# Waol B, a New Trihydrofuran Derivative with Cytocidal Activity, isolated from *Myceliophthora lutea*

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Multidrug resistance remains a serious problem in the chemotherapy of solid tumors[1]. Many tumors are either intrinsically resistant to the chemotherapeutic agent or develop resistance over the course of treatment. Mammalian cells which have acquired resistance to a single cytotoxic natural product drug can become not only resistant to the agent used but also cross-resistant to a wide range of structurally and functionally unrelated antibiotics and alkaloids[2]. As a result, treatment with chemotherapeutic agents generally results in temporary remission of tumor disease in the clinic. For this reason we have been looking for agents effective equally against parent and resistant mammalian cells.

In the course of our screening program for low molecular compounds effective against multi-drug resistant tumor cells using adriamycin resistant human promyelocytic leukemia cells (HL-60), we have discovered a new $\delta$ lactone FD-211 (waol A)[††] in the fermentation broth of *Myceliophthora lutea* TF-0409[3]. Our further[1] screening precedure led to the isolation of waol B as a congener of waol A from the fermentation broth of the same strain. Structures of waols A and B are shown in Fig. 1.

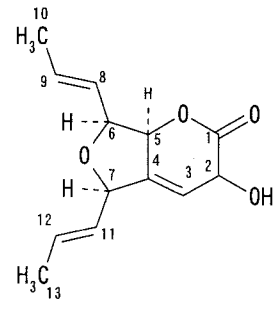
This paper describes physico-chemical properties, structure determination and biological activities of waol B.

## Results and Discussion

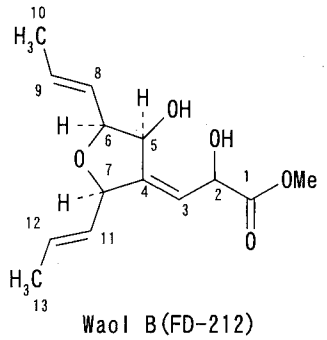
By isolation procedure guided by a bioassay for cytocidal activity against adriamycin-resistant HL-60 cells, waol B was obtained as colorless oil from the fermentation broth of *Myceliophthora lutea* TF-0409, a strain producing waol A (FD-211). The physico-chemical properties of waols A and B are described in Table 1. These physico-chemical properties are similar to each other. They are lipophilic, neutral in nature and gave positive color response to iodine, $H_2SO_4$ and vanillin-$H_2SO_4$, but negative to ninhydrin.

Their UV spectrum showed end absorption. The IR spectrum of waol B showed two bands at 3400 $cm^{-1}$ and 1718 $cm^{-1}$ characteristic of a hydroxy group and an ester carbonyl, respectively, whereas the absorption due to a $\delta$ lactone at 1767 $cm^{-1}$ in the spectrum of waol A disappeared. The molecular weight was determined to be 268 by the observation of its molecular ion in the EI mass spectrum. The molecular formula of waol B was

Fig. 1. Structures of waols A and B.




Waol A(FD-211)




Waol B(FD-212)

†† Waol A was previously designated as FD-211. Structure of FD-211 looks like a frog, which is called "wa" in Chinese. So we named it waol A.

Table 1. Physicochemical properties of waols A and B.

| | Waol A | Waol B |
|---|---|---|
| Appearance | Colorless oil | Colorless oil |
| $[\alpha]_D$ | -170.0° ( c=0.01, MeOH ) | |
| EIMS | m/z 236( $M^+$ ) | m/z 268 ( $M^+$ ) |
| FABMS ( + ) | m/z 237 ( M + H ) | m/z 269 ( M + H ) |
| FABMS ( - ) | m/z 235 ( M - H ) | m/z 267 ( M - H ) |
| HREIMS | m/z 263.1050 ( found ) | m/z 268.1314 ( found ) |
| | m/z 236.1049 calcd. for $C_{13}H_{15}O_4$ | m/z 268.1312 calcd. for $C_{14}H_{20}O_5$ |
| Molecular Formula | $C_{13}H_{16}O_4$ | $C_{14}H_{20}O_5$ |
| UV $\lambda$max nm ( $\varepsilon$ ) | end | end |
| IR $\nu$max $cm^{-1}$ | 3450 ( OH ), 1767 ( $\delta$ lactone ) | 3400 ( OH ), 1718 ( C=O ) |

established as $C_{14}H_{20}O_5$ by its molecular ion measurement ($M^+$) at *m/z* 268.1314 (calcd. 268.1312 for $C_{14}H_{20}O_5$) in the high resolution EI mass spectrum in combination with the $^1H$ and $^{13}C$ NMR spectra. The $^1H$ and $^{13}C$ NMR data of waols A and B are shown in Table 2.

These spectra were very similar to each other. The functionalities of the carbon signals of waol B were determined by the DEPT spectra. Consistent with its molecular formula, the $^{13}C$ NMR spectrum of waol B gave 14 lines, which were classified into $-CH_3\times2$, $-CHO-\times4$, $-OCH_3\times1$, $-CH=\times5$, $>C=\times1$ and $>C=O\times1$. The degree of unsaturation was estimated to be 5 by its molecular formula. These data indicated that three unsaturations were assigned to three double bonds and one to one carbonyl group, leaving the final one unsaturation to accommodate a ring. Although the $^1H$ NMR gave eighteen proton signals, two hydroxyl groups were likely to exist in the molecule. The presence of two hydroxyl groups was confirmed by treatment of waol B with acetic anhydride-pyridine to give waol B diacetate, of which the $^1H$ NMR spectrum showed two signals at $\delta$ 2.01 and 2.05 due to two acetyl groups.

The relation from a signal at $\delta_H$ 3.95 (H-2) to a line at $\delta_H$ 7.12 (H-3) was strightforward assigned. The latter in turn showed a long range coupling with a line at $\delta_H$ 4.76 (H-5). In the $^1H$-$^1H$ COSY spectrum a resonance at $\delta_H$ 4.60 (H-6) made up cross peaks with two signals at $\delta_H$ 4.76 (H-5) and at $\delta_H$ 5.44 (H-8), respectively. The latter was further correlated with a line at $\delta_H$ 5.65 (H-9), which in turn coupled to a methyl at $\delta_H$ 1.68 (H-10). Thus, the partial structure of H-5 to H-10 was established. Similarly by tracing a spin network from a line at $\delta_H$ 3.92 (H-7), the fragment of H-7 to H-13 was deduced. These three partial structures were assembled together by key HMBC correlations to establish structure of waol B as discussed below. H-3 ($\delta_H$ 7.12) showed long range correlations with C-1 ($\delta_C$ 165.8), C-5 ($\delta_C$ 132.7), and C-7 ($\delta_C$ 76.8), respectively. Coupling of C-1 with a singlet methyl at $\delta_H$

Table 2. $^{1}H$ and $^{13}C$ NMR data of waols A and B.

| | Waol A | | Waol B | |
|---|---|---|---|---|
| No. | $^{1}H(\delta H)$ | $^{13}C(\delta C)$ | $^{1}H(\delta H)$ | $^{13}C(\delta C)$ |
| 1 | | 166.4 | | 165.8 |
| 2 | 4.10 bd | 64.1 | 3.95 bd | 64.2 |
| 3 | 6.90 dd, J=2.4, 3.8Hz | 133.6 | 7.12 dd, J=6.3, 1.6Hz | 137.8 |
| 4 | | 132.9 | | 132.7 |
| 5 | 4.38 ddd, J=7.7, 2.4, 2.4Hz | 78.6 | 4.76 m | 77.3 |
| 6 | 4.60 dd, J=7.5, 7.7Hz | 82.9 | 4.60 m | 72.7 |
| 7 | 4.50 m | 79.8 | 3.92 d, J=6.5Hz | 76.8 |
| 8 | 5.61 ddq, J=15.4, 7.5, 1.6Hz | 125.9 | 5.44 ddq, J=15.3, 6.5, 1.4Hz | 128.5 |
| 9 | 6.02 dq, J=15.4, 6.5Hz | 134.0 | 5.65 dq, J=15.3, 6.5Hz | 128.8 |
| 10 | 1.80 d, J=6.5Hz | 17.9 | 1.68 d, J=6.5 | 17.7 |
| 11 | 5.70 ddq, J=15.4, 7.5, 1.6Hz | 125.7 | 5.67 ddq, J=15.4, 6.5, 1.4Hz | 126.7 |
| 12 | 5.91 dq, J=15.3, 6.5Hz | 131.2 | 5.84 dq, J=15.4, 6.5Hz | 130.2 |
| 13 | 1.80 d, J=6.5Hz | 18.1 | 1.77 d, J=6.5Hz | 18.0 |
| OMe | | | 3.76 | 51.9 |

3.76 indicated the presence of a methyl ester. Furthermore, cross peaks were observed not only from H-5 to C-3 and C-4, but also from H-7 to C-6 in the HMBC specturm, by which a five membered ring was formed. The location of two hydroxyl groups at C-2 and C-5 was unambiguously elucidated by acetylation shifts in the $^{1}H$ NMR spectrum of waol B diacetate. The coupling constants of 15.2 Hz between H-8 and H-9 and 15.2 Hz between H-11 and H-13 indicated that their geometrical relationships were all trans. These results led to the assignment of structure of waol B as a propanoic acid, 3-(dihydro-4-hydroxy-2,5-di-1-propenyl-3(2*H*)-furanylidene)-2-hydroxy-methyl ester as shown in Fig.1. The NOESY spectrum showed cross peaks from H-7 to H-6 and from H-6 to H-5, indicating that these protons are spatially near to each other and on the same side. The NOE from H-2 to H-5 could not be observed in a acetonide derivative formed between the two OH's at C-2 and C-5. Also the stereochemistry at C-2 failed to be determined by Mosher's rule[4] and benzoate rule[5].

As shown in Table 3, waols A and B exerted eight to ten times weaker cytocidal activities against various cultured cell lines than adriamycin. Both of waols A and B showed two times higher 50% inhibitory concentration against resistant cells of HL-60 than parent cells. No significant differences were observed for their inhibitory effect on the incorporation of the labeled precursors into the macromolecules (data not shown). Waols A and B did not show any antitumor effect on P388 leukemia in mice (data not shown).

Among test microbes tested, waols A and B showed a very weak activity only against *Staphyloccocus aureus* Smith. As these biological activities of waol B were equal to those of waol A, a 4-ethylidene-2,5-di(1,2-dehydro-*trans*-butene)-tetra-hydrofuran-3-ol might contibutue to their biological activity whereas a $\delta$ lactone might not participate in biological activity.

Table 3. Activity of waols A and B against various cultured tumor cells.

| | $IC_{50}$ ($\mu$g/ml) | | |
|---|---|---|---|
| Cell lines | Waol A | Waol B | Adriamycin |
| HL 60/ADM* | 0.1 | 0.2 | 2.0 |
| HL 60 | 0.2 | 0.2 | 0.02 |
| P388 | 4.0 | 4.0 | 0.03 |
| T-24 | 0.5 | 0.5 | 0.08 |
| Hela | 1.0 | 1.0 | 0.08 |
| A549 | 1.0 | 1.0 | 0.08 |

* : Adriamycin resistant cells

## Materials and Methods

### General

Optical rotation was measured on a Jasco DIP-360 polarimeter in 10 cm tube. IR spectrum was recorded on a Perkin-Elmer 1760 FT-IR spectrophotometer. UV spectrum measured on a Hitachi 220 spectrophotometer. EI-MS, HR-EI and FAB-MS spectra were determined with a Jeol JMX-SX 102 mass spectrometer. NMR spectra were obtained with a Jeol JMN-A500 at ambient temperature with $^{1}H$ NMR at 500 MHz and $^{13}C$ at 125 MHz using solvent peaks as internal references downfield of TMS at 0 ppm.

### HPLC

Preparative HPLC separations were performed using a Shenshu-pack ODS column (ODS-4251-N, 10 mm×25 cm) with a Water Model 600E system, maintained at 50°C and developed with 65% MeOH solution at a flow rate of 5 ml/minute, monitoring the the absorbance at 215 nm.

### The Producing Strain

The producing strain was isolated from a soil sample collected at the state of Guang-Xi in China and identified as *Myceliophthora lutea* TF-0409[1].

### Fermentation

A loopful of *Myceliophthora lutea* TF-0409 on oat meal agar slant was inoculated in two 500 ml Erlenmyer flasks each containing 100 ml of the medium consisting of glucose 2%, yeast extract 0.2%, NaCl 0.3%, polypeptone 0.5%, $Mg_2SO_4$ 0.05% and $KHPO_4$ 0.1%. The inoculated flasks were cultured at 26°C for 96 hours on the rotary shaker. 400 ml of the cultured broth were transferred into a 50-liter jar fermentor containing 30 liters of the same medium as in the seed culture. The fermentation was carried out at 26°C for 96 hours under air of 30 liters per minute and agitation speed 150 rpm.

### Isolation

The whole fractionation was guided by a bioassay for cytocidal activity against adriamycin resistant HL-60 cells. The cultured broth was separated into supernatant and mycelium by centrifugation. To 30 liters of supernatant was added 1 liter of HP-20 resins and then mixed for 2 hours to absorb active material on HP-20 resins. The HP-20 resins were washed with water and then eluted with 5 liters of 50% MeOH. After removal of MeOH *in vacuo*, the aqueous layer was extracted with 2 liters of ethyl acetate. The ethyl acetate layer was dried over $Na_2SO_4$ and concentrated *in vacuo* to give *ca.* 3.2 g of brownish syrup. The resultant material was chromatographed over a LH-20 column prepared with MeOH. Active fractions were collected and concentrated *in vacuo* to yield *ca.* 1.3 g of brownish oil. After application of this material to a silica gel column charged with $CHCl_3$, the column was eluted with $CHCl_3$-MeOH by a stepwise of 0.05% increase in MeOH concentration from 0~1.0%. The active material was eluted with 0.2% of MeOH in $CHCl_3$. Chromatogaphy of active

material over a Sephadex LH-20 column with $CHCl_3$-MeOH-*n*-hexane (5 : 1 : 5) led to the purification of about 100 mg of colorless oil, which was further separated by preparative HPLC. Thus, waols A (17 mg) and B (10 mg) were obtained as colorless oil, respectively.

Isolation and maintenance of adriamycin resistant HL-60 cell. Adriamycin resistant cells were isolated by stepwise selection in increasing concentrations of adriamycin starting with $2\times IC_{50}$ (0.02 $\mu$g/ml). Cells were grown and maintained in RPMI-1640 medium containing 10% fetal bovine serum and 1 $\mu$g/ml of adriamycin.

Cytocidal Activity

HL-60, HL-60/ADR and P388 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. A549 lung adenocarcinoma, Hela and T-24 renal carcinoma were grown in DULBECCO's modified EAGLE's medium supplemented with 10% calf serum. All cells were maintained at 37°C in a humidified 5% $CO_2$ atomospere cells were seeded into 96-well microtiter plates ($2\times10^4$ cells/well) and incubated for 24 hours. The test sample, dissolved in MeOH, was added in serial dilutions. After addition, the plates were incubated for 72 hours. For the evaluation of *in vitro* cytocidal activity, a microculture tetrazolium assay (MTT assay) method was used. The $IC_{50}$ value was calculated with PROBIT's method.

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