Bis(bibenzyls) from Liverworts Inhibit Lipopolysaccharide-Induced Inducible NOS in RAW 264.7 Cells: A Study of Structure−Activity Relationships and Molecular Mechanism

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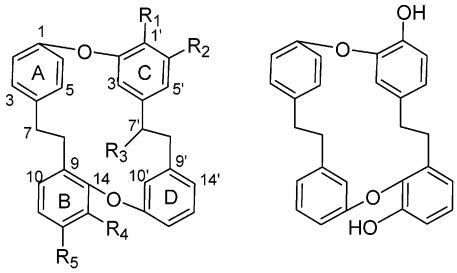
Received July 20, 2005

The inhibition of lipopolysaccharide-induced NOS by 19 bis(bibenzyls) isolated from liverworts in RAW 264.7 macrophages was evaluated. The presence of phenolic hydroxyls and saturation at 7,8 and/or 7′/8′ are required for inhibition of NO production. Among the compounds tested, marchantin A (**1**) was the most potent, and its inhibitory activity was consistent with the inhibition of LPS-induced iNOS mRNA.

Nitric oxide synthase (NOS), composed by three isoforms (nNOS, eNOS, iNOS), catalyzes the conversion of L-ariginine and oxygen stimulators into L-citrulline and nitric oxide (NO).[1] Overproduction of NO is involved in inflammatory response-induced tissue injury and the formation of carcinogenic *N*-nitrosamines.[2,3] Large amounts of NO were expressed and generated by induced iNOS upon stimulation of endotoxins or cytokins involved in pathological responses.[4] Therefore, inhibition of iNOS is very important to control inflammatory diseases. The use of LPS-stimulated RAW264.7 macrophage cells is one approach to evaluate inhibitory activity of chemical compounds in iNOS.

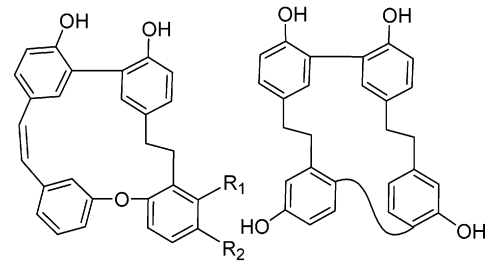
Bis(bibenzyls) are aromatic compounds mainly found in liverworts from genera such as *Riccardia*, *Marchantia*, *Plagiochila*, *Preissia*, *Reboulia*, *Monoclea*, *Ricciocarpos*, and *Blasia*.[5] These compounds are cyclic and have one or two diaryl ether or biphenyl bonds, or acyclic, possessing a diaryl ether or biphenyl bond, and have shown a wide range of cytotoxic, antibacterial, antifungual, and 5-lipoxygenase inhibitory activity.[5] Bis(bibenzyls) arise biogenetically from lunularic acid or lunularin, which is widely distributed in leafy and thalloid liverworts.[6] In our ongoing studies of biologically active compounds from liverworts, we investigated the inhibition of nitric oxide production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells by 19 bis(bibenzyls) (**1**−**19**). Inhibition of lipopolysaccharide-induced inducible NOS in RAW 264.7 macrophages by **1**, which was the strongest inhibitor among the tested compounds, was also performed in this study.

The inhibition of compounds **1**−**19** of LPS-induced NO production in culture media on RAW 264.7 cells was tested, and the IC_{50} of each compound is reported in Table 1. Marchantin A (**1**) showed the strongest inhibition (IC_{50}: 1.44 μM), while introduction of a hydroxyl group at C-12 (in the case of **2**) reduced activity. The only difference in the structure of **1** and marchantin D (**4**) is the presence of an additional hydroxyl group at C-7′. The activity of **4** was however 7 times less than **1** and 40 times less if the hydroxyl group at C-7′ was methylated (**5**). Perrotetin F (**13**) is an acyclic bis(bibenzyl) having a 1−2′ ether bond and four hydroxyl groups in the molecule. The strong NO inhibition of **13** (IC_{50} 7.4 μM) offers support that substituents of the A and C rings similar to that of **1** are necessary for activity. The inhibition of the methylated derivatives of **1** and **2** (compounds **18** and **19**) at 42.5 and 42.4 μM, respectively, indicates that the hydroxyl groups play an important role in the mechanism of inhibition of nitric oxide. However, riccardins A (**14**) and F (**16**), which have one methoxyl group at the 11- or 1′-position and a biphenyl bond, exhibited strong inhibitory activity (2.5 and 5 μM, respectively). This finding suggested that the strong activity was due to the presence of the hydroxyl group at the



1: R_1= R_2= R_4= OH, R_3= R_5= H
2: R_1= R_2= R_4= R_5= OH, R_3= H
3: R_1= R_4= OH, R_2= R_3= R_5= H
4: R_1= R_2= R_3= R_4= OH, R_5= H
5: R_1= R_2= R_4= OH, R_3= OMe, R_5= H
6: R_1= R_4= R_5= OH, R_2= H, R_3= H
18: R_1= R_2= R_4= OMe, R_3= R_5= H
19: R_1= R_2= R_4= R_5= OMe, R_3= H

7



8: R_1= H, R_2 = OH
9: R_1= OH, R_2 = OH

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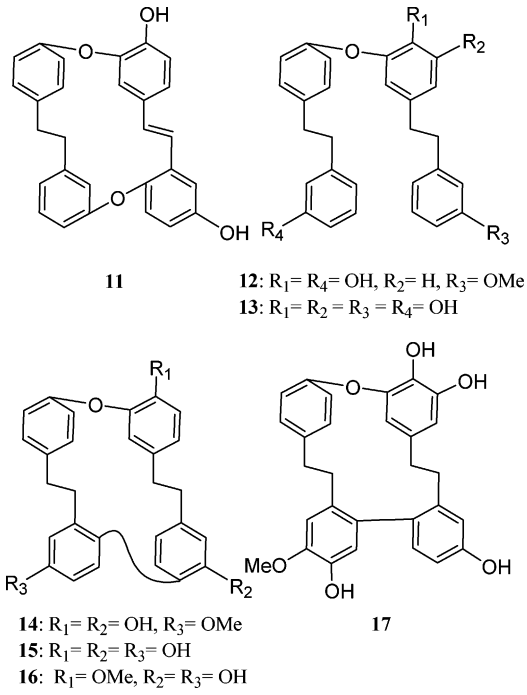
10.1021/np0502589 CCC: $30.25
Published on Web 11/23/2005

Table 1. NO Inhibition of Compounds **1**−**19**

compound	NO inhibition IC_{50} (μM)
1, marchantin A	1.44
2, marchantin B	4.10
3, marchantin C	13.28
4, marchantin D	10.18
5, marchantin E	62.16
6, marchantin H	15.34
7, isomarchantin C	>100
8, isoplagiochin A	>100
9, isoplagiochin B	>100
10, isoplagiochin D	14.32
11, ptychantol A	>100
12, perrottetin E 11′-methyl ether	49.85
13, perrottetin F	7.42
14, riccardin A	2.50
15, riccardin C	>100
16, riccardin F	5
17, plagiochin A	9.07
18, marchantin A trimethyl ether	42.50
19, marchantin B trimethyl ether	42.45

C-13′ position, the 14−12′ biphenyl bond, and the methoxyl group at the 11 or 1′ position. This suggestion was supported by the low inhibition of riccardin C (**15**). Interestingly, the structure of **16** is very similar to **10** with respect to rings B and D. The inhibitory activity of **10** was, however, 3 times weaker than **16**. This may be due to the 6−2′ biphenyl bond in **10** and the *ortho*-hydroxyl groups on rings A and C, which require a stable conformation of the molecule. This conclusion supports the high inhibition of **17** (IC_{50} 9 μM), in which the biphenyl bond is between C-14 and C-10′, with the absence of *ortho*-hydroxyl groups. Compounds **3**, **6**, and **13** showed moderate activity. Although **3** and **7** are tautomers, the latter showed no NO inhibition. The position of the ether linkage (14−11′) and the hydroxyl group in the B and D rings (at C-13 instead of C-11′ in the case of **7**) appears to be important for the improvement of the inhibitory activity. It is interesting to note that 7,8-unsaturation dramatically decreased the activity of compounds **8** and **9**.



To provide information on the possible mechanisms for inhibition of NO production by bis(bibenzyls), marchantin A (**1**), the strongest NO inhibitor among the compounds screened, was examined concerning the relationship to iNOS mRNA expression along with LPS-induced RAW 264.7 cells. Expression of iNOS mRNA could not be detected when RAW 267.4 cells were incubated in the medium without LPS for 24 h, and the basal level of iNOS mRNA was not affected when incubated with **1**. iNOS protein increased when the cells were treated with LPS for 24 h; however, dose-dependent variation of the iNOS protein level was observed when the cells were treated with both LPS and **1** at different concentrations (20, 10, and 0.1 μM) for 24 h. The amount of β-actin protein (the internal control) observed was constant (Figure 1).

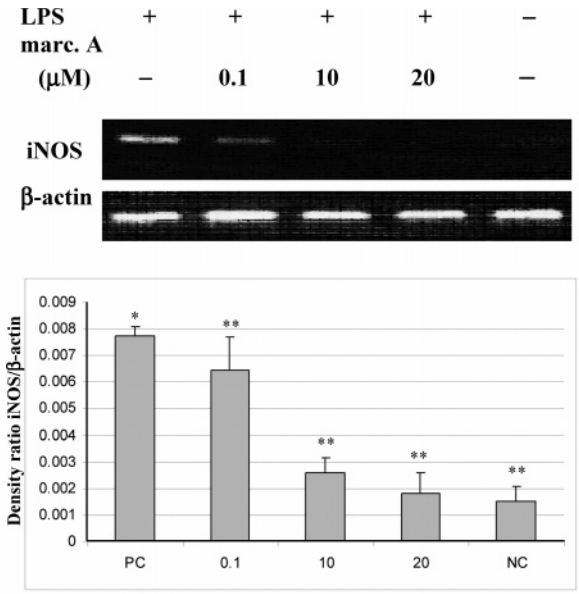


Figure 1. RT-PCR analysis for iNOS mRNA expression level in RAW 267.4 cells stimulated with LPS (1μg/mL) or plus treatment with **1**, respectively, as indicated at concentrations of 20, 10, and 0.1 μM. RT-PCR products of β-actin were used for internal control, and the relative iNOS mRNA was normalized with the respective amount of β-actin. Experiments were repeated three times, and individual values are mean ± SEM. *P < 0.005 compared to negative control (NC); **P < 0.005 compared to positive control (PC).

In this paper, we evaluated the inhibitory activity of 19 bis(bibenzyls) for NO production in LPS-induced RAW 264.7 macrophages. Our results indicate that the presence of 1−2′ and 14−11′ diaryl ether bonds is important for strong inhibition. The presence of phenolic hydroxyl groups also plays an important role in the activity. However, in the case of phenolic hydroxyls in the molecules containing one or two biphenyl bonds (isoplagiochin, plagiochin, or riccardin-type), the activity level appears to depend on stable conformations of these molecules. Compounds with 7,8-unsaturation dramatically decreased the inhibition of NO, while introduction of a hydroxyl group at C-7′ resulted in slightly decreased activity. The presence of a hydroxyl group at the C-13′ position, a 14−12′ biphenyl bond, and a methoxyl group at the 11 or 1′ position led to high levels of inhibition of NO production.

Experimental Section

General Experimental Procedures. Compounds and Sources. Compounds **1**−**5** were isolated from *Marchantia polymorpha* and *M. palmata*,[7,8] **6** and **7** from *Plagiochila barteri* and *Marchantia pappeana*, respectively, both collected from Madagascar (unpublished result), **8**−**10** from *Plagiochila fruticosa*,[9,10] **11** from *Ptychanthus striatus*,[11] **12** from *Pellia endiviifolia*,[12] **13** from *Radula perrottetii*,[13] **14** and **15** from *Riccardia multifida* and *Blasia pusilla* L.,[14,15] respectively, **15**

from *Mastigophora diclados*,[16] and **17** from *Plagiochila acanthophylla* subsp. *Japonica*;[17] **18** and **19** are the methyl ether derivatives of **1** and **2**.

Bioassay. Cell Culture and Sample Treatment. The RAW 264.7 cells were cultured in RPMI 1640 medium containing 10% FBS, kanamycin (50 μg/mL), and ampicillin (60 μg/mL). The cells (0.8×10^6 cells/mL in 96-well plates, 100 μL/well) were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air. Compounds **1**−**19** were solubilized in DMSO. The final DMSO concentration was below 0.1% in the culture medium. At this concentration, DMSO showed neither NO induction without stimulation with LPS nor inhibition of LPS-induced NO production. Cells were incubated with compounds **1**−**19** at 60 μM for 1 h and stimulated with LPS (purified from *Pantoea agglomerans*) at 1 μg/mL for 24 h.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of iNOS mRNA Expression. RAW 264.7 cells (10^6 cells/mL in 24-well plates, 500 μL/well) were incubated overnight before LPS (1 μg/mL) and/or compound treatment for 8 h. After washing with PBS twice, total RNA was isolated from the cell pellet, using an RNA isolation kit (Trizol treatment). Total RNA was determined by the absorbance at 260 nm. An amount of 2.5 μg of total RNA was reverse transcribed into cDNA using ReverTra Ace (MMLV reverse transcriptase Rnase H−) and oligo (dT) primers (Invitrogen Co.). The PCR samples, contained in 10 μL of the reaction mixture, comprised 5 μL of 2× Master mix, 1 μL of distilled H_2O, 1 μL of sense and antisense primer each, and 3 μL of prepared RT product. The sense primer for mouse iNOS was 5′-gTAgAAAgTCCAgCCgCAC-3′, and the antisense primer was 5′-gTAgCTgCCgCTCTCATCCAg-3′. For mouse β-actin the sense primer was 5′-CCAACCGTGAAAAGATGACC-3′ and the antisense was 5′-CAGGAGGAGCAATGATCTTG-3′. The PCR was carried out with 20 cycles for β-actin and 25 cycles for iNOS to obtain results within the exponential range. The PCR amplification was performed after 10 min incubation at 95 °C under the following conditions: 20 to 25 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 20 s, using a thermal cycler. The amplified PCR products, in lengths of 203 bp for iNOS and 660 bp for β-actin, were separated on 2% agarose for β-actin PCR product and 2.5% for iNOS PCR product. The bands in the agarose-gel were photographed, and the fluorescence intensities were analyzed.

Nitrite Assays. Nitrite, accumulated in the culture medium, was measured as an indicator of NO production by the Griess reaction [35 μL of cell culture medium was mixed with 35 μL of Griess reagent, prepared by addition of equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine HCl] and incubated at room temperature for 10 min, and then the absorbance at 550 nm was measured in a microplate reader (using a BIO-RAD model 550 microplate reader). In all experiments, fresh culture medium was used as control. The amount of nitrite in the sample was calculated by means of a $NaNO_2$ serial dilution standard curve, freshly prepared. The IC_{50} was calculated for each compound (**1**−**19**) and reported in Table 1.

Acknowledgment. The authors are grateful to the Japan Society for Promotion of Science (JSPS) for granting a post-doctoral fellowship to L.H. Thanks are also due to Dr. M. Tanaka for NMR measurements and Ms. Y. Okamoto (TBU) for recording mass spectra. This work was supported by a "Open Research Center" Project of the Ministry of Education, Culture, Sports, Science and Technology.

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NP0502589