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# Immunosuppressive auronol glycosides from *Artocarpus tonkinensis*

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Activity-guided fractionation of the *n*-butanol extract from the leaves of *Artocarpus tonkinensis* led to the isolation of the auronol glycosides maesopsin 4-*O*-glucoside (**1**), as well as the new alphitonin-4-*O*-glucoside (**2**). These structures were identified on the basis of MS and NMR spectroscopic data. The lymphocyte stimulation test showed both compounds having immunosuppressive activity.

## 1. Introduction

*Artocarpus tonkinensis* A. Cheval. (Moraceae) is a 15 m high tree growing in North Vietnam. The decoction of the leaves and roots is used in folk medicine to treat backache and rheumatism (Loi 1991). The crude extract from the leaves of this plant is used successfully in Vietnam to treat Myasthemia gravis autoimmune disease and as an immunosuppressive agent for the transplantation of skin (Ha et al. 1994). In our previous studies on Vietnamese *Artocarpus tonkinensis* we isolated from the bark oxyresveratrol, the flavonoids catechin and triterpenoids as well as the new benzofurane artotonkin (Lien et al. 1998). Our preliminary test of the methanol extract from the leaves showed immunosuppressive activity. More detailed bioassays indicated that the butanol extract was significantly active. In continuation of our phytochemical and pharmacological studies we isolated from the leaves two components possessing immunosuppressive effects. We here report the isolation and structural elucidation of the auronol glycosides hovetrichoside C (maesopsin 4-*O*-β-D-glucoside, **1**) (Yoshikawa et al. 1998), as well as the new 2,4,6-trihydroxy-2-[3′,4′-dihydroxyphenyl)-methyl]-3(2*H*)-benzofuranone-4-yl-β-D-glucopyranoside (alphitonin 4-*O*-β-D-glucoside, **2**) and their activity. These structures were elucidated by MS and various NMR techniques, including HMQC, HMBC, NOE-difference experiments.

## 2. Investigations, results and discussion

Two glycosides were isolated from the *n*-BuOH extract of the leaves using normal and reversed phase column chromatography. The HR ESI TOF MS of compound **1** gave the $[M + Na]^+$ peak at m/z 473.10543 leading to the molecular formula $C_{21}H_{22}O_{11}$ (M = 450). The sugar moiety was easily identified from its characteristic signals in the $^1H$ and $^{13}C$ NMR spectra (Table 1) as β-D-glucopyranose, thus giving $C_{15}H_{12}O_6$ as aglycone. In the $^{13}C$ NMR spectra many signals appeared as very close pairs due to an isomeric mixture, whereas some signals were very broad indicating a dynamic process. The presence of one carbonyl group ($\delta_C$ 196.15/196.03) and many aromatic signals together with the molecular formula of the aglycone with 10 double bond equivalents suggested a flavone skeleton with 4 hydroxy groups for the aglycone, only that the two aromatic carbon signals at about δ 174 had unusually high shifts. From the $^1H$ multiplicities of the signals at $\delta_H$ 7.01 and 6.59/6.58, which were parts of an AA′XX′-system of a *para*-disubstituted benzene-ring, their double integrals and their CH long-range correlations from the HMBC experiment (Table 1) a 1,4-*para*-disubstituted ring B was deduced. One substituent was identified as a hydroxy group by the high chemical shift of the connected carbon ($\delta_C$ 157.9), whereas the other substituent was a $CH_2$ group revealed by the strong CH long-range correlations from C-1′ (δ 124.72/124.74) and C-2′ (δ 132.51) to the methylene protons at δ 3.09-3.11. This suggested a modified skeleton of a dihydroaurone. The methylene protons additionally gave CH long-range correlations to the carbon at δ 107.60/107.57, revealing a hemiketal function at C-2. The occurrence of two isomers thus was due to the epimerisation of this hemiketal function. Full analysis of all CH long-range correlations (Table 1) resulted in the structure of maesopsin for the aglycone moiety and gave the signal assignment. The connection of the glucose at C-4 was deduced from the correlation of C-4 (δ 158.49/158.37) with the anomeric proton H-1″ (δ 4.85). The assignment of the signal at δ 158.49/158.37 to be C-4 and not C-6 was confirmed by its CH long-range correlations to only one of the aromatic protons of ring A, whereas C-6 should show correlations to both. A keto-enol-tautomerism was supposed to produce the tautomer **1a**. This dynamic equilibrium was able to explain the signal broadening of the involved nuclei C-5, C-6, C-7, H-5 and H-7 with half maximum intensity line widths $\Delta\nu_{1/2}$ up to 17 Hz (Table 1) and the high deshielding of C-6 ($\delta_C$ 174.22), whereas the deshielding of C-8 (δ 174.62) compared to C-8 of flavones was due to increased ring tension in the five-membered ring. Maesopsin-4-*O*-glucoside was already known from *Hovenia trichocarea* (Yoshikawa et al. 1998) whereas the 6-*O*-glucoside was found in *Ceanothus americanus* (Li et al. 1997).

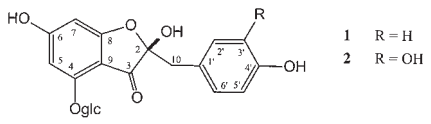
For the second glycoside **2** the molecular formula $C_{21}H_{22}O_{12}$ with one oxygen more than compound **1** was

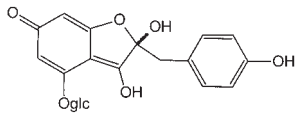
**Table 1: $^{13}C$ and $^{1}H$ NMR data of compounds 1–2 in $CD_3OD$ at 125/500 MHz (shifts of the minor compound in brackets, if different)**

| | **1**, $\delta_C$ | **1**, $\delta_H$ (J in Hz) | **2**, $\delta_C$ | **2**, $\delta_H$ (J in Hz) | HMBC correlations of **1** and **2** |
|---|---|---|---|---|---|
| 2 | 107.56 [107.60] | – | 107.60 [107.57] | – | $H_2$-10 (s)] |
| 3 | 196.15 ($\Delta\mu_{1/2}$ 6 Hz) 196.03, $\Delta\mu_{1/2}$ 6 Hz] | – | 197.01 [196.88] | – | $H_2$-10 (s) |
| 4 | 158.49 [158.37] | – | 158.37 [158.29] | – | H-5 (m), H-1“ (m) |
| 5 | 98.20 ($\Delta\mu_{1/2}$ 12 Hz) [98.57, $\Delta\mu_{1/2}$ 13 Hz] | 6.00 br s ($\Delta\mu_{1/2}$ 8 Hz) | 97.19 [97.75] | 6.07 d (1.7) [6.09 d (1.7)] | H-7 (m) |
| 6 | 174.22 ($\Delta\mu_{1/2}$ 17 Hz) | – | 171.49 | – | [e] H-5 (m), H-7 (m) |
| 7 | 93.96 [93.77] ($\Delta\mu_{1/2}$ 20 Hz for both signals together) | 5.88 br s ($\Delta\mu_{1/2}$ 5 Hz) | 93.20 [93.37] | 5.96 d (1.7) [5.98 d (1.7)] | H-5 (m) |
| 8 | 174.62 ($\Delta\mu_{1/2}$ 4 Hz) | – | 174.64 [174.59] | – | [e] H-7 (m) |
| 9 | 102.68 ($\Delta\mu_{1/2}$ 8 Hz) [102.51, $\Delta\mu_{1/2}$ 11 Hz] | – | 103.42 [103.65] | – | H-5 (m), H-7 (m) |
| 10 | 41.97 [42.17] | 3.10 s[c] [3.09 d [a] (14.1)/ 3.11 d [a] (14)][c] | 42.17 [42.30] | 3.05 s[c] [3.04 d [a] (13.9)/ 3.06 d [a] (14.1)][c] | H-2′ (m), H-6′ (m) |
| 1′ | 125.72 [125.74] | – | 126.17 [126.23] | – | $H_2$-10 (s), H-5′ (s) |
| 2′ | 132.51 | 7.01 ″d″ [b] (8.4) | 118.62 [118.73] | 6.65 d (2.0) [6.66 d (2.0)] | $H_2$-10 (s), H-6′ (s) |
| 3′ | 115.77 [155.73] | 6.59 ″d″ [b] (8.3) [6.58 ″d″ [b] (8.3)] | 145.62 [145.66] | – | H-2′ (w), H-5′ (s) |
| 4′ | 157.21 [157.24] | | 145.16 | – | H-2′ (s), H-3′ (w), H-5′ (w), H-6′ (s) |
| 5′ | 115.77 [155.73] | 6.59 ″d″ [b] (8.3) [6.58 ″d″ [b] (8.3)] | 115.90 [115.85] | 6.57 d (8.1) [6.55 d (8.0)] | H-3′ (m) |
| 6′ | 132.51 | 7.01 ″d″ [b] (8.4) | 123.08 [122.99] | 6.51 dd (8.1; 2.1) [6.50 dd (8.1; 2.0)] | $H_2$-10 (s), H-2′ (s) |
| 1″ | 101.63 | 4.85 d (7.6) [hidden under HDO] | 101.56 [101.67] | 4.89 d (7.6) [4.87 d (7.4)] | H-2″ (m) |
| 2″ | 74.02 [74.09] | 3.54 m[d] | 73.97 [74.08] | 3.53 m[d] [3.55 m[d]] | H-3″ (m) |
| 3″ | 77.37 [77.34] | 3.48 m[d] | 77.32 | ca 3.49 m[d] | H-2″ (m) |
| 4″ | 71.14 | 3.42 m[d] | 71.16 | ca 3.42 m[d] | H-3″ (m) |
| 5″ | 78.35 [78.29] | 3.43 m[d] | 78.37 [78.30] | ca 3.41 m[d] | H-4″ (m) |
| 6″ | 62.30 | 3.88 br d (12.3) / 3.70 m | 62.30 | 3.888 d (12.0) / 3.68 dd (12.1; 5.5) [3.892 d (12.1) / 3.70 dd (12.0; 5.0)] | |

[a] Very strong AB-effect
[b] AA'XX' system
[c] Exchangeable
[d] Overlapped
[e] Correlations from the signals δ 174.2 und 174.6 not clearly distinguishable

obtained from the high resolution of the $[M + Na]^+$ peak at m/z 489.10134 in the ESI TOF MS. The NMR spectra were very similar to those of **1**, showing also pairs of signals with mostly similar chemical shifts to **1**. From the integrals of the $^{1}H$ signal pairs δ 6.07/6.09 and δ 5.96/5.98 the ratio of isomers was roughly determined as 55 : 45. The difference between **1** and **2** was located at ring B, where the ABM spin system for a 1,2,4-trisubstituted benzene ring was revealed by the multiplicities of the proton signals at δ 6.50/5.51 (both dd, J = 8 and 2 Hz), δ 6.57/6.55 (both d, J = 8 Hz) and δ 6.65/6.66 (both d, J = 2 Hz). Thus, an additional hydroxy group at C-3′ was deduced for compound **2**. Analysis of the CH long-range correlations (Table 1), which were identical to the correlations of **1** under regard of the missing H-3′, confirmed this structure including the glycosylation site at C-4 which was revealed by the correlation C-4/H-1″. Further proof was gained from the NOE-difference spectra. The anomeric proton (δ 4.89/4.87) gave NOE interaction to H-5 (δ 6.07/6.09) and no effect to H-7, whereas the enhancement of the signals of H-2′ (δ 6.65/6.66) and H-6′ (δ 6.51/6.50) upon irradiation of $H_2$-10 (δ 3.04–3.06) confirmed the substitution pattern of ring B. No significant signal broadening was observed for **2**, which may be explained by an acceleration of the keto-enol tautomerism by the additional hydroxy group. The aglycone of **2** is known as alphitonin from the heart wood of the Australia “red ash” (*Alphitonia excelsa*) (Birch et al. 1960; Smith and Read 1922), whereas its 4-*O*-glucoside is a new natural product. Compounds **1** and **2** belong to the rare auronol glucosides group.




1a

The results of the lymphocyte stimulation tests of the fractions and isolated compounds were given in Tables 2 and 3, compared with cyclosporin A, a potent immunosuppressant widely used in clinic to prevent graft rejection or in the

**Table 2: Effect of fractions 6.1–6.6 from the BuOH extract of *Artocarpus tonkinensis* on the inhibition of transformation in lymphocytes**

| | Inhibition I[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Concentration (mg/ml) | 1.25 | 0.625 | 0.312 | 0.156 | 0.078 | 0.039 | 0.019 | 0.009 |
| Fraction 6.1 | 99.6 | 84.5 | 66.9 | 34.2 | 56.1 | 35.7 | 9.2 | 12.8 |
| Fraction 6.2 | 96.8 | 76.5 | 56.8 | 35.3 | 26.0 | 20.6 | 15.7 | 0 |
| Fraction 6.3 | 99.0 | 81.2 | 72.8 | 39.0 | 36.4 | 33.9 | 13.4 | 37.6 |
| Fraction 6.4 | 98.5 | 86.4 | 46.8 | 33.5 | 24.6 | 22.4 | 3.1 | 15.4 |
| Fraction 6.5 | 87.0 | 84.2 | 78.8 | 29.2 | 20.0 | 6.9 | 0 | 0 |
| Fraction 6.6 | 97.1 | 91.4 | 55.8 | 0 | 19.7 | 11.1 | 12.4 | 10.5 |
| Cyclosporin A[b] | 99.1 | 99.2 | 99.8 | 96.7 | 87.9 | 78.0 | 72.7 | 72.0 |

**Table 3: Effect of 1, 2, catechin and cyclosporin A on the inhibition of transformation in lymphocytes**

| | Inhibition I[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Concentration (mg/ml) | 1.25 | 0.625 | 0.312 | 0.156 | 0.078 | 0.039 | 0.019 | 0.009 |
| Compound 1 | 99.6 | 99.5 | 99.3 | 99.0 | 56.5 | 18.9 | 4.4 | 3.5 |
| Compound 2 | 99.3 | 92.9 | 82.9 | 23.0 | 21.3 | 19.5 | 15.9 | 0 |
| Catechin | 99.5 | 81.8 | 46.3 | 39.3 | 20.1 | 23.6 | 10.2 | 11.5 |
| Cyclosporin A[b] | 99.1 | 99.2 | 99.8 | 96.7 | 87.9 | 78.0 | 72.7 | 72.0 |

[a] Calculated according to the formula given in chapter 3
[b] Concentration reduced 10 times

treatment of various autoimmune diseases. In this test the immunosuppressive activity of investigated substances will be expressed by transformed blood cells (blast cells) in the test mixture (Trao et al. 1998). Both compounds **1** and **2** showed strong activity at higher concentrations and significantly reduced activity at low concentrations. These are the first representatives of auronol glycosides possessing immunosuppressive activity. This result gives evidence for the use of the crude extract from the leaves of *Artocarpus tonkinensis* to prevent graft rejection in skin transplantation in mice and in the treatment of autoimmune diseases like lupus and rheumatism in traditional medicine in Vietnam (Ha et al. 1994), as well as for the additional searching for new natural immunopharmacological agents.

## 3. Experimental

### *3.1. Instruments and general methods*

NMR: Bruker Avance 500. HR-ESI MS: QStar Pulsar (Applied Biosystems). $[\alpha]_D$: Digital Polarimeter Jasco Tip 1000. FT-IR: Infrared spectrophotometer Nicolet IMPACT 410, intensities were given as s = strong, m = medium, w = weak. TLC: Silica gel 60 F-254 (Merck); reversed phase (LiChroprep RP-8). CC: Silica gel 60 (230–400 mesh, Merck) and LiChropep RP-8 (Merck).

### *3.2. Plant material*

Leaves of *Artocarpus tonkinensis* were collected in Hanoi in April 2001. The species was identified by Dr. Nguyen Van Tap. A voucher specimen (Nr. 1482) was deposited in the herbarium of the Institute for Materia Medica, Hanoi, Vietnam.

### *3.3. Extraction and isolation*

The dried and powdered leaves (4.5 kg) were extracted with 85% aqueous MeOH at room temperature. Evaporation of MeOH *in vacuo* gave an aqueous solution which was subjected to liquid-liquid partition with $CHCl_3$ followed by *n*-BuOH. The *n*-BuOH solution was evaporated *in vacuo* and afforded 200 g residue of *n*-BuOH extract. 80 g of this extract were chromatographed over silica gel and gradually eluted with $CHCl_3$–MeOH (9 : 1–7 : 3) followed by $CHCl_3$–MeOH–$H_2O$ (60 : 35 : 5) and then MeOH 100% to give 7 fractions (fractions 1–7) of increasing polarity. Fraction 6 (3.19 g) was further chromatographed over silica gel with $CHCl_3$/MeOH/$H_2O$ (40 : 10 : 1–70 : 30 : 2 and then 65 : 35 : 5) to afford 6 fractions (fractions 6.1–6.6). Fraction 6.1 with high activity was subjected to CC over silica gel with EtOAc-MeOH–$H_2O$ (5 : 60 : 2) and finally purified by reversed phase CC over RP8 with MeOH–$H_2O$ (1 : 2) to afford **1** (163 mg, 0.0091%) and **2** (60 mg, 0.0033%).

*3.3.1. Maesopsin-4-O-β-D-glucopyranoside (**1**, 2,4,6-trihydroxy-2-[(4′-hydroxyphenyl)methyl]-3(2H)-benzofuranone-4-yl-β-D-glucopyranoside)*

Powder from MeOH; $[\alpha]_D^{25}$ −30° (MeOH, c 1.0); HR-ESI-TOF-MS (m/z): 473.10539 $[M + Na]^+$ ($C_{21}H_{22}O_{11}Na$ requires 473.10543). ESI-MS (negative ions): 449 $[M-H]^-$; IR $\nu_{max}^{KBr}$ ($cm^{-1}$): 3400–3300 (s), 2914 (w), 1684 (m), 1617 (s), 1518 (m), 1445 (s), 1357 (m), 1278 (w), 1122, 1109 (m), 1071 (m), 1005 (m), 929 (w), 834 (s), 708 (m); $^1H$ and $^{13}C$ NMR data see Table 1.

*3.3.2. Alphitonin-4-O-β-D-glucopyranoside (**2**, 2,4,6-trihydroxy-2-[3′,4′-dihydroxyphenyl)methyl]-3(2H)-benzofuranone-4-yl-β-D-glucopyranoside*

Powder from MeOH, m.p. 102-105 °C; $[\alpha]_D^{25}$ −87° (MeOH, c 0.02); HR-ESI-TOF-MS (m/z): 489.10134 $[M + Na]^+$ ($C_{21}H_{22}O_{12}Na$ requires 489.10035); ESI-MS (negative ions): 465 $[M-H]^-$; IR $\nu_{max}^{KBr}$ ($cm^{-1}$): 3500–3300 (s), 1692 (m), 1626 (s), 1459 (w), 1298 (m), 1109 (m), 1078 (m); $^1H$ and $^{13}C$ NMR data see Table 1.

### *3.4. Lymphocyte stimulation test*

The lymphocyte stimulation test (immunosuppressive activity) was caried out in the the National Hygiene and Epidemiology Institute, Hanoi, Vietnam in the following manner according to Trao et al. (1998) and Trao (2002):
Mononuclear cells were isolated from healthy human blood by gradient specific weight centrifuge over Ficoll layer (d = 1.007) with a speed of 2,000 rounds/minute; washed in PBS (pH = 7.2) and supplemented with 10% human AB serum (according to a routine procedure of the Immunology Lab of the National Hygiene and Epidemiology Institute, Hanoi, Vietnam). The cells were seeded into 96 wells of round-bottomed microtitre plates at $5–7.5 \times 10^4$ cells with twice diluted concentration (1/2–1/128) of test substance. All cultures were set up in triplicate.
After incubation at 37 °C for 72 h in a humidified 5% $CO_2$ atmosphere, 20 µl $^3H$-thymidine (specific activity 2.0 Ci/mmol) were added and the cultures incubated a further 18 h befor harvesting on a "Titertek" multiple automatic sample harvester and counting of the retained radioactivity in a "Beckman" liquid scintillation counter. Activation, as measured by $^3H$-thymidine incorporation, was expressed as counts per minute (cpm). The inhibition (% I) of the test substance was calculated as follows:

$$\%\,I = \frac{\text{cpm PHA control cell} + \text{test substance}}{\text{cpm PHA controll cell without test substance}} \times 100$$

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