# Inhibition of T-Lymphocyte Proliferation by Cucurbitacins from *Picrorhiza scrophulariaeflora*

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Two cucurbitacin aglycons were isolated from the dried rhizomes of *Picrorhiza scrophulariaeflora* and were identified as 25-acetoxy-2,3,16,20-tetrahydroxy-9-methyl-19-norlanosta-5,23-dien-22-one (picracin, **1**) and 2,3,16,20,25-pentahydroxy-9-methyl-19-norlanosta-5,23-dien-22-one (deacetylpicracin, **2**). Both compounds inhibit mitogen-induced T-lymphocyte proliferation at an $IC_{50}$ value of 1 $\mu$M.

*Picrorhiza scrophulariaeflora* Pennell and *Picrorhiza kurrooa* Royle ex Benth. (Scrophulariaceae) are extensively used in traditional medicine of China, Tibet, Nepal, and India for the treatment of various immune-related diseases. For instance, both plants are used in asthma, jaundice, and arthritis[1–3] which are T-cell-mediated diseases.[4–6]

On the basis of its use, *P. scrophulariaeflora* was selected in our search for immunomodulatory compounds. This paper describes the isolation and identification of two cucurbitacins, which strongly inhibit mitogen-induced T-cell proliferation.

Two cucurbitacins were isolated from the rhizomes of *P. scrophulariaeflora*, guided by inhibition in a mitogen-induced human T-lymphocyte proliferation assay. After sequential Soxhlet extraction using an eluotropic range of solvents, inhibitory activity was found to reside in the diethyl ether extract ($IC_{50}$ = 13 $\mu$g/mL). The latter extract was subjected to column chromatography on Si gel; fractions containing **1** yielded a pure compound (**1**) after crystallization. Other fractions containing **2** were further separated on Sephadex LH20; subsequent purification by HPLC yielded **2**.



**1**: R = OH
**2**: R = OAc
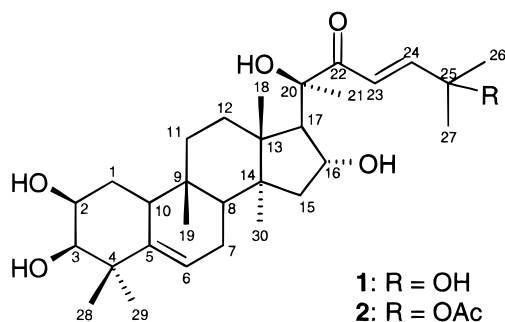
Both compounds were identified as aglycons of known 11-deoxycucurbitacin glucosides.[7,9] The compounds were named deacetylpicracin (**1**) and picracin (**2**). Their structures were determined by EIMS; FABMS; and IR, ¹H NMR, and ¹³C NMR spectroscopy (including APT, COSY, and HETCOR, Table 1).

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**Table 1.** ¹H–¹H and ¹H–¹³C Correlations for Picracin (**2**) in CDCl₃ (300 MHz)[a]

|  | correlation with ¹H (COSY) | correlation with ¹³C (HETCOR) |
| --- | --- | --- |
| H-17 (2.25) |  | C-17 (58.9) |
| H-3 (3.43) | H-2 | C-3 (79.2) |
| H-2 (3.96) | H-1a, H-1b | C-2 (68.7) |
| H-16 (4.30) | H-17 | C-16 (71.8) |
| H-6 (5.62) | H-7a, H-7b | C-6 (122.0) |
| H-23 (6.44) | H-24 | C-23 (120.7) |
| H-24 (7.00) |  | C-24 (151.4) |

[a] $\delta$ Values are in parentheses.

On the basis of NMR and EIMS spectral data as well as its melting point, picracin was found to be 25-acetoxy-2,3,16,20-tetrahydroxy-9-methyl-19-norlanosta-5,23-dien-22-one (**2**), which was reported previously as an aglucone obtained by hydrolysis of the corresponding 2-*O*-$\beta$-D-glucoside isolated from *P. kurrooa*.[7,8]

The ¹H NMR spectrum of deacetylpicracin (2,3,16,20,25-pentahydroxy-9-methyl-19-norlanosta-5,23-dien-22-one; **1**) showed also the characteristic signals of an 11-deoxy-cucurbitacin skeleton.[9,10] Compared with the ¹H NMR spectrum of picracin (recorded in CD₃OD), the methyl groups at C-25 (26-Me and 27-Me) were shifted upfield to $\delta$ 1.22 (−0.2 ppm), whereas the signal for OCO*Me* at $\delta$ 1.90 was missing, indicating the absence of the C-25 acetyl group. The ¹³C NMR spectra of deacetylpicracin (**1**) were very similar to those of picracin (**2**), except for some signals of the side chain. In contrast to picracin, only one carbonyl carbon signal was found (C-22 at $\delta$ 204.7, recorded in pyridine-$d_5$); downfield shifts were observed for 26-Me and 27-Me (ca. +3 ppm) and C-24 (+5.7 ppm), whereas the signal for C-25 showed a strong upfield shift to 70.1 (−9.6 ppm). The absence of the acetyl group at C-25 was also confirmed by the mass difference of 42 between picracin and its deacetylated derivative. In addition, it was shown by TLC analysis that methanolysis of picracin (**2**) with sodium methoxide resulted in the formation of deacetylpicracin (**1**). Concerning the stereochemistry of deacetylpicracin, the coupling constant of 2.2 Hz between H-2 and H-3 is consistent with the 2$\beta$,3$\beta$-diol configuration found in cucurbitacins so far.[7,11,12] In analogy with other cucurbitacins, it was assumed that the 20-OH of deacetylpicracin is $\beta$-oriented, whereas the 16-OH is in the $\alpha$ position.[10,13] The $\alpha$ orientation of 16-OH and the $\beta$ orientation of 20-OH have been confirmed by X-ray crystallographic analysis

of $(20R)$-$16\alpha$-acetoxy-$20$-hydroxy-$24,25,26,27$-tetranor-$2\beta$-($\beta$-D-tetraacetylglucopyranosyloxy)-$10\alpha$-cucurbit-5-ene-3,11,22-trion, a derivative of a cucurbitacin obtained from *P. kurrooa*, and the di-*p*-iodobenzoate of datiscoside, a cucurbitacin isolated from *Datisca glomerata* (Cucurbitaceae).[14,15]

Both compounds exhibited a dose-dependent inhibition of phytohemagglutinin-induced T-cell proliferation. The $IC_{50}$ values were determined to be 1 $\mu$M for both picracin (**2**) and deacetylpicracin (**1**).

Because cucurbitacins are well-known for their cytotoxic behavior,[16] cytotoxic properties of the two compounds were determined by means of two different assays. Labeling the phytohemagglutinin-stimulated lymphocytes with carboxyfluorescein diacetate and propidium iodide to distinguish between viable and dead cells, showed only minor cell death (<10%) for concentrations of picracin (**2**) and deacetylpicracin (**1**) below 60 $\mu$M. Another cytotoxicity experiment, using the lactate dehydrogenase (LDH) release assay, showed no toxic effects up to 50 $\mu$M for both cucurbitacins. As these concentrations are much higher than the $IC_{50}$ values of both compounds (1 $\mu$M), it can be concluded that the inhibitory activity of picracin (**2**) and deacetylpicracin (**1**) cannot be ascribed to cytotoxic effects.

Several cucurbitacins have been shown to inhibit incorporation of radioactive precursors into DNA, RNA, and protein in HeLa S3 cells. $ID_{50}$ values were similar to the $ED_{50}$ values of cucurbitacin-induced growth inhibition, suggesting a relationship between inhibition of intracellular metabolic activity and growth-inhibitory activity.[17] Furthermore, cucurbitacin E was shown to induce morphological changes and inhibit cell growth of prostate carcinoma cells and human endothelial cells, which were associated with disruption of the filamentous (F)-actin cytoskeleton.[18,19] The cytoskeleton has been implicated as a critical intermediate in signal transduction pathways controlling cell division.[20,21] Therefore, the mechanism of picracin (**2**) and deacetylpicracin (**1**) may also include interference with the cytoskeleton and subsequent abrogation of proliferative signal transduction, leading to inhibition of T-cell proliferation.

In conclusion, picracin (**2**) and deacetylpicracin (**1**), two cucurbitacins isolated from *P. scrophulariaeflora*, are potent inhibitors of mitogen-induced proliferation of T-lymphocytes. These findings provide a basis for the rational use of *P. scrophulariaeflora* in the treatment of T-cell-mediated diseases, such as asthma, jaundice, and arthritis. In addition, picracin and deacetylpicracin may serve as lead compounds for the development of future antiinflammatory drugs.

## Experimental Section

**General Experimental Procedures.** TLC analysis was carried out on precoated Si60 $F_{254}$ silica plates (Merck), developed using ethyl acetate/methanol/water (20:3:2), sprayed with vanillin/sulfuric acid reagent, and heated for 5 min at 110 °C.

HPLC separation was performed on a modular Gilson system (dual 306 pumps, 234 auto injector, 170 diode array detector, FC 204 fraction collector) provided with UniPoint software (version 1.65) for automated sample handling, and an Alltech Adsorbosphere silica column (5 $\mu$m, 250 × 10 mm), using a gradient of cyclohexane and ethanol (0−2 min: 100%; 2−10 min: 100% → 93%; 10−24 min: 93%; 24−30 min: 93% → 100%).

RPMI 1640 medium, fetal calf serum (FCS), and M199 medium were obtained from Gibco BRL, Life Technologies, Paisley, Scotland; phytohemagglutinin (PHA), from Murex

Diagnostics, Dartford, England; concanavalin A type IV, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-carboxyfluorescein diacetate (CFDA), and propidium iodide (PI), from Sigma Chemicals, St. Louis MO, and sodium dodecyl sulfate (SDS), from ICN Biomedicals, Zoetermeer, The Netherlands. LDH substrate mixture consisted of 1 mg NAD ($\beta$-nicotinamide adenine dinucleotide·4H$_2$O, Janssen Chimica, Geel, Belgium), 80 $\mu$g phenazine methosulfate (Janssen Chimica), 4.8 mg calcium lactate, and 0.5 mg MTT per ml 0.2 M Tris buffer [tris(hydroxymethyl)aminomethane, Merck, Darmstadt, Germany], adjusted to pH 7.2.

**Plant Material.** Dried rhizomes of *P. scrophulariaeflora* were purchased from Gorkha Ayurved Company, Gorkha, Nepal. These samples were collected within the Gorkha District. Field work showed only the presence of *P. scrophulariaeflora* in this area, at an altitude of 4000 m and upward, as identified by H. F. Smit, Department of Medicinal Chemistry, Universiteit Utrecht, The Netherlands, where herbarium specimens are deposited (Smit 9601).

**Isolation Procedure.** Dried rhizomes (200 g) were crushed, ground to powder, and subjected to sequential Soxhlet extraction, using light petroleum, diethyl ether, ethyl acetate, methanol, and water as the respective solvents. Organic solvents were removed under reduced pressure, water was added, and all aqueous suspensions were lyophilized. The diethyl ether extract (4 g) showed significant inhibitory activity in mitogen-induced T-cell proliferation ($IC_{50}$ = 13 $\mu$g/mL), whereas all other extracts displayed $IC_{50}$ values > 200 $\mu$g/mL. The diethyl ether extract (3 g) was subjected to silica column chromatography (50 cm × 5 cm diameter) using diethyl ether/ethyl acetate (1:1) as eluent. Active fractions were subjected to TLC analysis. Deacetylpicracin was directly obtained from the relevant fractions by crystallization (60 mg). Fractions containing picracin (**2**) were pooled and separated on Sephadex LH20 (70 cm × 2 cm diameter) with acetone as eluent. Picracin was further purified by HPLC, yielding 100 mg.

**Structure Elucidation.** The structures of both compounds were determined using EIMS, FABMS, IR, $^1$H NMR, and $^{13}$C NMR spectroscopy (including APT, COSY, and HETCOR).

**Deacetylpicracin (1)**: colorless needles (ethyl acetate); mp 224−228 °C (dec); IR (KBr) $\nu_{max}$ 3400, 2939, 1682, 1633, 1282 cm$^{-1}$; $^1$H NMR (CD$_3$OD, 300 MHz) $\delta$ 6.87 (1H, d, $J_{23-24}$ = 15.4 Hz, H-23), 6.69 (1H, d, H-24), 5.49 (1H, d, $J$ = 5.5 Hz, H-6), 4.32 (1H, t, $J$ = 7.5 Hz, H-16), 3.88 (1H, br d, $J$ = 10.7 Hz, H-2), 3.29 (1H, d, $J$ = 2.2 Hz, H-3), 1.29 (3H, s), 1.22 (6H, s, 26-Me, 27-Me), 1.07 (3H, s), 1.02 (3H, s), 0.96 (3H, s), 0.93 (3H, s), 0.85 (3H, s); $^{13}$C NMR (APT; pyridine-$d_5$, 75.5 MHz) $\delta$ 204.8 (C, C-22), 155.3 (CH, C-24), 142.0 (C, C-5), 121.0 (CH, C-23), 119.8 (CH, C-6), 79.8 (CH, C-3), 79.6 (C, C-20), 70.9 (CH, C-16), 70.1 (C, C-25), 68.9 (CH, C-2), 59.6 (CH, C-17), 48.9 (C, C-14), 48.9 (CH$_2$, C-15), 46.7 (C, C-13), 42.7 (CH, C-8), 41.9 (C, C-4), 37.2 (CH, C-10), 34.4 (C, C-9), 31.9 (CH$_2$, C-1), 30.7 (CH$_2$, C-12), 30.5 (CH$_2$, C-11), 29.8 (CH$_3$), 29.5 (CH$_3$), 27.9 (CH$_3$), 27.2 (CH$_3$), 26.2 (CH$_3$), 25.2 (CH$_3$), 24.5 (CH$_2$, C-7), 18.4 (CH$_3$), 18.3 (CH$_3$); $^{13}$C NMR (CD$_3$OD, 75.5 MHz) $\delta$ 205.4 (C-22), 155.3 (C-24), 141.9 (C-5), 121.7 (C-23), 121.5 (C-6), 80.5 (C-20), 80.4 (C-3), 72.4 (C-16), 71.6 (C-25), 69.9 (C-2), 60.0 (C-17), 49.9 (C-14), 47.0 (C-15), 44.1 (C-8), 42.6 (C-4), 38.3 (C-10), 35.3 (C-9), 32.9 (C-12), 31.5 (C-11), 30.5 (C-1), 29.2 (C-19), 29.2 (C-26), 28.5 (C-27), 27.5 (C-28), 26.1 (C-29), 25.2 (C-7), 25.1 (C-21), 19.2 (C-30), 18.6 (C-18), signal obscured by the solvent signal (C-13); FABMS *m/z* 505 [M + H]$^+$.

**Picracin (2):** colorless amorphous powder; mp 181−182 °C; IR (KBr) $\nu_{max}$ 3575, 2947, 1716, 1672, 1618, 1258 cm$^{-1}$; $^1$H NMR (CDCl$_3$, 300 MHz) $\delta$ 7.00 (1H, d, $J_{23-24}$ = 15.7 Hz, H-24), 6.44 (1H, d, H-23), 5.62 (1H, d, $J$ = 5.5 Hz, H-6), 4.30 (1H, br m, H-16), 3.96 (1H, br d, $J$ = 11.7 Hz, H-2), 3.43 (1H, br s, H-3), 2.25 (d, $J$ = 7.0 Hz, H-17), 1.97 (3H, s, OCO*Me*), 1.51 (6H, s), 1.39 (3H, s), 1.16 (3H, s), 1.04 (6H, s), 1.00 (3H, s), 0.90 (3H, s); $^1$H NMR (CD$_3$OD, 300 MHz) $\delta$ 6.87 (1H, d, $J_{23-24}$ = 15.8 Hz, H-23), 6.68 (1H, d, H-24), 5.48 (1H, d, $J$ = 5.1 Hz, H-6), 4.38 (1H, t, $J$ = 7.9 Hz, H-16), 3.87 (1H, br d, $J$ = 11.7 Hz, H-2), 3.29 (1H, s, H-3), 1.90 (3H, s, OCO*Me*), 1.46 (3H, s, 26-Me), 1.44 (3H, s, 27-Me), 1.29 (3H, s), 1.06 (3H, s), 1.00 (3H, s), 0.92 (6H, s), 0.84 (3H, s); $^{13}$C NMR (APT; pyridine-$d_5$,

75.5 MHz) $\delta$ 204.7 (C, C-22), 169.8 (C, O$C$OMe), 149.6 (CH, C-24), 142.0 (C, C-5), 122.7 (CH, C-23), 119.8 (CH, C-6), 80.1 (C, C-20), 79.8 (CH, C-3), 79.7 (C, C-25), 71.2 (CH, C-16), 68.9 (CH, C-2), 60.1 (CH, C-17), 48.9 (C, C-14); 48.8 (CH$_2$, C-15), 46.6 (C, C-13), 42.8 (CH, C-8), 41.9 (C, C-4), 37.3 (CH, C-10), 34.5 (C, C-9), 31.9 (CH$_2$, C-1), 30.6 (CH$_2$, C-11), 30.6 (CH$_2$, C-12), 27.9 (CH$_3$), 27.2 (CH$_3$), 26.5 (CH$_3$), 26.2 (CH$_3$), 26.0 (CH$_3$), 25.2 (CH$_3$), 24.5 (CH$_2$), 21.5 (CH$_3$, OCO$Me$), 18.5 (CH$_3$), 18.4 (CH$_3$); $^{13}$C NMR (CD$_3$OD, 75.5 MHz) $\delta$ 205.6 (C-22), 172.0 (O$C$OMe), 151.4 (C-24), 141.9 (C-5), 123.0 (C-23), 121.7 (C-6), 81.1 (C-20), 80.9 (C-25), 80.4 (C-3), 72.6 (C-16), 69.9 (C-2), 60.5 (C-17), 49.7 (C-14), 49.5 (C-13), 46.9 (C-15), 44.1 (C-8), 42.6 (C-4), 38.2 (C-10), 35.3 (C-9), 32.8 (C-12), 31.3 (C-11), 30.5 (C-1), 28.4 (C-19), 27.5 (C-28), 26.8 (C-27), 26.5 (C-26), 26.1 (C-29), 25.3 (C-7), 24.9 (C-21), 21.8 (OCO$Me$), 19.2 (C-30), 18.7 (C-18); EIMS $m/z$ 486 [M − CH$_3$COOH]$^+$ (1.5), 96 (100).

**T-Cell Proliferation Assay.** Peripheral blood lymphocytes (PBL) were obtained from buffycoat residues (Bloedbank Midden-Nederland) using Ficoll-Hypaque centrifugation, according to manufacturer's instructions (Amersham Pharmacia, Uppsala, Sweden). Under sterile conditions, cells were diluted to 2·10$^6$ cells/mL medium (RPMI 1640 supplemented with 10% FCS), dispensed in 96-wells microtiter plates (50 $\mu$L/well), activated with 50 $\mu$L PHA (0.1 mg/mL), and incubated at 37 °C with 50 $\mu$L of test samples in appropriate dilution ranges. After 4 days, T-cell proliferation was determined using a modified colorimetric MTT assay.[22] The formazan product formed was dissolved by shaking for 1 h on a microtiter plate shaker. The extinction was measured by an ELISA reader (SLT Labinstruments) at 550 nm. Controls consisted of PBL with PHA (100% activity), PBL with medium (0% activity), or samples with nonstimulated PBL.

**T-Cell Toxicity Assays.** For the assessment of cytotoxic effects of picracin (**2**) and deacetylpicracin (**1**) toward T-lymphocytes, LDH-release[23] and CFDA/PI-labeling[24] assays were performed. Cell suspensions (50 $\mu$L) were incubated at 37 °C in U-well microtiter plates with 50 $\mu$L medium and appropriate dilution ranges of test samples. To determine the amount of LDH released after 24 h, plates were spun (5 min, 300$g$), supernatants (50 $\mu$L) were transferred to flat-bottom microtiter plates, and 25 $\mu$L of LDH substrate mixture was added. After incubation (10 min, 37 °C), the optical densities at 550 nm were measured. Controls consisted of cells incubated with test samples and 50 $\mu$L Triton X-100 (0.1% v/v), cells incubated with 50 $\mu$L Triton X-100 (0.1% v/v), or cells incubated with medium only.

To assess cytotoxicity after 4 days, stimulated lymphocytes were labeled with CFDA and PI to distinguish between viable and dead cells. After 4 days of incubation (37 °C, 5% CO$_2$), 50 $\mu$L of a 1 $\mu$L/mL CFDA dilution in RPMI medium was added. After incubation in darkness (15 min, room temperature), 50 $\mu$L PI in ink was added. The plates were inspected under an upside-down fluorescence microscope, and the ratio of viable-to-dead cells was assessed. Controls consisted of cells incubated with medium only.

## References and Notes

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