3. Extraction

Dried powdered roots of *Arenaria juncea* (639 g) were defatted with 5 l *n*-hexane and extracted successively with 5 l $CHCl_3$ and 5 l MeOH. After removal of the solvent by evaporation under reduced pressure, the MeOH extract (30 g) was suspended in H_2O (400 ml) and submitted to partition between H_2O saturated *n*-BuOH (3×200 ml). After evaporation of the solvent under reduced pressure, 23 g of the *n*-BuOH fraction was obtained. The *n*-BuOH extract was solubilized in MeOH (10 ml) and precipitated in Et_2O (3×250 ml) yielding 7 g of a crude saponin fraction of which 4 g was dialysed for 3 days and submitted to column chromatography on Sephadex LH-20 eluted by MeOH yielding four fractions (F1–F4).

4. Isolation and characterization of 1

Five saponins were obtained from F2 (810 mg) (Gaidi et al. 2001). F3 (226 mg) was first fractionated by column chromatography on Sephadex LH-20 eluted by MeOH, then by MPLC on reversed phase material, Lichroprep RP-18, Merck (40–63 μm) eluted with MeOH–H_2O (linear gradient 50–60%) to give compound **1**. Final purification was carried out on Sephadex LH-20 eluted by MeOH to obtain compound **1** (10 mg).
Junceoside D (**1**): Yellow powder. $[\alpha]_{20}^{D} + 16°$ (c 0.10, MeOH); IR ν_{max} KBr cm^{-1} 3398, 2928, 1735, 1718, 1615, 1386, 1220; negative FABMS glycerol matrix: m/z 807 $[M-H]^-$, 727 $[(M-H)-80]^-$, 565 $[(M-H)-80-162]^-$, 485 [(M−H)-80-162-80]; positive HRESIMS: m/z 831.2568 $[M + Na]^+$ (Calcd for 831.2544, $C_{35}H_{52}O_{17}NaS_2$). MALDITOFMS: m/z 807 $[M-H]^-$, 845 $[(M + K-H)-H]^-$, 727 $[M-H-80]^-$. ^1H and ^{13}C NMR: Table.

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Boehringer Ingelheim Pharma GmbH & Co. KG[1], Biberach an der Riss, Germany, and Shanghai Institute of Materia Medica[2], Shanghai, China

Clausine Z, a new carbazole alkaloid from *Clausena excavata* with inhibitory activity on CDK5

O. Potterat[1], C. Puder[1], W. Bolek[1], K. Wagner[1], Changqiang Ke[2], Yang Ye[2], F. Gillardon[1]

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Dr. Olivier Potterat, Boehringer Ingelheim Pharma GmbH & Co. KG, Birkendorfer Strasse 65, D-88397 Biberach an der Riss, Germany
Olivier.potterat@unibas.ch

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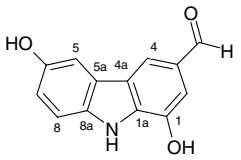
A new carbazole alkaloid, named clausine Z, has been isolated from the stems and leaves of *Clausena excavata* Burm. (Rutaceae). Its structure was established by spectroscopic methods. The compound exhibits inhibitory activity against cyclin-dependent kinase 5 (CDK5) and shows protective effects on cerebellar granule neurons *in vitro*.

CDK5 is a member of the family of cyclin dependent kinases (CDKs). Contrarily to the other members of this family, CDK5 is not involved in the cell cycle control but plays a crucial role in the development of the central nervous system and the regulation of neuronal signal transduction. Experimental studies have shown that over-activation of CDK5 might contribute to the pathology of neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis and cerebral ischemia (Dhavan and Tsai 2001; Shelton and Johnson 2004).
In the course of our search for new CDK5 inhibitors, we noticed that an EtOAc extract of the stems and leaves of *Clausena excavata* Burm. (Rutaceae) showed *in vitro* CDK5 inhibitory activity. *C. excavata* is a shrub growing in Southeast Asia known to contain, among others, coumarins and carbazole alkaloids (Ito et al. 1996, 1997, 2000; Wu et al. 1999). Semipreparative fractionation of the bioactive extract by HPLC-UV and subsequent testing of the fractions enabled the activity to be attributed to a main peak (Rt 8.9 min) with a UV spectrum corresponding to a 9H-carbazole ring system. The compound (**1**) was isolated from an ethanolic extract of the stems and leaves by a combination of liquid/liquid partition and CC on silicagel and Sephadex LH20. The ESI TOF MS (positive ion mode, $[M + H]^+$ m/z 228.0630) allowed the molecular formula to be established as $C_{13}H_9NO_3$. 1D and 2D NMR data revealed the presence of a formyl group (δ^1H 9.91, $\delta^{13}C$ 191.6) and two hydroxy groups (δ^1H 10.24, δ^1H 9.07). The ^1H and ^{13}C resonances of the formyl group, derived from a HC-HSQC experiment, were used as starting point for the assignment of the regiochemistry of compound **1**. The close neighbourhood of the formyl proton with two aromatic protons (H-2 d, $J = 1.3$ Hz and H-4 d, $J = 1.3$ Hz) was confirmed in the HH-ROESY spectrum by the two corresponding intense cross peaks, as well as in the HC-HMBC spectrum, which showed HC

long range couplings between the formyl carbon and the aromatic protons H-2 and H-4. The connection of the spin system of the first aromatic ring with that of the second was achieved by the intense cross peak between protons H-4 and H-5 in the HH-ROESY spectrum. The signal pattern of proton H-5 (d, J = 2.3 Hz) led to the assignment of the second aromatic spin system, in which proton H-7 (dd, J = 2.3 Hz, 8.6 Hz) is located in the *m*-position and proton H-8 (d, 8.6 Hz) in the *p*-position to proton H-5. Additional proof was given in the HH-ROESY spectrum by the intense cross peaks between the aniline proton to proton H-8 and the phenolic proton (OH-1). Thus, **1** is 1,6-dihydroxy-9*H*-carbazole-3-carboxaldehyde, a new alkaloid.



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Compound **1** showed potent inhibition of recombinant CDK5 in a filter plate assay with IC_{50} of 0.51 μM (Fig. A). As a reference, the known CDK5 inhibitor butyrolactone I (Leclerc et al. 2001) exhibited an IC_{50} of 0.11 μM in our assay. We have recently demonstrated that, in some paradigms, neuroprotection correlates with inhibition of neuron-specific CDK5 (Weishaupt et al. 2003), and several small molecule non-selective CDK inhibitors (e.g. butyrolactone, roscovitine, flavopiridol) have already been reported to show neuroprotective effectiveness in various *in vitro* and *in vivo* models (Walker 1998; Shelton and Johnson 2004). Testing of compound **1** in cell cultures revealed that it protects cerebellar granule neurons against cell death induced by free radical stress with an EC_{50} of 1.1 μM (Fig. 1B). In this assay, butyrolactone I showed similar neuroprotective effectiveness with an EC_{50} of about 3 μM.

Experimental

1. General

NMR spectra were recorded on a Bruker Avance DPX400 NMR spectrometer at 303 K. For 2D experiments [HH-ROESY, HC-HSQC, HC-HMBC] standard pulse sequences contained in the software release XWINNMR 2.6 PL5 (Bruker) were employed. IR: ThermoNicolet ATR-FTIR Avatar 370 System. UV: Perkin Elmer Lambda 2S. ESI TOF MS spectra were obtained on a LCT mass spectrometer (Micromass Waters) equipped with a lockspray source. For accurate mass determination, lincomycin ($[M + H]^+$: m/z 407.2216) was used as lock mass.

2. Plant material

Stems and leaves of *Clausena excavata* Burm. were collected in Hainan Province, China in September 2001. The species was authenticated by Professor Yi Zhong from Hainan Normal University. A voucher specimen has been deposited in the herbarium of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

3. Screening extract

Stems and leaves (250 g) were extracted with CH_2Cl_2 (1.9 L) followed by EtOAc (1.9 L). To localize the bioactivity, a small amount of the EtOAc extract (2.0 g) was separated on a Waters Alliance 2690 HPLC System equipped with a Symmetry C-18 column (5 μm, 3.9 × 150 mm) and a pre-column. A gradient of MeCN in aq. NH_4OAc (1 mM, pH 4) was used (5 : 95 to 1 : 0 in 30 min; 1 ml/min). 300 μg of extract dissolved in 30 μL DMSO were injected. Detection was performed with a Waters 996 photodiode array detector. Fractions were collected at one minute intervals into a 96 deepwell plate, evaporated to dryness and redissolved in DMSO (50 μl) for biological testing.

4. Isolation procedure

Dried stems and leaves (4.75 kg) were soaked in 95% EtOH (20 L) at room temperature (3 × 3 days). After evaporation, the brown residue was suspended in water and extracted with Et_2O. After evaporation to dryness, the Et_2O extract (132 g) was applied onto a silica gel column and eluted with petroleum ether-acetone (4 : 1, 2 : 1, 1 : 1, 1 : 2, and 0 : 1) stepwise to give 6 fractions. Fraction 3 (16 g) was further subjected to a silica gel column and eluted with petroleum ether-acetone (3 : 1 and 1 : 1) to give 0.8 g of a fraction, which was further purified by CC on silica gel with $CHCl_3$–MeOH (20 : 1) to afford 230 mg of crude **1**. Final purification by Sephadex LH-20 with MeOH afforded 115 mg of **1**.

Clausine Z (1,6-dihydroxy-9H-carbazole-3-carboxaldehyde) (**1**): Amorphous brownish powder. ESI TOF MS (positive ion mode), m/z: $[M + H]^+$ 228.0630; calcd. for $C_{13}H_{10}NO_3$: 228.0661. ^{1}H NMR (DMSO-d_6) δ 11.37 (br s, NH), 10.24 (br s, HO–C-1), 9.91 (s, HC=O), 9.07 (br s, HO–C-6), 8.13 (d, J = 1.3 Hz, H-4), 7.45 (d, J = 2.3 Hz, H-5), 7.35 (d, J = 8.6 Hz, H-8), 7.25 (d, J = 1.3 Hz, H-2), 6.94 (dd, J = 8.6, 2.3 Hz, H-7). ^{13}C NMR (DMSO-d6) δ 191.6 (C=O), 151.3 (C-6), 143.6 (C-1), 134.4 (C-1a), 134.0 (C-8a), 128.7 (C-3), 123.8 (C-5a), 123.1 (C-4a), 118.8 (C-4), 115.7 (C-7), 112.3 (C-8), 106.4 (C-2), 104.9 (C-5). UV (EtOH): λ nm (log ε) = 223 (4.33), 243 (4.31), 256 (4.30), 279 (4.45), 299 (4.41), 340 (sh), 357 (4.12). IR: ν (cm^{-1}) = 3407, 3175, 2844, 2713, 1649, 1579, 1333, 1193, 1136.

5. CDK5 assay

Human recombinant CDK5 and its regulatory subunit p25 were expressed as described elsewhere (Weishaupt et al. 2003). CDK5/p25 activity was assessed in 96-well filter plates (MultiScreen, Millipore, Eschborn, Germany) in assay buffer (25 mM MOPS, 4 mM $MgCl_2$, 1 mM DTT, pH 7.0) containing 30 mU CDK5/p25, 5 μg histone H1, 7.5 μM cold ATP, and 1 μCi [γ-^{33}P]ATP. For screening 1 μM biotinylated peptide (biotin-PKTPKKAKKL) derived from the CDK5 phosphorylation site of histone H1 was used as substrate in a scintillation proximity assay (Amersham Bioscience, Uppsala, Sweden). Following incubation at room temperature for 40 min, incorporated radioactivity was monitored in a liquid scintillation counter.

6. Assessment of cell viability

Cerebellar granule neurons (CGNs) were isolated from 7 days old Wistar rat pups and cultured as described in detail elsewhere (Weishaupt et al. 2003). Seven days after plating, the cells were washed once and subsequently incubated in BME containing 25 mM KCl. CGNs were treated with the glutamylcysteine synthetase inhibitor, L-buthionine-sulfoximine (0.5 mM), which causes glutathione depletion, free radical stress and subsequent neuronal cell death (Wullner et al. 1999).
Mitochondrial metabolic activity was tested using the Alamar Blue Assay (Serotec, Oxford, UK). CGNs were incubated in 5% Alamar Blue solution for 2 h, thereafter fluorescence was monitored at 530 nm excitation and 590 nm emission wavelength in a Millipore CytoFluor Reader 2350.

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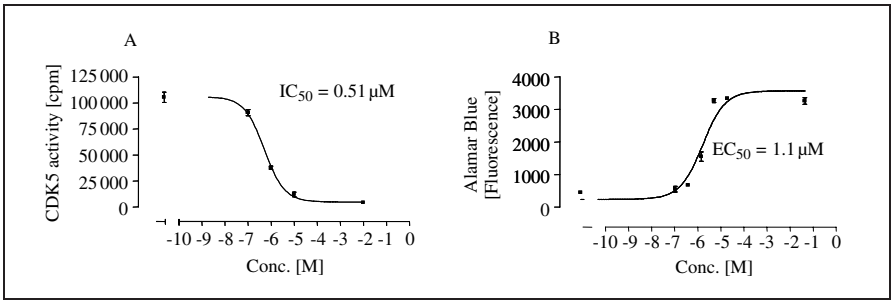

Fig.:
(A) Inhibition of recombinant CDK5 by clausine Z in a filter plate assay. (B) Effect of clausine Z on survival of cultured cerebellar granule neurons treated with buthionine-sulfoximine

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