Inhibitory Activity of Tryptanthrin on Prostaglandin and Leukotriene Synthesis

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Abstract

The indolo[2,1-*b*]quinazoline alkaloid tryptanthrin has previously been identified as the cyclooxygenase-2 inhibitory principle in the extract ZE550 prepared from the medicinal plant *Isatis tinctoria*. We here investigated the potential inhibitory activity of tryptanthrin and ZE550 on cyclooxygenase -2 and cyclooxygenase -1 in cellular and cell-free systems. A certain degree of selectivity towards cyclooxygenase -2 was observed when cyclooxygenase -1 dependent formation of thromboxane B_2 in HEL cells and cyclooxygenase -2 dependent formation of 6-keto prostaglandin $F_{1\alpha}$ in Mono Mac 6 and RAW 264.7 cells were compared. Preferential inhibition of cyclooxygenase -2 by two orders of magnitude was found in phorbol myristate acetate activated bovine aortic coronary endothelial cells. Assays with purified cyclooxygenase isoenzymes from sheep confirmed the high selectivity towards cyclooxygenase -2. The leukotriene B_4 release from calcium-ionophore-stimulated human granulocytes (neutrophils) was used as a model to determine 5-lipoxygenase activity. Tryptanthrin and the extract ZE550 inhibited leukotriene B_4 release in a dose dependent manner and with a potency comparable to that of the clinically used 5-lipoxygenase inhibitor zileuton.

Key words

tryptanthrin, *Isatis tinctoria*, Brassicaceae, cyclooxygenase, 5-lipoxygenase, 6-keto-$PGF_{1\alpha}$, TxB_2

Introduction

There is still an unmet need for medicinal plants and phytopharmaceuticals with scientifically proven anti-inflammatory and antirheumatic efficacy. Although herbal drugs such as devil's claw (*Harpagophytum procumbens* DC., Pedaliaceae), willow bark (*Salix alba* L., Salicacae) and others have been positively reviewed in the 1980's by the subcomittee for phytotherapy (the so-called Kommission E) of the German Drug Agency [1], none of these herbs nor phytopharmaceuticals obtained from their extracts satisfies the criteria of orthodox medicine. Due to the good acceptance of herbal drugs among the population, phytopharmaceuticals with demonstrated clinical efficacy could become a suitable therapeutic alternative to current medication for specific indications such as the adjuvant treatment of mild or chronic forms of rheumatic and other inflammatory ailments [2].

In a survey of historical accounts on old folk medicines with potential antiinflammatory properties, woad (*Isatis tinctoria* L., Brassicaceae) was selected for further investigation. Subsequently, lipophilic woad extracts such as ZE550 were found to display a promising *in vitro* pharmacological profile. In cell culture assays a pronounced inhibition was found for cyclooxygenase-2 (COX-2) only, but not for cyclooxygenase-1 (COX-1). Furthermore, a distinct effect on purified 5-lipoxygenase (5-LOX), and inhibition of histamine and serotonin release from mast cells was shown. With the aid of activity directed fractionation tryptanthrin (Fig. **1**) was identified as the COX-2 inhibitory principle in the extracts [3, 4]. Given the compound's rather remarkable potency in a cell based assay for COX-2 activity with Mono Mac 6 cells, we conducted more detailed investigations to elucidate the *in vitro* pharmacological effects of trypthantrin on key enzymes involved in the eicosanoid synthesis.

Etc.

Results

The inhibition of eicosanoid formation in LPS-stimulated Mono Mac 6 cells was determined by the measurement of 6-keto-$PGF_{1\alpha}$ synthesis. Preliminary experiments had established the comparability of data with those obtained in a PGE_2 assay. First, dose-inhibition curves for tryptanthrin and a number of known COX-inhibitors were obtained (Fig. **2A**). The IC_{50} value of tryptanthrin (0.037 µM) was comparable to that of nimesulide (IC_{50} 0.027 µM), a clinically used preferential COX-2 inhibitor. The potency of acetyl salicylic acid was lower by two orders of magnitude (IC_{50} 3.8 µM). This finding is in line with the fact that the latter compound is a selective COX-1 inhibitor [5]. Diclofenac, a clinically used non-selective COX inhibitor, and NS 398, a selective COX-2 inhibitor, showed IC_{50} values of 0.002 and 0.003 µM, respectively. The relative inhibitory potencies of tryptanthrin, nimesulide and NS 398 on 6-keto-$PGF_{1\alpha}$ synthesis in RAW 264.7 cells (Fig. **2B**) was comparable to those in Mono Mac 6 cells, although all IC_{50} values were shifted by about one order of magnitude (IC_{50} of 0.25 µM, 0.21 µM and 0.013µM, respectively).

Etc.

Discussion

With respect to the mode of action of tryptanthrin there is evidence of its inhibitory activity on key enzymes of the arachidonic acid cascade in various cell types. Tryptanthrin strongly inhibited prostaglandin synthesis in stimulated Mono Mac 6, RAW 264.7 cells and BAECs expressing COX-2, whereas significantly weaker inhibition was observed in HEL cells and thrombocytes which both constitutively express COX-1. The difficulties arising with assessment of isoenzyme selectivity due to a lack of standard *in vitro* assays has been pointed out earlier [14]. The consistency of our findings in a range of assay systems using known reference compounds and different endpoint measures,

however, strongly corroborate the selectivity of the compound.

Etc.

Materials and Methods

Cell lines, chemicals and biochemicals

HEL (human erythroleukemia) and Mono Mac 6 (human acute monocytic leukemia) cell lines were purchased from the DMSZ. Tryptanthrin was synthetized according to Friedlaender and Roschdestwensky [20]. Purity was assessed by HPLC, NMR and ESI-MS as > 99%. Extract ZE550 was prepared according to Danz et al. [4]. The tryptanthrin content of ZE550 was 0.10%. Nimesulide (purity >98% by HPLC), indometacin, diclofenac (purity >99% by HPLC), acetylsalicylic acid, arachidonic acid, OPI media supplement Hybri-Max, glutathione, hematin, epinephrine and lipopolysaccharide (LPS, from *E. coli*) were purchased from Sigma Aldrich. ELISA kits for thromboxane B_2 (TxB_2), 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) and leukotriene B_4 (LTB_4) were purchased from R&D Systems. The assays were performed according to manufacturer's instructions. Unless stated otherwise, all other reagents were from Sigma.

Plant material

Leaves of *Isatis tinctoria* L. were collected in Martigny, Switzerland, in June 2004, and identified by Dr. C. Mustermann, University of Basel. A voucher specimen (U 36251) is deposited at the Herbarium of the Department of Botany, University of Basel, Switzerland.

Extraction and isolation

Air-dried leaves were ground in a cross beater mill equipped with a 1 mm sieve. An aliquot (100 g) was extracted at r.t. with CH_2Cl_2 or EtOH (3 × 1000 mL, 24 h each). Frozen fresh material was thawed, and an aliquot (100 g) extracted in a Waring blender model 38BL45 with EtOH (3 × 750 mL, 20 min each).

The EtOH extract (25 g) was submitted to vacuum liquid chromatography on silica gel (40-63 µm, 5 x 50 cm; Macherey & Nagel) using a step gradient of $CHCl_3$-MeOH 100:0 (3 L) and 97:3 (2 L). Fractions of 250 mL were collected and monitored by TLC (system 1) with respect to tryptanthrin

content. The fractions 4-9 (750-2250 mL, 3.5g) were combined and separated on a silica gel column (25-40 µm, 3 × 50 cm, eluent $CHCl_3$, flow rate 3 mL/min). 14 fractions (I-XIV) were collected. Fraction IV (600-800 mL, 101 mg) was subjected to solid phase extraction on a Bakerbond C18 cartridge (1 × 3cm) eluted with MeOH-H_2O 80:20, approx. flow 1 mL/min. Fractions 2-5 (5-20 mL, 16 mg) were combined. Pure tryptanthrin (**1**) (5 mg) was obtained by preparative HPLC on a LiChrosorb RP-18 column (7 µm, 2 × 30 cm i.d.; Merck) eluted with MeOH-H_2O 80:20 at 10 mL/min. (t_R = 7.5 min).

LC-DAD-MS analysis

LC-DAD-MS data were obtained with an Agilent 1100 series HPLC system consisting of an auto sampler, high-pressure mixing pump, column oven and DAD detector connected to a Perkin Elmer API 165 single quadrupole instrument equipped with a PE Sciex Turbo ion probe.

HPLC conditions: LiChrospher 100 RP18 cartridge, (5 µm, 4 × 125 mm; Merck), solvent system: A - H_2O, B - MeCN; isocratic 10% B for 5 min, 10-90% B over 45 min, 90% B for 15 min; flow rate: 1 mL/min; injection volume: 20 µL; Sample concentration: 10 mg/mL in CH_2Cl_2. DAD conditions: 220, 254, 328, 387, 540 nm, or spectra 190-700 nm (step 2 nm).

ESI-MS conditions: positive ion mode; split ratio: 1 : 4; scan range: 140-1000 amu; source temperature: 350°C; ion spray voltage 5 kV; focussing potential: 230 V; declustering potential: 20 V.

COX-1 and COX-2 assays with HEL, RAW 264.7 and Mono Mac 6 cells

The protocol by Berg et al. [21] was used for the assay with Mono Mac 6 cells, with some modifications. Cells were cultured in VLE RPMI 1640 medium with 10% fetal calf serum, 1% of penicillin/streptomycin stock solution, 2 mM L-glutamine, non-essential amino acid stock solution, supplemented with OPI media supplement Hybri-Max (0.5 x). Cells were cultured at a density of approx. 2 x 10^5 cells/mL, harvested and resuspended in fresh medium at 1 x 10^6 cells/mL. After addition of test solutions, cells were incubated for 30 min at 37°C. An LPS solution (10 µL, final LPS concentration in assay 100 ng/mL) was added and the cells were incubated for additional 6 h. The

further steps were carried out as for HEL cells, with the exception that formation of 6-keto-$PGF_{1\alpha}$ was determined by ELISA. Preliminary comparative experiments using a PGE_2 ELISA kit (R&D Systems) instead were carried out. The IC_{50} values found for tryptanthrin and reference compounds in both assays were comparable (data not shown). Diclofenac and nimesulid were used as positive controls.

COX-1 and COX-2 assays with purified enzymes

The procedure of Mitchell et al. [22] was employed with some modifications. Briefly, 40 µL of enzyme solution (100 U/mL) were diluted with 140 µL Tris buffer, and 80 µL of test compound solution in Tris buffer (final DMSO concentration 1.25%) were added and incubated for 10 min at 37°C. 140 µL of a substrate solution consisting of an ethanolic stock solution of arachidonic acid and a cofactor solution containing epinephrin, glutathione and hematin in Tris buffer were added. The final volume of the reaction mixture was 400 µL, the concentrations in the assay were 6.6 µM for arachidonic acid, 5 mM for epinephrin and glutathione, 1 µM for hematin, 0.25% for DMSO and 0.16% for ethanol. After incubation for 10 min at 37°C, the reaction was stopped by addition of 20 µL 1 N HCl and immediately centrifuged (13'000 rpm for 3 min). The supernatant was removed and neutralized with 1 N NaOH prior to determination of the 6-keto-$PGF_{1\alpha}$ concentration by ELISA.

Statistical analysis

Statistical calculations were carried out with the SPSS 10.0 for Windows software package (Statistica). Results are expressed as the mean ± S.E.M. of 5 independent experiments. Student's *t*-test was used for statistical analyses; P values > 0.05 were considered to be signficant.

Helveticosine A (1): yellow needles ($CHCl_3$); mp 132-138°C; R_f 0.70, silica gel 60 F_{254}, $CHCl_3$/MeOH/H_2O (80:20:2); R_f 0.15, RP-18 F_{254s}, MeOH/0.02M CH_3COONH_4 (pH 5) (9:1); $[\alpha]^{25}_D$ – 74.4 (*c* 0.24, MeOH); UV (MeOH) λ_{max} (log ε) 253 (4.43), 384 (4.55) nm; CD (CH_3CN) $\Delta\epsilon_{211}$ +17.1, $\Delta\epsilon_{231}$ –10.6; IR (KBr) ν_{max} 1680, 1540, 1035, 940 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.26 (1H, d, *J* = 8.1 Hz, H-4), 6.42 - 6.05 (2H, m, H-8, H-12), 3.77 (3H, s, NCOOC*H*$_3$), 2.28 (1H, dd, *J* = 7.3, 2.1

Hz, H-15); ^{13}C NMR ($CDCl_3$, 125 MHz) d 176.4 (C, C-4), 127.8 (CH, C-9), 100.9 (CH_2, OCH_2O), 52.5 (CH_3, NCOO*C*H_3), 39.2 (CH_2, C-5; ESI MS (pos. ion mode) *m/z* 444.2 $[M+Na]^+$, 422.3 $[M+H]^+$; ESI MS (neg. ion mode) *m/z* 420.3 $[M-H]^-$; HR ESIMS *m/z* 444.21526 $[M+Na]^+$ (calcd for $C_{26}H_{31}NaNO_4$ 444.21508); *anal.* C 71.89%, H 12.13%, calc. for $C_{30}H_{64}O_5$, C 72.12%, H 12.65%.

Supporting information

Microscopic sections of seeds of *Unknownia helvetica*, ^{1}H NMR spectra of compound **1**, and COSY correlations for compound **1** are available as Supporting Information.

Acknowledgements

Thanks are due to Prof. C. Unknown, University of Appenzell, for the gift of RAW 264.7 cells and information on culturing. Financial support by Herbal Bonanza AG, Irgendwo, Switzerland, for part of this work is acknowledged.

Conflict of Interest

The authors declare no conflict of interest.

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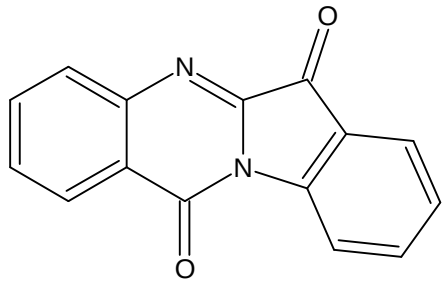
Legends for Figures

Fig. **1** Chemical structures of tryptanthrin (**1**), rutaecarpine (**2**), indigo (**3**) and indirubine (**4**).

Fig. **2** Effect of tryptanthrin, acetyl salicylic acid, diclofenac, nimesulide and NS 398 on 6-keto-$PGF_{1\alpha}$ synthesis in (**A**) LPS-stimulated Mono Mac 6, and (**B**) LPS-stimulated RAW 264.7 cells. Each symbol and bar indicates the mean ± S.E.M. of nine experiments.

Fig. **1**



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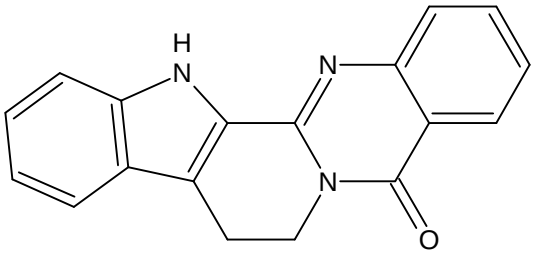

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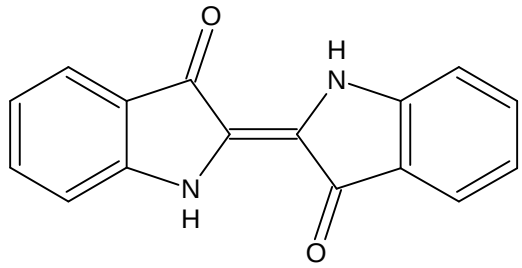

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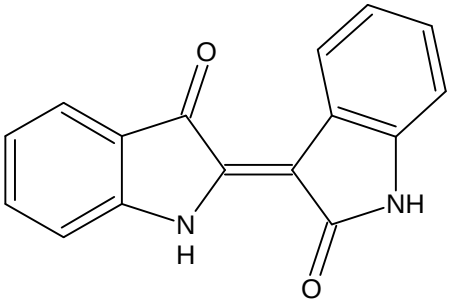

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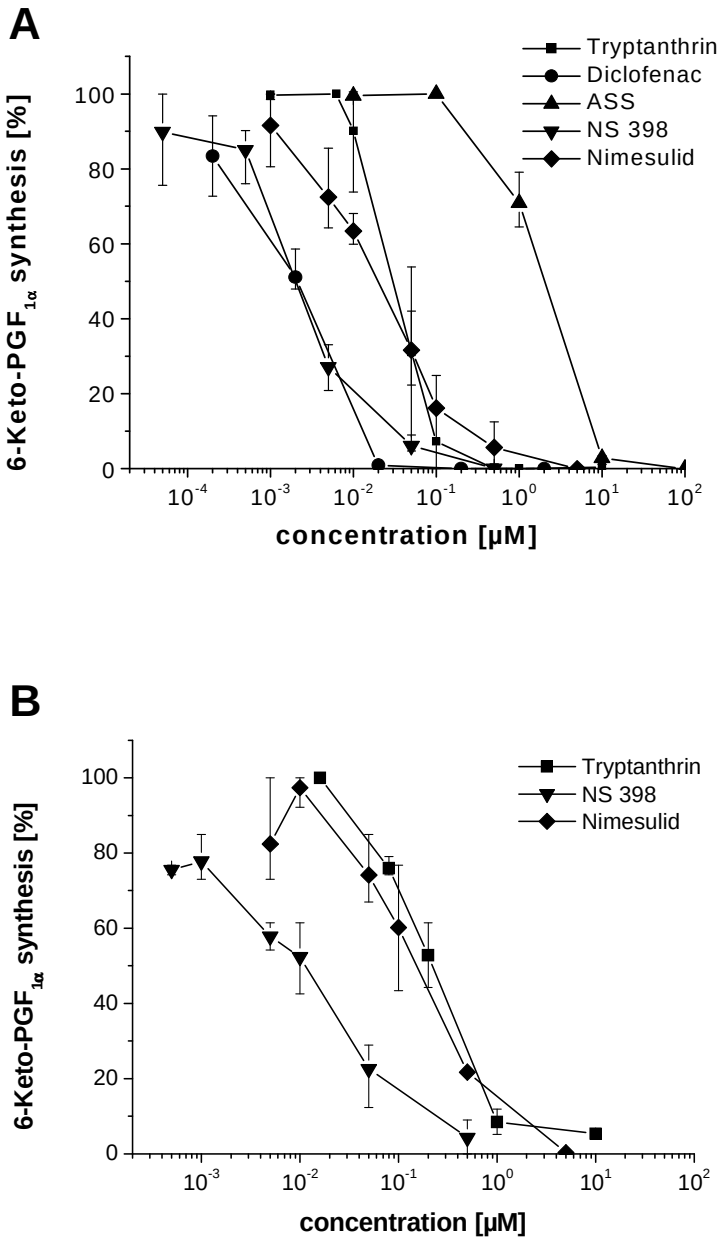
Fig. **2**


A
Tryptanthrin
Diclofenac
ASS
NS 398
Nimesulid
6-Keto-PGF1α synthesis [%]
concentration [µM]
B
Tryptanthrin
NS 398
Nimesulid
6-Keto-PGF1α synthesis [%]
concentration [µM]

Supporting Information

Inhibitory Activity of Tryptanthrin on Prostaglandin and Leukotriene Synthesis

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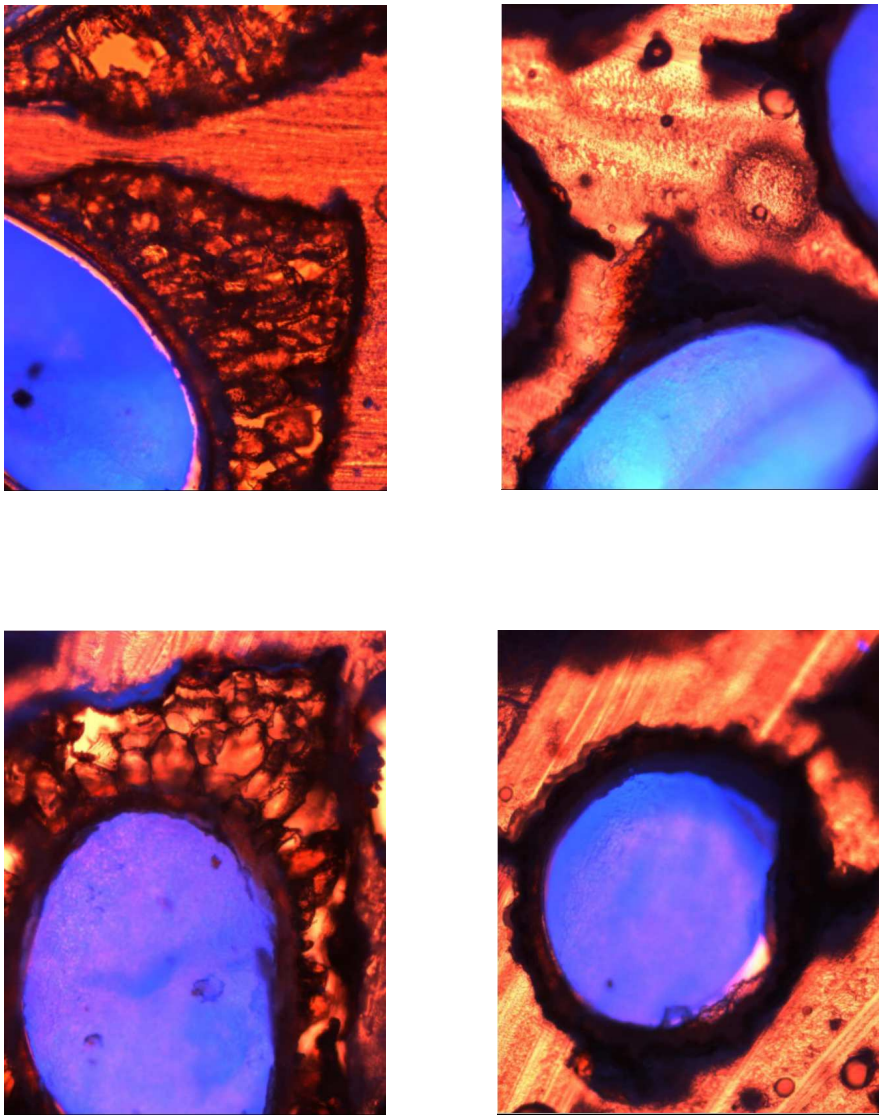

Fig. 1S Cross section of *Unkownia helvetica* (left, top and bottom) and *U. pannonica* (right, top and bottom) seeds. Fluorescence microscopy with DAPI filter 365 nm. The width of the photographic image corresponds to approx. 0.7 mm. Embryos display intense blue fluorescence. The testa of *U. helvetica* is irregular in thickness, and the large cells of the mesotesta are clearly discernible. The testa of *U. pannonica* shows uniform thickness.

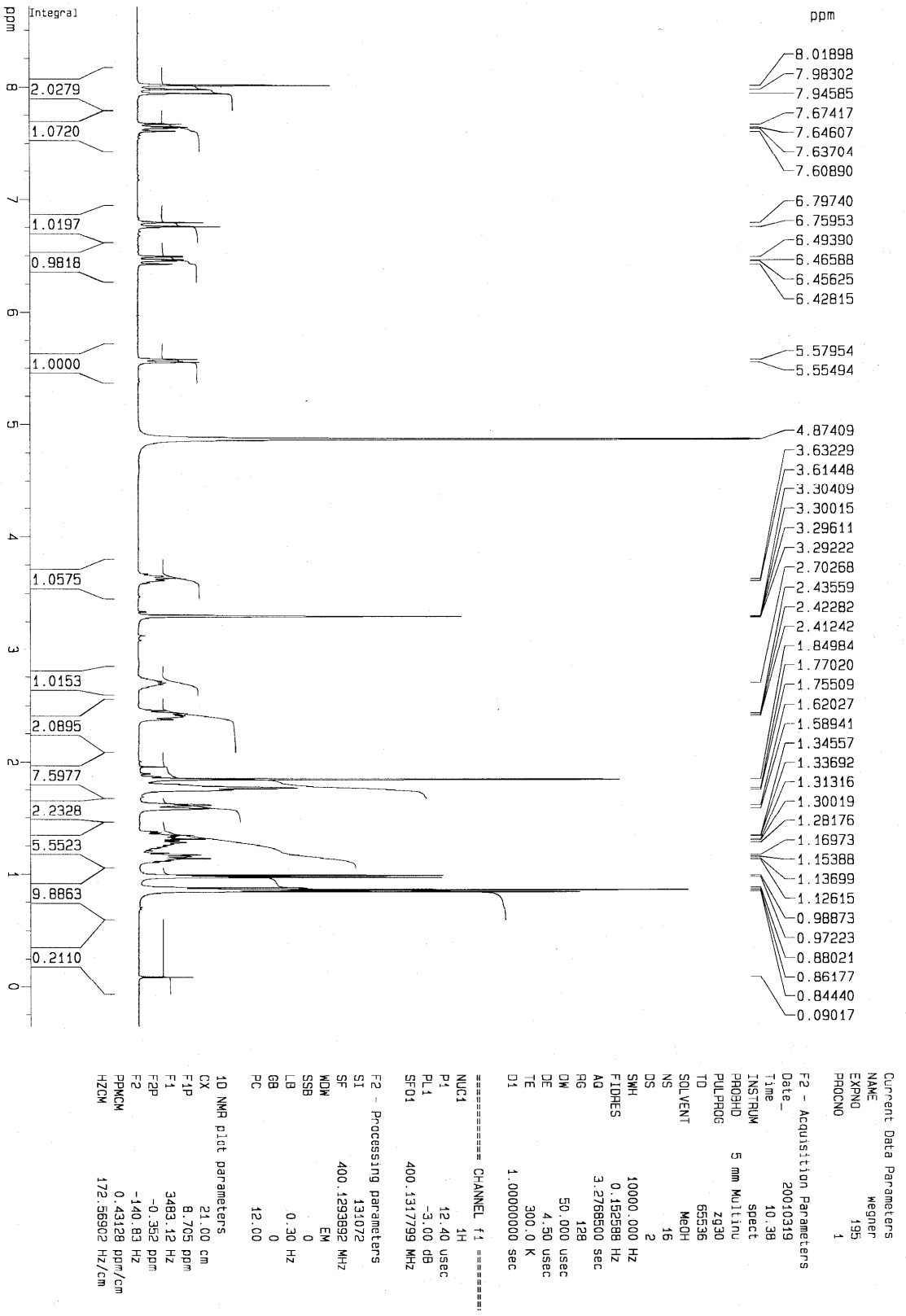

Fig. 2S ^{1}H NMR spectrum of compound **1** (CD_3OD, 400.13 MHz)

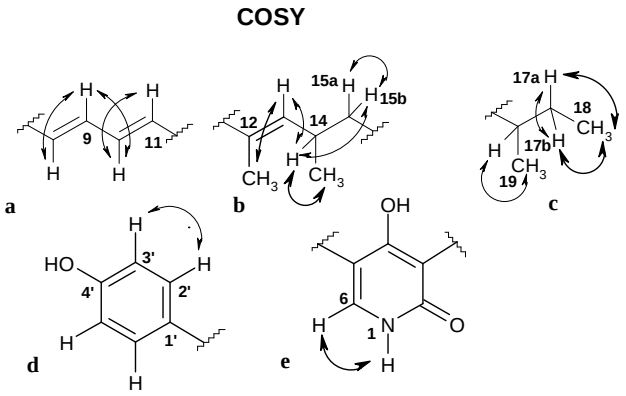

Fig. 3S Partial structures of **1** assembled with the aid of COSY correlations.