Measurement of cytokines concentration

For quantitative determination of human TNF-α, IL-1β, IL-6, and GM-CSF, the commercial kit "Quantikine" (R&D system, MN, USA) for each cytokine was used. Using standard cytokines and reagents for ELISA provided by the commercial kit, the measurement of different cytokine concentrations was performed exactly following the instruction manual. Both standard cytokine and sample to be tested were added to an ELISA plate which was precoated with a cytokine-specific monoclonal antibody. The plate was incubated at 37 °C for two hours, washed, and then a cytokine-specific polyclonal antibody conjugated with enzyme was added. The plate added with antibody and enzyme was incubated at 37 °C for another two hours and then washed again. After the substrate solution was added, the reaction went on at room temperature for 30 minutes and was finally stopped with 2N sulfuric acid. The optical density of each well on the ELISA plate was determined by an ELISA reader set at 450 nm.

Statistical analysis

Comparison of the results between different groups throughout the study was based on the Wilcoxon rank-sum test.

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A Novel Anthranilic Acid Derivative from *Isatis tinctoria*

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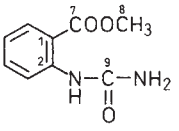
Isatis tinctoria L. (Brassicaceae) was used for centuries to produce the blue dye indigo due to its indigo precursors isatan B and indican. Recently, we reported on fungicidal and insecticidal compounds (1), which explain the wood preservative properties (2) of the sap of *I. tinctoria*. Ficht et al. (3) described the successful use of its leaves as a wound and allergic prophylaxis in the form of tea preparations. Only a few chemical investigations have been made on the chemical composition of *I. tinctoria* (see, e.g., 4–8). Our study on the organic phase of its leaves resulted in the isolation of the fungicidal and insecticidal compounds tryptanthrin, indole-3-acetonitrile, and *p*-coumaric acid methyl ester (9). The present research on the leaves was carried out to identify further compounds, especially glycosides, contributing to the plant's pharmacological effects. We isolated one novel anthranilic acid derivative *N*-carbamoylanthranilic acid methyl ester (**1**) and twelve known compounds [two β-phenylethyl glycosides: martynoside (**2**) (10), β-phenylethyl-β-D-glucopyranoside (**3**) (11); four ionone derivatives: roseoside (**4**) (12), 3-oxo-α-ionyl-β-D-glucopyranoside (**5**) (13), icariside B_2 (**6**) (14), blumenol C-β-D-glucopyranoside (**7**) (11); one lignan compound: icariside E_4 (**8**) (15); one phenol glycoside: 4-hydroxy-2-methoxy-phenyl-β-D-glucopyranoside (**9**) (16); two nucleosides: adenosine (**10**), uridine (**11**) (4); anthranilic acid (**12**) and its derivative: *N*-formylanthranilic acid methyl ester (**13**) (17)].

The methanolic extract of the leaves was fractionated into a $CHCl_3$ layer and an H_2O layer. Both layers were evaporated in vacuo and separated by repeated column chromatography on silica gel and/or reverse phase. Thirteen compounds were obtained. Uridine (**11**) was already identified in the roots of *I. tinctoria* (4). Compounds **2**–**10** and **12**–**13** are known compounds, but isolated for the first time from this plant. They were assigned respectively by comparison with published data (^{1}H-NMR, ^{13}C-NMR, MS, if necessary: UV, CD). All data and spectra are obtainable on request from the author of correspondence.



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Compound **1** was obtained as a colourless crystalline solid and is reported for the first time from a natural source. Its high resolution mass spectrum exhibited a molecular ion peak at *m/z* 194 $[M]^+$ consistent with a molecular formula of $C_9H_{10}N_2O_3$. Other prominent fragment ions were observed at *m/z* 177 (M − 17) $[M - NH_3]^+$, 162 (M − 32) $[M - MeOH]^+$, 146 (M − 48) $[M - MeOH - NH_2]^+$, and 119 (M − 75) $[M - MeOH - NH_2 - CO + H]^+$. The ^{1}H-NMR spectrum ($CDCl_3$-CD_3OD, 300 MHz) showed the existence of four aromatic proton signals. Their coupling patterns were consistent with a 1,2-disubstituted aromatic ring. The APT-NMR and the ^{13}C-^{1}H COSY experiment revealed the presence of four aromatic methines, two aromatic quaternary carbon atoms, two further quaternary carbon atoms (δ 156.6, 168.6), and a methyl carbon atom due to a methoxy group. Based on the fragmentation pattern and the spectral evidence, the structure was confirmed as *N*-carbamoylanthranilic acid methyl ester. The melting point and the spectral data of **1** were in good agreement with those of the synthetic compound (18).

Materials and Methods

Plant material

Plants of *I. tinctoria* L. were cultivated from seeds (Botanical Garden Oldenburg, Germany) in the garden of the Institute for Plant Biochemistry Halle, Germany. A voucher specimen of *I. tinctoria* is deposited in the herbarium of the Institute.

Extraction and isolation

Dried and powdered leaves (5.5 kg) were extracted 3 times with methanol (30 l). The solvent was evaporated, and the residue was fractionated into a $CHCl_3$ layer and an H_2O layer. The $CHCl_3$ layer was dried under reduced pressure (220 g), and then subjected to a column of silica gel (4 kg). The mobile phase (40 l) was a linear gradient starting from 100 % of $CHCl_3$ to 100 % of MeOH, to afford 9 fractions. The appropriate fraction was further purified by repeated TLC on silica gel with $CHCl_3$/MeOH, 95 : 5, to yield compound **1** (7 mg, R_f 0.47).

The H_2O layer was evaporated, and the residue (170 g) was chromatographed on a silica gel column as described above. The fractions of interest were subjected to CC on reverse phase (LiChroprep RP-8, 250 g) and eluted with an MeCN-H_2O-mixture (solvent A: MeCN, solvent B: H_2O, gradient: 5 to 100 % A in 7 h). The appropriate fractions were combined and evaporated, the residue was chromatographed 2 times on preparative TLC (silica gel) with $CHCl_3$/MeOH/H_2O, 74 : 23 : 3, to obtain three fractions (**I** R_f 0.20, **II** R_f 0.30, **III** R_f 0.46). Further purification of fraction **I** on HPLC (LiChroprep RP-18, 5 – 20 µm, solvent A: MeCN, solvent B: H_2O, gradient: 5 to 25 % A in 0 – 20 min, isocratic: 25 % A in 20 – 32 min, flow: 2.0 ml/min, UV detection: λ 312 nm) resulted in martynoside (**2**, 10 mg, t_R 25.5 min). HPLC of **II** (same conditions as **I**, gradient: 5 – 27 % A in 0 – 10 min, 27 – 30 % A in 10 – 24 min, UV detection: λ 230 nm) yielded roseoside (**4**, 2 mg, t_R 12.3 min). The HPLC of **III** (same conditions as **I**, gradient: 5 – 40 % A in 0 – 25 min, flow: 5.0 ml/min, UV detection: λ 230 nm) gave icariside B_2 (**6**, 6 mg, t_R 14.4 min), β-phenylethyl-β-D-glucopyranoside (**3**, 10 mg, t_R 15.3 min), 3-oxo-α-ionyl-β-D-glucopyranoside (**5**, 13 mg, t_R 17.2 min) and blumenol C-β-D-glucopyranoside (**7**, 8 mg, t_R 18.6 min). Without HPLC, indole-3-acetonitrile (550 mg, R_f 0.61) as the main fraction and *p*-coumaric acid methyl ester (5 mg, R_f 0.25), which were already isolated from the organic phase (9), were obtained purely by repeated TLC on reverse phase (1. RP-18, 2. RP-8, MeOH/H_2O/HOAc, 68 : 30 : 2). Icariside E_4 (**8**, 2 mg, R_f 0.30), 4-hydroxy-2-methoxyphenyl-β-D-glucopyranoside (**9**, 2 mg, R_f 0.70), adenosine (**10**), 20 mg, R_f 0.55), uridine (**11**, 33 mg, R_f 0.83), anthranilic acid (**12**, 14 mg, R_f 0.27), and *N*-formylanthranilic acid methyl ester (**13**, 0.5 mg, R_f 0.22) were chromatographed in the same way.

Since acetone extraction (excluding methanol) also resulted in the isolation of methyl esters, we can reasonably assume that the methyl esters of compound **1** and **13** are primary constituents of the plant, and not artifacts derived during extraction and isolation.

N-Carbamoylanthranilic acid methyl ester (**1**)

Colourless crystals (7 mg), m.p. 172 °C; Lit. (18): 175 – 177 °C. ^{1}H-NMR ($CDCl_3$ + CD_3OD, 300 MHz): δ = 8.20 (dd, $J_{4,6}$ = 1.1 Hz, $J_{5,6}$ = 8.7 Hz, 1H, 6-H), 7.81 (dd, $J_{3,5}$ = 1.7 Hz, $J_{3,4}$ = 8.1 Hz, 1H, 3-H), 7.32 (ddd, $J_{3,5}$ = 1.7 Hz, $J_{5,6}$ = 8.7 Hz, $J_{4,5}$ = 7.3 Hz, 1H, 5-H), 6.83 (ddd, $J_{4,6}$ = 1.1 Hz, $J_{4,5}$ = 7.3 Hz, $J_{3,4}$ = 8.1 Hz, 1H, 4-H), 3.85 (s, 3H, 8-H_3). ^{13}C-NMR ($CDCl_3$ + CD_3OD, 75 MHz): δ = 168.6 (C-7), 156.6 (C-9), 142.0 (C-2), 134.0 (C-4), 130.5 (C-6), 120.9 (C-5), 119.6 (C-3), 114.3 (C-1), 51.8 (C-8). MS (70 eV), *m/z* (%): 194 (25) $[M]^+$, 177 (6), 162 (52), 146 (100), 119 (5), 118 (7), 92 (4), 90 (7). Synthesis of **1** (18): 500 mg anthranilic acid methyl ester, dissolved in 7 ml HOAc/H_2O (1 : 1) at 22 °C, were cooled to 10 °C and stirred with aqueous KOCN (290 mg in 1 ml H_2O). The precipitate was evaporated in vacuo. The solid residue was recrystallized from absolute methanol at 30 °C.

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