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New Quinones and Hydroquinones from *Malbranchea cinnamomea* HKI 286 and HKI 296 and Interaction with Tax/CREB Expression System in Yeast

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In addition to malbranicin (**1**) and dihydromalbranicin (**5**), new substituted quinones **2**, **3**, **6** and hydroquinone **4** were isolated from the culture brothes of two strains of *Malbranchea cinnamomea*. The chemical constitutions of new metabolites **2**, **3**, **4** and **6** were elucidated by optical spectroscopy, mass spectrometry and 1D/2D NMR spectroscopy. **2** (7-methoxymalbranicin) at a concentration of 42 μM inhibited by 67% Tax/CREB-mediated expression of β-galactosidase in a recombinant strain of *Saccharomyces cerevisiae*.

The Human T-cell lymphotrophic retrovirus type I (HTLV-I) is the ethiological agent for adult T-cell leukemia (ATL) and various human mycopathies/neuropathies[1,2]. HTLV-1 encodes 40 kDa phosphoprotein, Tax, which has been implicated in cellular transformation. In similarity with several other onco-proteins such as Myc, Jun, and Fos, Tax is a transcriptional activator. HTLV-I Tax protein activates transcription from three 21-base-pair repeat sequences in the viral enhancer. The HTLV-I 21-bp repeat contains a TGACGT motif that is homologous to the cAMP-responsive element (CRE) and crucial for tax transactivation[3]. Tax exhibits marginal affinity for DNA but rather interacts with cellular CRE-binding proteins such as CREB (CRE binding protein) to enhance their affinity for the HTLV-I 21-bp repeats[4]. Consequently, one mechanism by which Tax activates transcription is mediated through the direct interaction with CREB to stabilize their assembly on the Tax-responsive CRE motifs in the HTLV-I enhancer[5]. For that reason the inhibition of mutual interaction of Tax and CREB and their binding to DNA has been proposed as a new target in the search for new drugs with activity against HTLV-I.

Recently we assayed the inhibitory activity of chromatographic microfractions obtained by HPLC from the crude extracts of two fungal strains, *Malbranchea cinnamomea* HKI 286 and *Malbranchea cinnamomea* HKI 296. Several of these samples displayed inhibition of Tax/CREB-mediated expression of β-galactosidase in a recombinant *Saccharomyces cerevisiae*[6,7]. This finding led us to isolate the major metabolites of the above strains and to check the inhibitory activity of the purified compounds. Here we report the isolation and structures of novel metabolites **2**, **3**, **4** and **6**, which occurred along with the known metabolites malbranicin (**1**) and dihydromalbranicin (**5**)[8] and their effects on the Tax/CREB-dependent functional transcription assay in *Saccharomyces cerevisiae*.

Results and Discussion

Isolation and Physico-chemical Properties

Malbranchea cinnamomea HKI 286 and *Malbranchea cinnamomea* HKI 296 were obtained from the fungal strain collection of the Hans-Knöll-Institute for Natural Products Research, Jena Germany. Both strains were cultivated at 32°C (see Experimental).

After cultivation 14 liters of the culture broth of both strains were extracted twice with ethyl acetate in ratio 1 : 1. The extracts from each strain were evaporated to dryness. The residues of both extracts were fractionated by several subsequent chromatographic steps such as column

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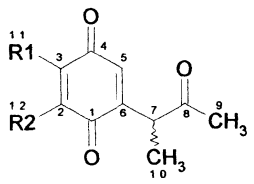
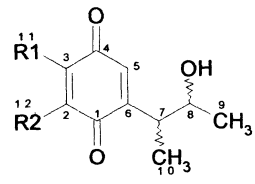
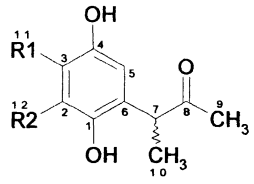
Fig. 1. Chemical constitutions of malbranicin (**1**) and new metabolites **2**, **3**, **4** and **6** and dihydromalbranicin (**5**).



1 R1 = H, R2 = OCH_3
2 R1 = OCH_3, R2 = OCH_3



3 R1 = OCH_3, R2 = OCH_3



4 R1 = OCH_3, R2 = OCH_3
5 R1 = H, R2 = OCH_3

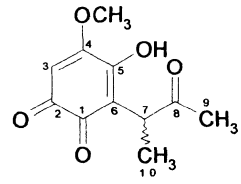


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Table 1. Physico-chemical properties of new compounds **2**, **3**, **4** and **6**.

	2	**3**	**4**	**6**
Appearance	reddish-brownish wax	reddish-brownish wax	slightly brownish solid	reddish solid
Melting point	-	-	108 - 110 °C	> 150 °C (decomp)
Molecular weight (HREI-MS; M^+)	238.0825 calcd. 238.0841	240.0980 calcd. 240.0998	240.0991 calcd. 240.0998	224.0693 calcd. 224.0685
Chemical formula	$C_{12}H_{14}O_5$	$C_{12}H_{16}O_5$	$C_{12}H_{16}O_5$	$C_{11}H_{12}O_5$
UV-VIS λ_{max} (nm MeOH) ε (cm^2/mmol)	300; $2.3 \cdot 10^4$ 402; $1.6 \cdot 10^4$	300; $2.4 \cdot 10^4$	300; $2.5 \cdot 10^4$	300; $5.9 \cdot 10^5$ 340; $3.1 \cdot 10^5$
IR (ν_{max}/cm^{-1}, KBr)	930,945,1048, 1097,1192,1252, 1310,1350,1434, 1460,1602,1655, 1707,2940,3462	751,914,925,976, 1050,1056,1098, 1190,1370,1466, 1602,1654,1714, 2852,2931,3520	881,945,1046, 1090,1095,1189, 1275,1357,1434, 1438,1464,1496, 1601,1703,2941, 3415	726,793,843, 1009,1220,1314, 1355,1383,1439, 1530,1605,1647, 1705
$[\alpha]^{22}_D$ (*c* 0.05, MeOH)	- 42.3 °	- 18.2 °	- 126.4 °	- 49.5 °
R_f on TLC (Merck silica gel aluminium sheets; $CHCl_3$/MeOH; 9:1	0.95	0.81	0.45	0.15

chromatography on silica gel 60 (Merck, 0.063~0.1 mm; elution by chloroform), and preparative TLC on silica gel aluminium sheets (Merck F_{254}, $CHCl_3$/MeOH; 95 : 5; v/v). The isolation of the compounds was guided chemically following either the natural colors of the given fraction, or staining behaviour with 1% vanillin in conc. H_2SO_4. Both strains produced a qualitatively identical but quantitatively different metabolite spectrum. Thereby six compounds (**1**~**6**, Fig. 1) were isolated. Compound **1** was readily identified as malbranicin (**1**) by physico-chemical measurements (MS, NMR). Malbranicin was reported earlier as a metabolite of *Malbranchea cinnamomea* TAIM13T54[8]. Another recurrent metabolite was identified as dihydromalbranicin (**5**) which was also previously reported as the reduction product of malbranicin[8].

The physico-chemical properties of the new metabolites **2**, **3**, **4** and **6** are shown in Table 1.

Structure Elucidation

The molecular weights of compounds **2**, **3**, **4** and **6** and their chemical formulae were readily suggested by positive ion ESI-MS and HREI-MS, respectively (Table 1). Compounds **3** and **4** appeared to be isomers due to identical molecular masses and formulae. The FTIR spectra of **1**, **3** and **6** showed the presence of carbonyl absorbances, and this was supported by UV-visible spectra attributable to quinoid chromophores. Conclusive evidence for the chemical constitutions of **2**, **3**, **4** and **6** was furnished by 1D and 2D NMR measurements (^{1}H, ^{13}C, ^{1}H,^{1}H-COSY, DEPT, HMQC, HMBC, NOESY). The C,H long-range couplings for **2** are shown in Fig. 2 as an example. The assignment of chemical shift and coupling data is given in Table 2. By this way, too, co-isolated compounds **1** and **5** were identified as malbranicin and dihydromalbranicin[8]. The constitution of **6** as an ortho-quinone was confirmed unambiguously by the occurrence of quinone carbonyls in the ^{13}C NMR spectrum (173.4 ppm, 187.1 ppm) and observable C,H long-range couplings ($^3J_{H\text{-}7,C\text{-}1}$ and $^3J_{H\text{-}7,C\text{-}5}$; Fig. 3) in the HMBC spectrum.

Novel compounds **2**, **3**, **4**, **6** and the known metabolites **1** and **5** from both strains of *Malbranchea cinnamomea* thus appear as products of a branched pathway leading to monoalkyl-substituted quinones and hydroquinones. In addition to variations in the ring substitutions, a further source of structural diversity is the occurrence of either a keto or a hydroxyl group in the side chain.

As for malbranicin (**1**) and dihydromalbranicin (**5**)[8],

Fig. 2. C,H long-range couplings from the HMBC spectrum of **2**.

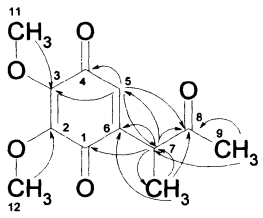


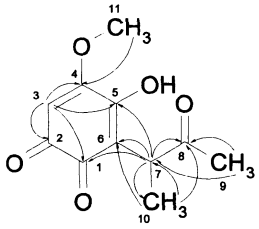
Table 2. Assignment of ^{1}H and ^{13}C NMR spectra of **2**, **3**, **4** and **6** (in $CDCl_3$; δ in ppm, coupling constants in Hz).

Position	2		3		4		6	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	183.5 (s)	-	184.3 (s)	-	140.2 (s)	-	173.4 (s)	-
2	149.9 (s)	-	144.5	-	139.5 (s)	-	187.1 (s)	-
3	144.9 (s)	-	145.1	-	138.4 (s)	-	101.6 (d)	5.48 s
4	183.7 (s)	-	184.4 (s)	-	142.4 (s)	-	164.6 (s)	-
5	131.2 (d)	6.39 s	130.9 (d)	6.44 s	108.8 (d)	6.43 s	169.9 (s)	-
6	146.3 (s)	-	149.6 (s)	-	122.0 (s)	-	114.1 (s)	-
7	45.3 (d)	3.84 q, 7.0	39.3 (d)	3.01 m	46.6 (d)	3.99 q, 7.1	42.4 (d)	3.49 q, 7.1
8	206.9 (s)	-	71.4 (d)	3.82 m	209.7 (s)	-	209.5 (s)	-
9	28.9 (q)	2.25 s	21.6 (q)	1.18 d, 7.2	28.2 (q)	2.06 s	26.9 (q)	1.80 s
10	14.4 (q)	1.25 d, 7.0	16.0 (q)	1.12 d, 7.1	15.5 (d)	1.31 d, 7.1	19.9 (q)	0.94 (d), 7.1
11	61.2 (q)	3.98 s	61.1 (q)	4.01 s	60.7 (q)	3.87 s	55.6 (q)	3.67 s
12	61.2 (q)	4.00 s	61.2 (q)	3.80 s	60.83 (q)	3.91 s	-	-

Abbreviations: s: singlet, d: doublet, t: triplet, q: quartet, br: broad.

compounds **2**, **3**, **4** and **6** displayed negative optical rotations (Table 1). However, the stereochemistry at C-7 and C-8 in **3** and at C-7 in **2**, **4** and **6** was not determined.

Fig. 3. C,H long-range couplings from the HMBC spectrum of **6**.



Biological Properties

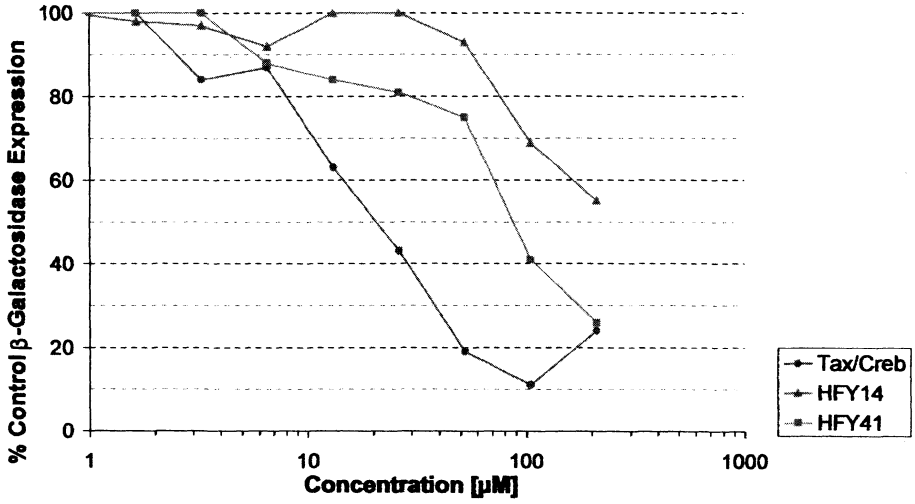
The Tax/CREB expression system in *Saccharomyces cerevisiae* was described earlier in detail[6,7]. β-Galactosidase production was taken as a measure of Tax/CREB interactions and positive transcriptional regulation. Table 3 displays the effect of 42 μmolar concentrations of **1**~**6** on β-galactosidase production by the recombinant *Saccharomyces* strain.

Compound **2** (7-methoxymalbranicin) displayed the only specific inhibition of Tax/CREB expression as measured by a coupled β-galactosidase reduction by 67%. The specific inhibition of the Tax/CREB dependent transactivation by compound **2** is depicted in Fig. 4. The dose-response curves show that increasing concentrations

Table 3. Specific inhibition of the Tax/CREB dependent transactivation of a β-galactosidase reporter *via* 21-bp repeats in *Saccharomyces cerevisiae* by compounds malbranicin (**1**), **2**, **3**, **4**, dihydromalbranicin (**5**) and **6**.

	1	**2**	**3**	**4**	**5**	**6**
Inhibition of control β-galactosidase expression (in %) by 42 μmolar concentrations	0	67	0	0	0	0

Fig. 4. Specific inhibition of the Tax/CREB dependent transactivation of a β-galactosidase reporter *via* 21-bp repeats in *Saccharomyces cerevisiae* by compound **2**.



The percent of control β-galactosidase expression inhibited by 1.6~210 μM of compound **2** in cultures of the Tax/CREB reporter strain[6] and two control strains HFY14 and HFY41[11] was plotted to generate a dose-response curve.

of **2** in the range from 13 to 50 μM inhibited the expression of the β-galactosidase reporter gene only in the Tax/CREB-reporter strain and not or only to a significantly lower extent in the control strains HFY14 and HFY41 (see Experimental). At higher concentrations **2** has an overtly toxic effect on the yeast cells (100 μM and above). The relatively high concentrations of **2** necessary for the specific inhibition of the β-galactosidase reporter gene in the Tax/CREB-reporter strain and the lower toxicity of **2** in comparison with mammalian cells[8] may be caused by a limited drug uptake. Compounds **1**, **3**, **4**, **5** and **6** were inactive. Obviously the presence of both a *p*-quinoid structure and a C-7 keto group at C-8 appears to be needed for inhibitory activity towards Tax/CREB interactions. Inhibition of human T-cell leukemia virus proliferation by quinoid structures such as avarone[9] and phenylmethylbenzoquinones[10] was reported. However, interaction of these antiviral agents with Tax/CREB proteins and Tax/CREB DNA complexes has not been shown.

Experimental

General

Melting points (uncorrected) were determined with a Kofler-type melting point apparatus. UV spectra were recorded on a Specord 2000 double beam spectrometer (Analytik Jena, Germany), IR spectra on a satellite FT-IR instrument equipped with ATR device (Mattson, Chicago, USA), optical rotations on a Propol polarimeter (Dr. Kernchen Optics, Seelze, Germany), ESI-mass spectra on a Quattro triple quadrupole mass spectrometer (VG Biotech, Altrincham, England), HREI mass spectra on a high resolution sector field instrument Finnigan MAT 95 XL (Finnigan, Bremen, Germany) and NMR spectra on a Bruker Avance DRX 500 instrument. The microfractionation was performed on a Shimadzu LC-10 HPLC system consisting of two LC-10AT pumps, a SCL-10A *VP* system controller, an SIL-10AD *VP* auto injector, a SPD-M10A *VP* diode-array detector, a FRC-10A fraction collector and the *CLASS-VP* soft ware.

Fermentation

Similar secondary profiles were observed with both strains in two different media. A small piece of a nature slant culture of the fungal strains grown on a malt extract agar (malt extract 4%, yeast extract 0.4%, and agar 1.5%; distilled water, pH 6.0) was used to inoculate 1 liter Erlenmeyer flasks containing 250 ml of the producing medium consisting of malt extract 2%, glucose 1%, yeast extract 0.1%, soy bean meal 0.2%, KH_2PO_4 0.1%, and $MgSO_4 \times 7H_2O$ 0.05%, pH 5.5. The cultivation was done on rotary shakers (110 r.p.m.) for 15 days at 32°C. An alternative route for cultivation was carried out also for 15 days at 32°C by surface culture using malt extract 2%, yeast extract 0.2%, glucose 1%, and $(NH_4)_2HPO_4$ 0.05 %, pH 6.0.

Microfractionation

The residue of the extract from each strain was dissolved in MeOH (1 mg/ml) and separated by HPLC. A 20-μl aliquot was injected onto a HPLC column (125×4.6 mm) equipped with a guard column (20×4.6 mm) which were packed with Nucleosil-120 C-18, 5 μm (GROM, Herrenberg, Germany). The fractions were eluted by a linear gradient elution using ACN and 0.1% aqueous TFA solution starting at 0% ACN to 100% ACN within 15 minutes at a flow rate of 2 ml/minute with 1 minute hold at 100% ACN, followed by a 5-minute post-time under initial conditions for re-equilibration. The UV-absorbance of the elutes was monitored simultaneously at 210 and 230 nm. The ACN was removed under vacuum and the remaining aqueous solutions by freezing drying.

Determination of Tax/CREB Dependent Transactivation[6,7]

The assay followed the procedure described in the literature[6]. Two copies of the HTLV-I 21-bp repeat were inserted upstream of the yeast minimal cytochrome c1 oxidase promoter at bp-178 to control the expression of bacterial *lacZ*. This construct was integrated into the yeast genome of *S. cerevisiae* the strain TFY176, resulting in strain MSY9, which was subsequently transformed with Tax and CREB expression vectors pMS8 and pMS11[6]. The test strain was cultivated over night in YNB-medium supplemented with leucine and tryptophan and diluted by sterile water to $OD_{600}=1.0$. 50 μl aliquots of the diluted yeast culture were dispensed in 96-well microtiter plates, 50 μl of medium supplemented with 1 μl **1**~**6** (1 mg/ml in DMSO) were added and the microtiter plate was incubated for 3 hours at 30°C. As controls the yeast two-hybrid strain HFY14 expressing a carboxy-terminal protein fragments of the oncoprotein c-Myc and an amino-terminal protein fragment of its interacting partner protein Max and HFY41 expressing carboxy-terminal protein fragments of c-Myc and its interacting partner protein Miz-1[11] were used and processed in the same way. β-Galactosidase expression was quantitatively detected by *o*-nitrophenyl-β-D-galactopyranoside hydrolysis measured photometrically at 420 nm upon permeabilization of cells[7].

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