglycerol acyltransferase,[7] and antimicrobial activities[8] have been discussed. Our previous study has demonstrated the cancer preventive effects of beer consumption on azoxymethane-induced rat colonic carcinogenesis.[9] We then focused on the evaluation of cancer preventive effects of beer components by using in vitro studies and found that the inhibitory activity of hops extract on NO production is much stronger than that of beer or malt. Therefore, we have been studying the chemical constituents of hops in an effort to identify the NO production inhibitory agents and found that the active compounds were extracted into the ethyl acetate soluble fraction.[10] In the present study, we describe the isolation and structural determination of 13 compounds from the active fraction. Of these compounds, one prenylchalcone (**5**) and six phloroglucinol derivatives (**8**−**13**) are reported as new natural products. We also report on the inhibition of NO production by these compounds.

Results and Discussion

Hops CAS pellet was extracted as described in the Experimental Section. Through bioactivity-guided fractionation, several NO production inhibitors were purified from the ethyl acetate fraction of hops. Compounds **1**−**5** were characterized as chalcones. By comparison with the previously published data,[1,11,12] compounds **1**−**4** and **7** were identified as xanthohumol (**1**), xanthohumol D (**2**), dihydroxanthohumol (**3**), xanthohumol B (**4**), and lupulone (**7**), respectively. The molecular formula of compound **5** was established as $C_{22}H_{26}O_7$ (*m/z* 402.16714 $[M]^+$) by HREIMS. Its chemical structure was identified from the HMBC and HMQC data and by comparison with ^1H and ^{13}C shift data for xanthohumol. Long-range correlations from H-1″ (δ 2.79, 2.47) of the substituted prenyl group to C-2′ (δ 163.3), C-3′ (δ 106.4), and C-4′ (δ 164.9) were observed in the HMBC spectrum, which provided evidence for the C-3′ attachment of the substituted prenyl moiety to the chalcone. Long-range correlation from the 3″-OCH_3 protons (δ 3.16) to C-3″ (δ 76.8) provided evidence for the substitution of methoxy at C-3″ of the prenyl group. Although compound **5** was identified to be a new component from hops, it is likely to be an oxidation product of xanthohumol at the double-bond position of the prenyl group.

The molecular formula of compound **6** was established as $C_{15}H_{20}O_4$ (*m/z* 264.13588 $[M]^+$) by HREIMS. The ^{13}C NMR spectrum exhibited only eight carbon signals, which indicated the possibility of a symmetrical structure. Analysis of the DEPT spectrum showed the presence of two methyl, one methylene, one methine, and four quaternary carbon signals. Extensive analysis of the ^1H and ^{13}C NMR spectra together with HMBC spectra indicated the presence of two prenyl (3-methylbut-2-enyl) groups in a symmetrical environment. Long-range correlations in the prenyl group were observed in the HMBC spectrum between the following: H-5′ (δ 1.54) to C-2′ (δ 118.9), C-3′ (δ 136.6), and C-4′ (δ 26.1); H-4′ (δ 1.59) to C-2′ (δ 118.9), C-3′ (δ 136.6), and C-5′ (δ 17.8); H-2′ (δ 4.81) to C-1′ (δ 33.5), C-4′ (δ 26.1), and C-5′ (δ 17.8); H-1′ (δ 2.31) to C-2′ (δ 118.9), C-3′ (δ 136.6), and C-4′ (δ 26.1, weak). The connections of prenyl groups were confirmed by the observation of long-range couplings from H-1′ (δ 2.31) to C-1 (δ 200.0, C=O), C-2 (δ 55.4), and C-1″ (δ 33.5, for another prenyl group). Compound **6** is thus hulupinic acid.[13]

Compounds **8**−**13** were identified as new derivatives of lupulone (**7**), a main constituent from hops. The molecular formula of compound **8** was determined as $C_{26}H_{36}O_4$ (*m/z* 412.26134 $[M]^+$) by HREIMS. In the IR spectrum, absorption bands attributable to carboxyl (C=O, 1660 cm^{-1}) and hydroxyl (−OH, 3447 cm^{-1}) groups were observed. The ^{13}C NMR spectrum exhibited 26 carbon signals, which were classified into eight methyl, three methylene, and five methine groups and 10 quaternary carbons by analysis of the DEPT spectra. The ^1H NMR spectrum displayed 17 proton signals. The connectivity of proton and carbon atoms was confirmed by the HMQC spectrum. Extensive analysis of the ^1H and ^{13}C NMR spectra together with HMBC spectra indicated the presence of one 3-methylbutyryl and two prenyl (3-methylbut-2-enyl) groups. Long-range correlations were observed in the HMBC spectrum between the following: 4a-(3-methylbut-2-enyl) [H-4′ (δ 1.47) to C-2′ (δ 117.3), C-3′ (δ 136.8), and C-5′ (δ 25.9); H-5′ (δ 1.62) to C-2′ (δ 117.3), C-3′ (δ 136.8), and C-4′ (δ 17.7); H-2′ (δ 4.89) to C-1′ (δ 44.5), C-4′ (δ 17.7), and C-5′ (δ 25.9); H-1′ (δ 2.37

* To whom correspondence should be addressed. Tel: 81-47-4655356. Fax: 81-47-4655440. E-mail: kitanaka@pha.nihon-u.ac.jp.
† Kirin Brewery Co.
‡ Nihon University.

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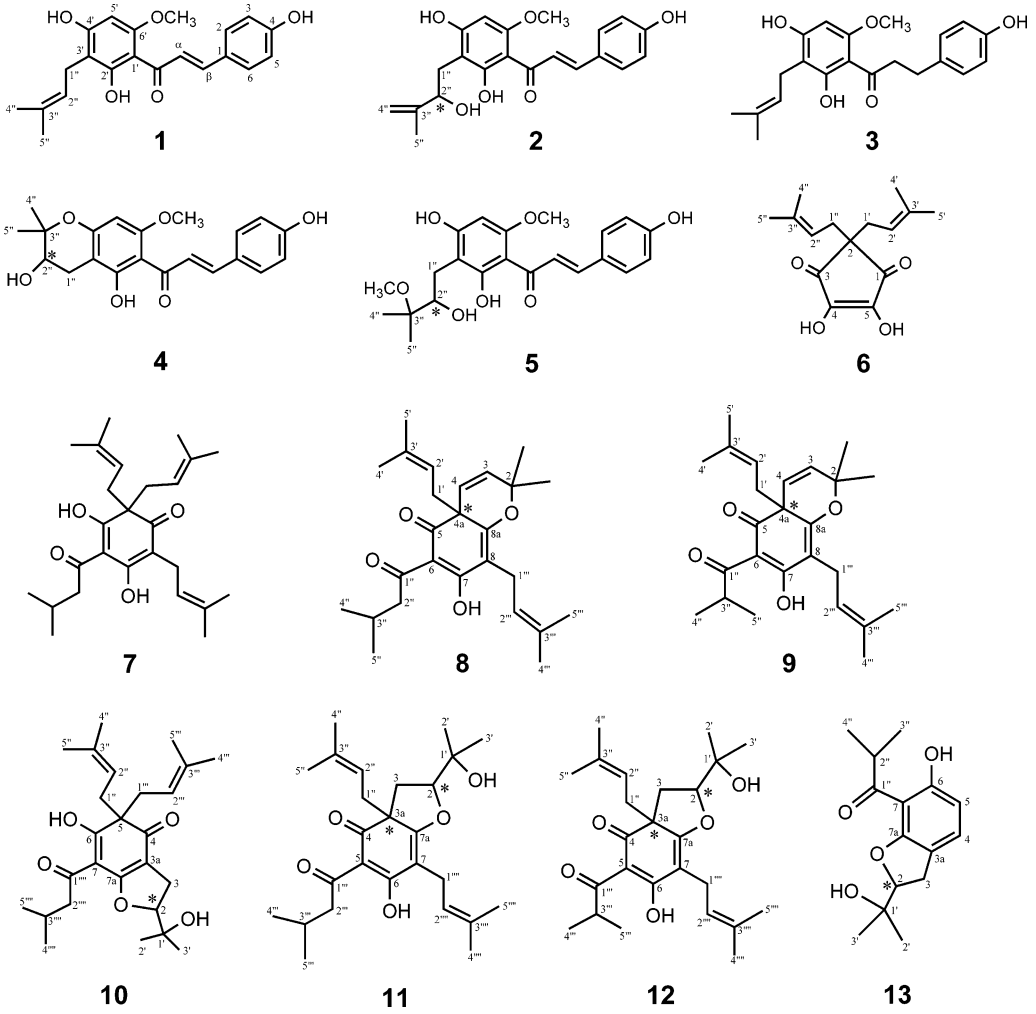
Chart 1



2.56) to C-2′ (δ 117.3) and C-3′ (δ 136.8)], 6-(3-methylbutyryl) [H-4″ (δ 0.97) to C-2″ (δ 48.0) and C-3″ (δ 25.7); H-5″ (δ 0.99) to C-2″ (δ 48.0) and C-3″ (δ 25.7); H-2″ (δ 2.88) to C-1″ (δ 202.4), C-4″ (δ 22.7), and C-5″ (δ 22.8)], 8-(3-methylbut-2-enyl) [H-4‴ (δ 1.75) to C-2‴ (δ 121.9), C-3‴ (δ 131.7), and C-5‴ (δ 25.7); H-5‴ (δ 1.68) to C-2‴ (δ 121.9), C-3‴ (δ 131.7), and C-4‴ (δ 17.9); H-2‴ (δ 5.13) to C-1‴ (δ 21.2), C-4‴ (δ 17.9), and C-5‴ (δ 25.7); H-1‴ (δ 3.07, 3.15) to C-2‴ (δ 121.9) and C-3‴ (δ 131.7)]. As shown in Figure 1, the partial structure A is supported by observation of long-range couplings from H-1′ (δ 2.37, 2.56) to C-4a (δ 52.7), C-5 (δ 193.6), C-4 (δ 123.4), and C-8a (δ 167.8) in the HMBC spectrum. The partial structure B is supported by observation of long-range couplings from H-3 (δ 5.75) to C-2 (δ 82.2) and C-4a (δ 52.7) and from H-4 (δ 6.24) to C-4a (δ 52.7) and C-8a (δ 167.8). The partial structure C is supported by observation of long-range couplings from H-2″ (δ 2.88) to C-6 (δ 115.5). The partial structure D is supported by observation of long-range couplings from H-1‴ (δ 3.07, 3.15) to C-7 (δ 190.1), C-8 (δ 108.5), and C-8a (δ 167.8). The two partial structures C and D could be connected by observation of long-range couplings from 7-OH (δ 19.04) to C-6 (δ 115.5) and C-1″ (δ 202.4). The connection of partial structures A, B, and D could be confirmed by the presence of the same carbon atoms C-4, C-4a, and C-8a in different partial structures. Because of the ambiguities whether the attachment of the heterocyclic ring should be reversed, an additional NOESY experiment was performed. Correlations were observed between H-4 (δ 6.24) and H-1′ (δ 2.37 2.56), which confirmed the structure of compound **8** (4a-C−, 8a-O−) and eliminated the alternative substructure (4a-O−, 8a-C−).

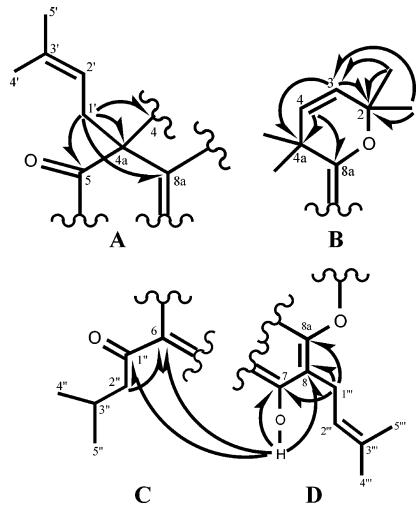


Figure 1. HMBC correlations of compound **8**.

The molecular formula of compound **9** was determined as $C_{25}H_{34}O_4$ (*m/z* 398.24569 $[M]^+$) by HREIMS. The ^{13}C NMR spectrum showed 25 carbon signals, containing eight methyl, two methylene (of which three were in compound **8**), five methine groups, and 10 quaternary carbons. The ^1H NMR spectrum displayed 16 proton signals, one proton less than that of compound **8**. The UV, IR, and ^1H and ^{13}C

Table 1. ^{1}H NMR Data of Compounds **1**−**5** (in DMSO-d_6, 400 MHz)[a]

position	**1**	**2**	**3**	**4**	**5**
α	7.77 d (15.1)	7.78 d (15.5)	3.17 t (8.1)	7.77 d (17.4)	7.75 d (15.1)
β	7.68 d (15.1)	7.65 d (15.5)	2.76 t (8.1)	7.69 d (17.4)	7.67 d (15.1)
2,6	7.57 d (8.8)	7.57 d (8.6)	7.01 d (8.3)	7.58 d (8.8)	7.57 d (8.8)
3,5	6.84 d (8.8)	6.84 d (8.6)	6.66 d (8.5)	6.84 d (8.8)	6.84 d (8.8)
5′	6.09 s	6.06 s	6.05 s	6.01 s	6.09 s
6′-OCH_3	3.87 s	3.87 s	3.79 s	3.88 s	3.88 s
1″	3.14 d (7.2)	2.75 dd (13.4, 6.7)	3.12 d (7.1)	2.71 dd (16.5, 5.2)	2.79 dd (13.7, 2.2)
		2.65 dd (13.4, 6.7)		2.39 dd (16.5, 5.2)	2.47 dd (13.7, 9.6)
2″	5.14 t (7.2)	4.22 t (6.6)	5.11 t (7.1)	2.50 m	3.60 dd (9.6, 2.2)
3″-OCH_3					3.16 s
4″	1.61 s	4.65 s	1.60 s	1.30 s	1.12 s
		4.61 s			
5″	1.70 s	1.73 s	1.69 s	1.23 s	1.11 s
2′-OH	14.64 s	14.65 s	14.18 s	14.76 s	14.60 s
4′-OH	10.56 s	10.52 s	10.51 s		10.47 s
4-OH	10.06 s	10.03 s	9.12 s	10.06 s	10.05 s

[a] Chemical shifts δ_H mult. (*J* in Hz).

Table 2. ^{13}C NMR Data of Compounds **1**−**5** (in DMSO-d_6, 100 MHz)[a]

position	**1**		**2**		**3**		**4**		**5**	
C=O	191.6	(C)	191.6	(C)	204.1	(C)	191.8	(C)	191.7	(C)
α	123.7	(CH)	123.8	(CH)	45.5	(CH_2)	123.5	(CH)	123.8	(CH)
β	142.4	(CH)	142.8	(CH)	29.5	(CH_2)	142.9	(CH)	142.5	(CH)
1	125.9	(C)	126.0	(C)	131.4	(C)	125.9	(C)	126.0	(C)
2,6	130.4	(CH)	130.4	(CH)	129.0	(CH)	130.5	(CH)	130.4	(CH)
3,5	115.9	(CH)	115.9	(CH)	115.0	(CH)	115.9	(CH)	115.9	(CH)
4	159.8	(C)	159.8	(C)	155.3	(C)	159.7	(C)	159.9	(C)
1′	104.5	(C)	104.5	(C)	104.0	(C)	100.4	(C)	104.8	(C)
2′	164.5	(C)	165.1	(C)	163.8	(C)	164.7	(C)	163.3	(C)
3′	107.2	(C)	105.1	(C)	107.1	(C)	104.9	(C)	106.4	(C)
4′	162.3	(C)	163.2	(C)	162.2	(C)	160.5	(C)	164.9	(C)
5′	90.9	(CH)	91.0	(CH)	90.6	(CH)	91.7	(CH)	91.4	(CH)
6′	160.4	(C)	160.6	(C)	160.7	(C)	160.0	(C)	160.6	(C)
6′-OCH_3	55.7	(CH_3)	55.6	(CH_3)	55.5	(CH_3)	56.0	(CH_3)	55.8	(CH_3)
1″	20.9	(CH_2)	28.8	(CH_2)	20.9	(CH_2)	25.0	(CH_2)	24.3	(CH_2)
2″	122.9	(CH)	73.6	(CH)	122.9	(CH)	67.3	(CH)	74.9	(CH)
3″	129.8	(C)	148.0	(C)	129.8	(C)	78.7	(C)	76.8	(C)
3″-OCH_3									48.7	(CH_3)
4″	25.4	(CH_3)	109.7	(CH_2)	25.4	(CH_3)	25.3	(CH_3)	21.3	(CH_3)
5″	17.6	(CH_3)	17.3	(CH_3)	17.5	(CH_3)	20.9	(CH_3)	20.1	(CH_3)

[a] Chemical shifts δ_C (mult.).

NMR spectra of compound **9** were nearly identical to those of compound **8** (Table 3), except for the loss of H-2″ and C-2″ signals and chemical shifts of signals for 1″, 3″, 4″, and 5″ observed in the ^{1}H and ^{13}C NMR spectra. Extensive analysis of the ^{1}H and ^{13}C NMR spectra together with HMQC and HMBC data indicated that it has similar partial structures A, B, and D. The partial structure F (Figure 2, right) is supported by observation of long-range couplings from H-4″ (*δ* 1.11) to C-1″ (*δ* 207.3) and C-3″ (*δ* 35.3); from H-5″ (*δ* 1.18) to C-1″ (*δ* 207.3) and C-3″ (*δ* 35.3); and from H-3″ (*δ* 3.89) to C-1″ (*δ* 207.3), C-4″ (*δ* 18.5), and C-5″ (*δ* 19.3). In the NOESY experiment, correlations were observed between H-4 (*δ* 6.25) and H-1′ (*δ* 2.38 2.56), which confirmed that the attachment of the heterocyclic ring in compound **9** should not be reversed.

The molecular formula of compound **11** was determined as $C_{26}H_{38}O_5$ (*m/z* 430.27167 $[M]^+$) by HREIMS. The ^{13}C NMR spectrum showed 26 carbon signals, which were classified into eight methyl, four methylene, and four methine groups and 10 quaternary carbons by analysis of the DEPT spectra. The ^{1}H NMR spectrum displayed 17 proton signals. The connectivity of proton and carbon atoms was confirmed by the HMQC spectrum. Extensive analysis of the ^{1}H and ^{13}C NMR spectra together with HMBC spectra indicated the presence of one 3-methylbutyryl and two prenyl (3-methylbut-2-enyl) groups. Long-range correlations were observed in the HMBC spectrum between the following: 3a-(3-methylbut-2-enyl) [H-4″ (*δ* 1.72) to C-2″ (*δ* 117.1), C-3″ (*δ* 137.0), and C-5″ (*δ* 25.9); H-5″ (*δ* 1.68) to C-2″ (*δ* 117.1), C-3″ (*δ* 137.0), and C-4″ (*δ* 17.8); H-2″ (*δ* 5.01) to C-1″ (*δ* 38.7), C-4″ (*δ* 17.8), and C-5″ (*δ* 25.9); H-1″ (*δ* 2.42 2.53) to C-2″ (*δ* 117.1) and C-3″ (*δ* 137.0)], 5-(3-methylbutyryl) [H-4‴ (*δ* 1.00) to C-2‴ (*δ* 46.1) and C-3‴ (*δ* 26.6); H-5‴ (*δ* 0.96) to C-2‴ (*δ* 46.1) and C-3‴ (*δ* 26.6); H-2‴ (*δ* 2.77, 2.71) to C-1‴ (*δ* 198.6), C-4‴ (*δ* 22.9), and C-5‴ (*δ* 22.5)], 7-(3-methylbut-2-enyl) [H-4⁗ (*δ* 1.54) to C-2⁗ (*δ* 121.6), C-3⁗ (*δ* 132.1), and C-5⁗ (*δ* 25.7); H-5⁗ (*δ* 1.58) to C-2⁗ (*δ* 121.6), C-3⁗ (*δ* 132.1), and C-4⁗ (*δ* 17.9); H-2⁗ (*δ* 5.11) to C-1⁗ (*δ* 21.5), C-4⁗ (*δ* 17.9), and C-5⁗ (*δ* 25.7); H-1⁗ (*δ* 3.09, 3.00) to C-2⁗ (*δ* 121.6) and C-3⁗ (*δ* 132.1)]. Further analysis of the HMBC spectra indicated that it has the partial structures A, C, and D similar to those of compound **8**. The partial structure E (Figure 2, left) is supported by observation of long-range couplings from H-2′ (*δ* 1.15) to C-1′ (*δ* 71.2), C-3′ (*δ* 26.8), and C-2 (*δ* 90.4); H-3′ (*δ* 1.33) to C-1′ (*δ* 71.2), C-2′ (*δ* 23.7), and C-2 (*δ* 90.4); H-2 (*δ* 4.51) to C-3′ (*δ* 26.8); and H-3 (*δ* 2.18, 2.11) to C-1′ (*δ* 71.2), C-2 (*δ* 90.4), C-3a (*δ* 60.5), C-7a (*δ* 174.7), C-4 (*δ* 195.8), and C-1″ (*δ* 38.7). The connection of partial structures A, C, D, and E could be confirmed by the observations of the long-range couplings from 6-OH (*δ* 18.90) to C-6 (*δ* 192.7), C-7 (*δ* 106.8), and C-1″ (*δ* 38.7) in further analysis. Because of the ambiguities whether the attachment of the heterocyclic ring should be reversed, an additional NOESY experiment was performed. Correlations were observed between H-3 (*δ* 2.18 2.11) and H-1″ (*δ* 2.42,

Table 3. ^{13}C and ^{1}H NMR Data of Compounds **8** and **9** (in $CDCl_3$)

position	**8** δ_C (mult.)		**8** δ_H mult. (*J*)	**9** δ_C (mult.)		**9** δ_H mult. (*J*)
2	82.2	(C)		82.2	(C)	
3	131.6	(CH)	5.75 d (8.2)	131.6	(CH)	5.76 d (8.2)
4	123.4	(CH)	6.24 d (8.2)	123.3	(CH)	6.25 d (8.2)
4a	52.7	(C)		52.8	(C)	
5 C=O	193.6	(C)		193.4	(C)	
6	115.5	(C)		115.3	(C)	
7	190.1	(C)	19.04 s (−OH)	190.2	(C)	19.10 s (−OH)
8	108.5	(C)		107.1	(C)	
8a	167.8	(C)		167.9	(C)	
2-methyl a	28.7	(CH_3)	1.32 s	28.7	(CH_3)	1.32 s
2-methyl b	29.5	(CH_3)	1.55 s	29.5	(CH_3)	1.55 s
4a-(3-methylbut-2-enyl)						
1′	44.5	(CH_2)	2.37 dd (13.2, 8.3) 2.56 dd (13.2, 8.3)	44.5	(CH_2)	2.38 dd (13.2, 8.3) 2.56 dd (13.2, 8.3)
2′	117.3	(CH)	4.89 t (8.3)	117.4	(CH)	4.91 t (8.3)
3′	136.8	(C)		131.7	(C)	
4′	17.7	(CH_3)	1.47 s	17.7	(CH_3)	1.48 s
5′	25.9	(CH_3)	1.62 s	25.9	(CH_3)	1.62 s
6-(3-methylbutyryl)						
1″C=O	202.4	(C)		207.3	(C)	
2″	48.0	(CH_2)	2.88 m			
3″	25.7	(CH)	2.17 m	35.3	(CH)	3.89 m
4″	22.7	(CH_3)	0.97 d (6.9)	18.5	(CH_3)	1.11 d (6.3)
5″	22.8	(CH_3)	0.99 d (6.4)	19.3	(CH_3)	1.18 d (6.3)
8-(3-methylbut-2-enyl)						
1‴	21.2	(CH_2)	3.07 dd (6.8, 13.7) 3.15 dd (7.3, 14.2)	21.2	(CH_2)	3.08 dd (6.8, 14.2) 3.15 dd (7.3, 14.2)
2‴	121.9	(CH)	5.13 t	121.9	(CH)	5.08 t
3‴	131.7	(C)		131.0	(C)	
4‴	17.9	(CH_3)	1.75 s	17.9	(CH_3)	1.74 s
5‴	25.7	(CH_3)	1.68 s	25.7	(CH_3)	1.62 s

E **F**

Figure 2. HMBC correlations of compounds **9** and **11**.

2.53), which confirmed the structure of compound **11** (3a-C−, 7a-O−) and eliminated the alternative substructure (3a-O−, 7a-C−).

The molecular formula of compound **12** was determined as $C_{25}H_{36}O_5$ (*m/z* 416.25663 $[M]^+$) by HREIMS. The ^{13}C NMR spectrum showed 25 carbon signals, containing eight methyl, three methylene (compared to four in compound **11**), four methine, and 10 quaternary carbons. The ^{1}H NMR spectrum displayed 16 proton signals, one proton less than that of compound **11**. The ^{1}H and ^{13}C NMR spectra of compound **12** were nearly identical to those of compound **11** (Table 5), except for the loss of H-2‴ and C-2‴ signals and chemical shifts of signals for 1‴, 3‴, 4‴, and 5‴ observed in the ^{1}H and ^{13}C NMR spectra. Extensive analysis of the ^{1}H and ^{13}C NMR spectra together with HMQC and HMBC data indicated that it has similar partial structures A, D, E, and F. In the NOESY experiment, correlations were observed between H-3 (δ 2.20, 2.13) and H-1″ (δ 2.53, 2.54), which confirmed that the attachment of the heterocyclic ring in compound **12** should not be reversed. By the same method, the structures of compounds **10** and **13** were determined. Long-range correlations were observed in the HMBC spectrum of compound **13** between the following: H-3 (δ 3.06 3.15) to C-2 (δ 91.5), C-3a (δ 117.5), C-7a (δ 160.0), and C-1′ (δ 71.6);

Table 4. ^{13}C and ^{1}H NMR Data of Compound **10** (in $CDCl_3$)

position	δ_C (mult.)		δ_H mult. (*J*)
2	92.3	(CH)	4.73 dd
3	26.7	(CH_2)	2.93 dd (10.3, 15.0) 2.80 dd (8.1, 15.0)
3a	109.4	(C)	
4 C=O	205.8	(C)	
5	61.9	(C)	
6	191.9	(C)	18.03 s (−OH)
7	103.9	(C)	
7a	169.5	(C)	
2-(1-hydroxyl-1-methyl)ethyl			
1′	71.9	(C)	
2′	24.3	(CH_3)	1.24 s
3′	25.2	(CH_3)	1.30 s
5-(3-methylbut-2-enyl) a			
1″	37.7	(CH_2)	2.67 d
2″	117.9	(CH)	4.76 t
3″	134.8	(C)	
4″	17.9	(CH_3)	1.56 s
5″	17.8	(CH_3)	1.52 s
5-(3-methylbut-2-enyl) b			
1‴	39.1	(CH_2)	2.62 d
2‴	118.2	(CH)	4.79 t
3‴	134.8	(C)	
4‴	17.9	(CH_3)	1.56 s
5‴	17.8	(CH_3)	1.52 s
7-(3-methylbutyryl)			
1⁗C=O	192.3	(C)	
2⁗	45.8	(CH_2)	2.75 d (7.3)
3⁗	27.7	(CH)	2.13 m
4⁗	22.6	(CH_3)	0.98 d (6.6)
5⁗	22.5	(CH_3)	0.98 d (6.6)

H-4 (δ 7.20) to C-3 (δ 29.3), C-7a (δ 160.0), and C-6 (δ 163.2); H-5 (δ 6.45) to C-3a (δ 117.5), C-6 (δ 163.2, weak), and C-7 (δ 106.0); 6-OH (δ 12.85) to C-5 (δ 109.7), C-6 (δ 163.2), and C-7 (δ 106.0); H-2′ (δ 1.27) to C-1′ (δ 71.6), C-3′ (δ 26.2), and C-2 (δ 91.5); H-3′ (δ 1.38) to C-1′ (δ 71.6), C-2′ (δ 24.7), and C-2 (δ 91.5); H-3″ (δ 1.20) to C-4″ (δ 19.1) and C-2″ (δ 39.3); H-4″ (δ 1.21) to C-3″ (δ 18.5), C-2″ (δ

Table 5. ^{13}C and ^{1}H NMR Data of Compounds **11** and **12** (in $CDCl_3$)

position	**11** δ_C (mult.)		**11** δ_H mult. (*J*)	**12** δ_C (mult.)		**12** δ_H mult. (*J*)
2	90.4	(CH)	4.51 dd (6.2, 9.9)	90.4	(CH)	4.51 dd (6.2, 9.9)
3	30.7	(CH_2)	2.18 dd (9.9, 13.2)	30.6	(CH_2)	2.20 dd (9.9, 13.2)
			2.11 dd (6.2, 13.2)			2.13 dd (6.2, 13.2)
3a	60.5	(C)		60.7	(C)	
4 C=O	195.8	(C)		195.7	(C)	
5	107.8	(C)		106.2	(C)	
6	192.7	(C)	18.90 s (−OH)	192.7	(C)	19.05 s (−OH)
7	106.8	(C)		106.6	(C)	
7a	174.7	(C)		174.7	(C)	
2-(1-hydroxy-1-methyl)ethyl						
1′	71.2	(C)		71.2	(C)	
2′	23.7	(CH_3)	1.15 s	23.8	(CH_3)	1.16 s
3′	26.8	(CH_3)	1.33 s	26.7	(CH_3)	1.33 s
3a-(3-methylbut-2-enyl)						
1″	38.7	(CH_2)	2.42 dd (13.9, 7.3)	38.5	(CH_2)	2.53 dd (14.3, 7.4)
			2.53 dd (13.9, 8.0)			2.54 dd (14.3, 8.0)
2″	117.1	(CH)	5.01 t (7.7)	117.2	(CH)	5.01 t (7.7)
3″	137.0	(C)		136.9	(C)	
4″	17.8	(CH_3)	1.72 s	17.9	(CH_3)	1.54 s
5″	25.9	(CH_3)	1.68 s	25.9	(CH_3)	1.69 s
5-(3-methylbutyryl)						
1‴C=O	198.6	(C)		203.6	(C)	
2‴	46.1	(CH_2)	2.77 dd (6.6, 13.2)			
			2.71 dd (7.7, 13.2)			
3‴	26.6	(CH)	1.71 m	34.0	(CH)	3.72 m
4‴	22.9	(CH_3)	1.00 d (6.6)	19.8	(CH_3)	1.20 d (7.0)
5‴	22.5	(CH_3)	0.96 d (6.6)	18.6	(CH_3)	1.11 d (7.0)
7-(3-methylbut-2-enyl)						
1⁗	21.5	(CH_2)	3.09 dd (6.6, 14.6)	21.5	(CH_2)	3.09 dd (6.6, 14.6)
			3.00 dd (8.0, 14.6)			3.00 dd (8.0, 14.6)
2⁗	121.6	(CH)	5.11 t (7.3)	121.6	(CH)	5.11 t (7.3)
3⁗	132.1	(C)		132.1	(C)	
4⁗	17.9	(CH_3)	1.54 s	17.8	(CH_3)	1.72 s
5⁗	25.7	(CH_3)	1.58 s	25.7	(CH_3)	1.68 s

39.3), and C-1″ (δ 210.1); H-2″ (δ 3.75) to C-1″ (δ 210.1), C-3″ (δ 18.5), and C-4″ (δ 19.1). In the COSY experiment of compound **13**, correlations were observed between H-2 (δ 4.74) and H-3 (δ 3.06 3.15), H-4 (δ 7.20), and H-5 (δ 6.45). In the NOESY experiment, correlations were observed between H-4 (δ 7.20) and H-2 (δ 4.74), H-4 (δ 7.20), and H-5 (δ 6.45). Compounds **2**, **4**, **5**, and **8**−**13** all possess one or more stereocenters (*), but additional experiments indicated that these compounds are racemic because the $[\alpha]^{25}_D$ values were zero.

Macrophages play major roles in inflammation and host defense mechanisms against bacterial and viral infections.[14] During acute and chronic inflammation, excessive production of NO may cause severe injury to host cells and tissues.[15] Excessive and prolonged NO generation mediated by an inducible NO synthetase (iNOS) has attracted much attention because of its relevance to epithelial carcinogenesis[16,17] and the production of vascular epidermal growth factor (VEGF).[18,19] In the previous study, the inhibitory effects of compounds **1**−**7** and **10**−**12** on the production of NO and the expression of iNOS have been reported.[10] Inhibitory effects of compounds **8**, **9**, and **13** on the production of NO induced by LPS/IFN-γ were examined. The results are summarized in Table 6. Chalcones (**1**−**5**) significantly inhibited NO production without showing cytotoxicity at concentrations lower than 10 μM (cell viability > 95%). Compounds **1**, **2**, **4**, and **5** have the same backbone structure but differ in the prenyl side chain. Their inhibitory activities were almost the same (IC_{50} values are 8.3, 9.4, 5.6, 6.5 μM, respectively), which indicated that the prenyl chain may not be necessary for the NO production inhibitory activities. Compound **3**, lacking the double bond between the α and β positions, did not show cytotoxicity even at the concentration of 20 μM, but it exhibited much weaker inhibitory activity than other chalcones (**1**, **2**, **4**, **5**), suggesting that the double bond is important for the inhibitory activity of chalcones. Hulupinic acid (**6**) did not show cytotoxicity at the concentration of 200 μM (cell viability > 95%), and no significant inhibitory activity was observed. Although compounds **7**−**12** inhibited the production of NO, these compounds showed very strong cytotoxicity. Especially in the case of compounds **11** and **12**, cytotoxicity was observed even at a low concentration of 2 μM (80% < cell viability < 95%). When the RAW 264.7 cells were treated with compounds **7**−**12** at concentrations that do not induce cytotoxicity, there was no significant inhibitory activity observed (NO inhibitory rates were all lower than 40%). A disposition could be considered that oxidation at different side chain positions of lupulone (**7**) may induce either much weaker inhibitory activities or false inhibitions with strong cytotoxicity. Compound **13** did not show significant inhibitory activity at the concentration of 100 μM.

Table 6. Inhibitory Activities of Compounds **1**−**13** on the Production of NO in RAW 264.7 Cells

compound	IC_{50} (μM)	compound	IC_{50} (μM)
1	8.3	**7**	17
2	9.4	**8**	20
3	23	**9**	14
4	5.6	**10**	63
5	6.5	**11**	11
6	>100	**12**	15
		13	>100

Experimental Section

General Experimental Procedures. IFN-γ was purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). LPS and MTT were from Sigma Chemical Co. (St.

Louis, MO). RPMI 1640 medium and fetal bovine serum were purchased from Gibco RBL (Grand Island, NY). UV spectra were obtained on a Hitachi 200-10 spectrophotometer, and IR spectra were recorded on a JASCO IR A-2 spectrophotometer. The NMR spectra were collected on a JEOL GL-500 spectrometer, using tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained on a Hitachi M-80B spectrometer. Column chromatography was carried out by using silica gel (Wako gel C-300, Wako Pure Chemical Ind., Ltd.) and Sephadex LH-20 (20−100 μm, Pharmacia Fine Chemical Co., Ltd.). TLC was performed on Merck TLC plates (0.25 mm thickness), and spots were visualized by 10% (v/v) H_2SO_4 in ethanol solution.

Extraction and Isolation. A hops CAS pellet (2.5 kg) was extracted with EtOAc to obtain a dark green extract (PEE, 329.17 g). The pellet was then extracted with 80% acetone (3 × 3 L), and the extract was suspended in H_2O and partitioned with *n*-BuOH. Evaporation of the solvent yielded the *n*-BuOH fraction (PEB, 84.19 g) and the aqueous fraction (PEW, 282.52 g). A 262.7 g portion of the PEE fraction was separated by a silica gel column chromatography eluted with a hexane−EtOAc gradient (0 → 100%) of increasing polarity to give 15 fractions: PEE-1 (1.9 g), PEE-2 (5.1 g), PEE-3 (41.8 g), PEE-4 (19.1 g), PEE-5 (8.6 g), PEE-6 (39.1 g), PEE-7 (22.8 g), PEE-8 (13.6 g), PEE-9 (8.1 g), PEE-10 (33.1 g), PEE-11 (18.1 g), PEE-12 (21.6 g), PEE-13 (7.4 g), PEE-14 (5.2 g), PEE-15 (4.3 g). Fractions 9−11 showed strong inhibitory activities but had no cytotoxic effects in our previous study.[10] Fraction PEE-9 was separated by a combination of normal-phase silica gel column chromatography, Sephadex LH-20 column chromatography, and HPLC to obtain **6**. Fraction PEE-10 was separated by a combination of normal-phase silica gel column chromatography, Sephadex LH-20 column chromatography, and HPLC to obtain **7**−**13**. Fraction PEE-11 was separated by a combination of normal-phase silica gel column chromatography, Sephadex LH-20 column chromatography, and HPLC to obtain **1**−**5**.

Compound 5. 1-[2,4-Dihydroxy-3-(3-hydroxy-2-methoxy-3-methylbutyl)-6-methoxyphenyl]-3-(4-hydroxyphenyl)propenone: yellow powder; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HREIMS *m/z* 402.16714 (calcd for $C_{22}H_{26}O_7$, 402.16782).

Hulupinic acid (6): colorless needles (MeOH); ^{1}H NMR (CD_3OD, 400 MHz) δ 2.31 (4H, d, J = 7.8 Hz, 1′ and 1″ −CH_2), 4.81 (2H, t, J = 7.8 Hz, 2′ and 2″ =CH−), 1.59 (6H, s, 4′ and 4″ −CH_3), 1.54 (6H, s, 5′ and 5″ −CH_3); ^{13}C NMR (CD_3OD, 100 MHz) δ 200.0 (C, C-1 and C-3, C=O), 55.4 (C, C-2), 151.4 (C, C-4 and C-5), 33.5 (CH_2, C-1′ and C-1″), 118.9 (CH, C-2′ and C-2″), 136.6 (C, C-3′ and C-3″), 26.1 (CH_3, C-4′ and C-4″), 17.8 (CH_3, C-5′ and C-5″); HREIMS *m/z* 264.13588 (calcd for $C_{15}H_{20}O_4$ [M]$^+$, 264.13614), 221, 195 [M$^+$ − prenyl], 178, 141, 69 [prenyl].

Lupulone A (8): yellow oil; $[\alpha]^{25}_D$ 0° (*c* 0.3, MeOH); UV (CH_3OH) λ_{max} (ϵ) 369.5 (170), 281.0 (142), 255.5 (170), 211.5 (810) nm; IR (KBr) ν_{max} 1620, 1660 (C=O), 3447 (−OH) cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 3; HREIMS *m/z* 412.26134 (calcd for $C_{26}H_{36}O_4$, 412.26135).

Lupulone B (9): yellow oil; $[\alpha]^{25}_D$ 0° (*c* 0.4, MeOH); UV (CH_3OH) λ_{max} (ϵ) 372.5 (159), 281.0 (125), 256.0 (182), 211.0 (796) nm; IR (KBr) ν_{max} 1729 (C=O), 3440 (−OH) cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 3; HREIMS *m/z* 398.24569 (calcd for $C_{25}H_{34}O_4$, 398.24571).

Lupulone C (10): yellow oil; $[\alpha]^{25}_D$ 0° (*c* 0.4, MeOH); ^{1}H and ^{13}C NMR data, see Table 4; HREIMS *m/z* 430.27123 (calcd for $C_{26}H_{38}O_5$ [M]$^+$, 430.27190), 375, 361 [M$^+$ − prenyl], 343, 307, 289, 69 [prenyl].

Lupulone D (11): yellow oil; $[\alpha]^{25}_D$ 0° (*c* 0.4, MeOH); ^{1}H and ^{13}C NMR data, see Table 5; HREIMS *m/z* 430.27167 (calcd for $C_{26}H_{38}O_5$, 430.27190).

Lupulone E (12): yellow oil; $[\alpha]^{25}_D$ 0° (*c* 0.5, MeOH); ^{1}H and ^{13}C NMR data, see Table 5; HREIMS *m/z* 416.25663 (calcd for $C_{25}H_{36}O_5$, 416.25626).

Lupulone F (13): yellow oil; $[\alpha]^{25}_D$ 0° (*c* 0.7, MeOH); UV (CH_3OH) λ_{max} (ϵ) 284.5 (122), 239.0 (95), 214.5 (54) nm; IR (KBr) ν_{max} 1734 (C=O), 3441 (−OH) cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.74 (1H, t, J = 7.6 Hz, H-2), 3.06 (1H, dd, J = 7.6, 12.0 Hz, H-3a), 3.15 (1H, dd, J = 7.6, 12.0 Hz, H-3b), 7.20 (1H, d, J = 6.6 Hz, H-4), 6.45 (1H, d, J = 6.6 Hz, H-5), 12.85 (1H, s, 6-OH), 2-(1-hydroxy-1-methyl)ethyl: 1.27 (3H, s, H-2′), 1.38 (3H, s, H-3′), 7-(2-methylpropyl): 3.75 (1H, m, H-2″), 1.20 (3H, d, J = 8.9 Hz, H-3″), 1.21 (3H, d, J = 8.9 Hz, H-4″); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 91.5 (CH, C-2), 29.3 (CH_2, C-3), 117.5 (C, C-3a), 131.5 (CH, C-4), 109.7 (CH, C-5), 163.2 (C, C-6), 106.0 (C, C-7), 160.0 (C, C-7a), 2-(1-hydroxy-1-methyl)-ethyl: 71.6 (C, C-1′), 24.7 (CH_3, C-2′), 26.2 (CH_3, C-3′), 7-(2-methylpropyl): 210.1 (C, C-1″, C=O), 39.3 (CH, C-2″), 18.5 (CH_3, C-3″), 19.1 (CH_3, C-4″); HREIMS *m/z* 264.13614 (calcd for $C_{15}H_{20}O_4$, 264.13604).

Assay of Inhibitory Activities on NO Production. The RAW 264.7 cells were incubated in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell concentration was adjusted to 5 × 10^5 cells/mL, and 200 μL of cell suspension was seeded in each well of a 96-well plate. After a 1 h incubation, cells were treated with LPS (100 ng/mL), IFN-γ (100 units/mL), and test samples dissolved in DMSO (final DMSO concentration 0.2%, v/v) for 16 h at 37 °C. A 100 μL sample of the culture supernatant was placed in a well in duplicate 96-well flat-bottomed plate. Standard solutions of $NaNO_2$ were also placed in other wells on the same plate, and the levels of NO_2^- in the sample supernatant were determined by Griess assay.[20,21] Griess reagent (50 μL of 1% sulfanilamide in 5% H_3PO_4, and 50 μL of 0.1% *N*-1-naphthylethylenediamine dihydrochloride) was added to each well. After 10 min, the reaction products were colorimetrically quantitated at 570 nm using a microplate reader. The inhibitory rate on NO production was calculated by the NO_2^- levels as follows: Inhibitory rate (%) = 100 × (LPS/IFN − LPS/IFN/sample)/(LPS/IFN − untreated). Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[10] An MTT solution (200 μg/mL) was added after the 16 h treatment and then incubated for another 4 h at 37 °C. The reduced MTT-formazan was solubilized with 150 μL of DMSO, and the absorbance of the MTT-formazan solution at 540 nm was measured by an immunoreader. The percentage of suppression was calculated by comparing the absorbance of sample-treated cells with that of nontreated cells.

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