Phytochemistry Reviews **1:** 333–344, 2002.
© 2003 *Kluwer Academic Publishers. Printed in the Netherlands.*

Isatis tinctoria – From the rediscovery of an ancient medicinal plant towards a novel anti-inflammatory phytopharmaceutical

Matthias Hamburger
Institute of Pharmacy, Friedrich-Schiller-University Jena, Semmelweisstrasse 10, D-07743 Jena, Germany (Tel: +49 3641 949840; Fax: +44 3641 949842; E-mail: b7hama@rz.uni-jena.de)

Key words: anti-inflammatory, Brassicaceae, breeding, cyclooxygenase inhibition, 5-lipoxygenase inhibition, *Isatis tinctoria*, phytopharmaceutical, standardisation, supercritical fluid extraction, tryptanthrin

Abstract

An account on the reinvestigation of the old dye and medicinal plant *Isatis tinctoria* as an anti-inflammatory and current research on the active principles in woad is given. In a broad-based screening, a dichloromethane extract from the leaves displayed significant activities on several clinically relevant targets of inflammation. The cyclooxygenase-2 inhibitory principle was identified with the aid of HPLC-based activity profiling as the alkaloid tryptanthrin. In cell based assays, tryptanthrin strongly inhibited eicosanoid synthesis catalyzed by cyclooxygenase-2 and 5-lipoxygenase. A supercritical carbon dioxide extraction process was developed to replace the dichloromethane extract. Dichloromethane and carbon dioxide extracts showed *in vivo* anti-inflammatory activity in topical and oral application. With the aid of electrospray ionization liquid chromatography-mass spectrometry coupled skin microdialysis, tryptanthrin was found to penetrate the skin. The penetration from the extract was better than for the pure alkaloid. A screening of 67 woad samples of different geographic origin revealed up to 30-fold differences in tryptanthrin content in leaves.

Abbreviations: COX – cyclooxygenase; DCM – dichloromethane; ESI LC-MS – electrospray liquid chromatography-mass spectrometry ; iNOS – inducible nitric oxide synthase; LOX – lipoxygenase ; NSAIDs – nonsteroidal anti-inflammatory drugs; PLE – pressurized liquid extraction; SFE – supercritical fluid extraction; SIM – single ion monitoring.

Introduction

The number of medicinal plants and phytopharmaceuticals with reasonably proven clinical efficacy in inflammatory and rheumatic diseases is small. Herbs such as Harpagophyti Radix (tubers of *Harpagophytum procumbens*, Pedaliaceae), Salicis Cortex (bark of *Salix alba* and other *Salix* species, Salicaceae), Urticae Radix and Folium (roots and leaves of *Urtica dioica*, Urticaceae), Guaiaci Lignum (heartwood of *Guaiacum sanctum*, Burseraceae) have received positive monographs by the German Commission E (the expert commission on phytotherapy of the German Drug Agency) (Blumenthal, 1998), and numerous products derived from these drugs are being sold as pharmaceuticals or as botanical dietary supplements. Nonetheless, the current state of knowledge on the active principles of these herbal drugs, their mode(s) of action, and the evidence regarding the clinical efficacy of the finished products are not satisfactory (Ernst and Chrubasik, 2000).

Inflammatory diseases, in particular rheumatic disorders, affect a large number of people worldwide. Anti-inflammatory drugs are, therefore, among the most widely used pharmaceuticals, whereby the so-called NSAIDs are typically given as first line medication. NSAIDs inhibit COX, and, consequently, the conversion of arachidonic acid into prostaglandins (Mutschler, 2001). Extended use of NSAIDs is associated with well-known adverse effects, such as gastric ulcers and bronchospasms. While there is currently no therapeutic alternative for NSAIDs in acute inflam-

matory disease, the side effects invariably associated with long-term use raises serious concerns about their suitability in non-acute inflammatory and rheumatoid symptoms. There is, hence, an unmet need for adequate treatments of non-acute disease states which could be possibly filled by rational phytopharmaceuticals with a favorable benefit/risk ratio.

Recent investigations into the anti-inflammatory activity of various medicinal plants from European as well as non-European traditions have demonstrated the potential of plant derived natural products. Selected compounds were shown to act on various inflammation related targets of current interest, supporting traditional use of these herbs as anti-inflammatories. Examples include sesquiterpenoids from *Arnica montana* (Lyss et al., 1997) curcuminoids from *Curcuma* sp. (Zhang et al., 1999), and alkaloids such as rutaecarpine from the Chinese medicinal plant *Evodia rutaecarpa* (Moon et al., 1999). While these findings provided scientific support for long established uses of these herbs as anti-inflammatories, we felt that lesser known or forgotten medicinal plants would be of higher interest under the perspective of a possible developmental project for a new phytopharmaceutical.

Isatis – Medicinal uses, phytochemistry and biological activity

Isatis tinctoria L. (woad, Brassicaceae) has a long and well documented history as an indigo dye plant in temperate climates, and as a medicinal herb. The original habitat of woad is probably in the grasslands of southeastern Russia; however, the plant was introduced early to the rest of Europe and to eastern Asia including China and Japan. The first written records in Europe about the medicinal uses of woad were by Galen and Pliny. From the Middle Ages up to the 18th century, *Isatis tinctoria* was the most important indigo dye in Europe. Woad was grown in Germany (Thuringia, Jülich), France (Languedoc, Somme, Normandy), England (Somerset, Lincolnshire) and Italy (Tuscany), but lost importance with the access to less expensive indigo sources. Its medicinal properties have been extensively described in a number of Renaissance and Baroque herbals. Woad was recommended for the treatment of wounds, ulcers and tumours, haemorrhoids, snake bites and various inflammatory ailments (Hurry, 1930). With the declining importance as a dye and the disappearance of woad cultures, the plant also fell into oblivion as a medicinal herb.

In China, woad has an equally rich history as dye and medicinal herb. Even today, the Chinese Pharmacopoeia contains monographs for *Isatis* root (Banlangen) and leaf (Daqingye), and natural indigo (Qingdai), a preparation derived from various indigo plants (Zhu, 1998). There are conflicting views on the taxonomy of Chinese woad. Initially described as a separate species *Isatis indigotica* FORT., it was later considered by some taxonomists as a variety of *I. tinctoria* (Index Kewensis, 1997). A recent analysis of European and Chinese genotypes by AFLP analysis revealed a high degree of genetic diversity in woad and supports the separation into two distinct species (Gilbert et al., 2002).

A considerable number of phytochemical, biological and pharmacological investigations have been carried out, mostly over the last four decades. More than 100 secondary metabolites have been identified in *I. tinctoria* and *I. indigotica*. They include numerous indole derivatives, such as isatin (**1**), tryptanthrin (**2**) (Honda et al., 1980), deoxyvasicinone (**3**), isaindigotone (**4**), isaindigotidione (**5**) (Wu et al., 1997b), quinazolines **6** and **7**, indolinone **8**, benzodiazepine **9** (Wu et al., 1997a), the indigo dyes indigo (**10**) and indirubin (**11**), and their natural precursors. Unlike in other indigo plants, such as *Indigofera indica* or *Polygonum tinctorium*, the main indigo precursor is not indican (**12**), but isatan B (indoxyl-5-ketogluconate, **13**) (Epstein et al., 1967; Strobel and Gröger, 1989; Kokobun et al., 1998) (Figure 1). The plant is a rich source for glucosinolates (Goetz and Schraudolf, 1983; Lockwood and Belkhiri, 1991; Fréchard et al., 2001). Representative compounds include glucoraphanin (**14**), progoitrine (**15**) and glucobrassicine (**16**). Other compound classes reported include aromatic and aliphatic carboxylic acids (Hartleb and Seifert, 1995), various glycosides (Hartleb and Seifert, 1994), amino acids (Zhu, 1998), isoprenoids (Hartleb and Seifert, 1994; Zhu, 1998), flavonoids and anthranoids (Wu et al., 1997b).

Isatis extracts and selected constituents have been screened for antiviral, antifungal, antibacterial and cytoinhibitory activities. Tryptanthrin was originally isolated by Honda et al. (1980) as an antidermatophytic. The antimycobacterial activity of the compound against *Mycobacterium smegmatis* prompted a synthetic programme on indoloquinazoline derivatives. Some compounds had good *in vitro* activity but were later found to lack *in vivo* efficacy (Mitscher and

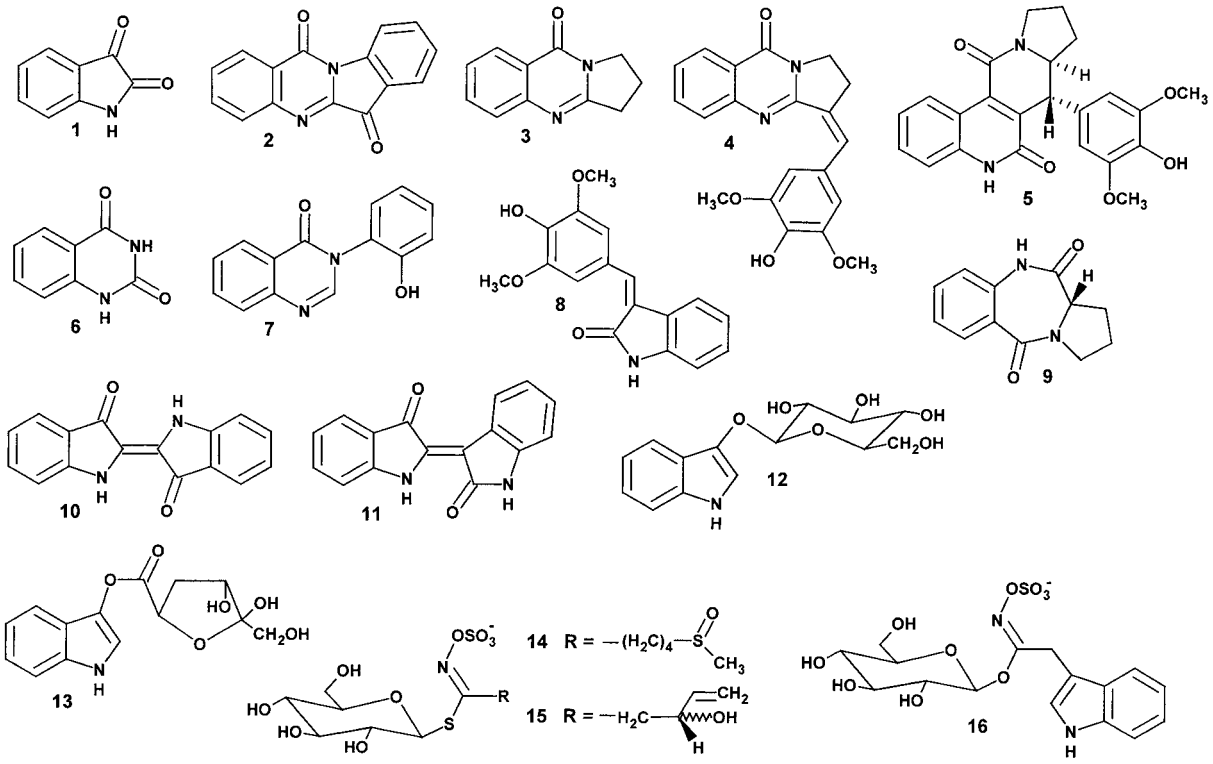

Figure 1. Structures of selected metabolites in *Isatis tinctoria* and *I. indigotica*.

Baker, 1998). An aqueous woad decoction had positive effects in a chronic pulmonary infection model in rats and was deemed promising for human studies (Song et al., 1996). In the context of the development of wood protecting varnishes from woad, fermented woad juice was tested for activity against wood rotting fungi (Bugge and Heiner, 1995), and tryptanthrin was shown to be weakly insecticidal (Seifert and Unger, 1994). In a clinical pilot trial with allergic patients, woad decoctions had some effects on immunological parameters, but the beneficial effects were considered as not being conclusive (Diehl et al., 1992). Indirubin (**11**) underwent extensive preclinical development in China as an anticancer drug (Tang and Eisenbrand, 1992). In animal leukemia and lung carcinoma models, the compound showed good inhibitory effect and low toxicity. Indirubin inhibits cyclin dependent kinases and, hence, cell division (Hoessel et al., 1999). Cytoinhibitory activity was also reported for tryptanthrin (Kimoto et al., 1999). These findings are in disagreement with our own cytotoxicity studies in Mono Mac and RAW cells, where we found no inhibitory effects at concentrations up to 10 μg/ml and moderate inhibition only at 100 μg/ml (Danz et al., 2001). We were also not able to reproduce the antidermatophytic and some of the antibacterial activities reported for woad extracts and tryptanthrin (Danz, 2000).

Exploring the pharmacological profile of Isatis tinctoria as an anti-inflammatory

Irrespective of these inconclusive bioactivity data, two aspects aroused our particular interest in *Isatis*: the well documented use over centuries as an important medicinal plant in two geographically distant cultural settings, and a certain common thread in the historical uses, in particular the association of woad preparations with ailments involving inflammatory processes. Given the need outlined above for rational plant-derived anti-inflammatory agents, we deemed the plant of sufficient promise for a broad-based evaluation of its potentialities as an anti-inflammatory. The lack of any previous scientific investigations on woad in that respect was a strong incentive.

Several approaches can be pursued in the assessment of a plant's pharmacological potential. Instead of

classical *in vivo* models for acute and chronic inflammation, such as carrageenan-induced rat paw oedema, croton oil ear oedema, or cotton wool granuloma (Vogel and Vogel, 1997), we felt that a broad-based *in vitro* screening directed at a multitude of clinically relevant targets would be a more suitable approach for our purpose. Our rationale was that modern *in vitro* assay technologies would provide higher sensitivity and allow the identification of defined mechanisms of action at a very early stage. We considered that knowledge of the pharmacological profile would be crucial for making informed decisions during the progression of the project, and would facilitate the activity-guided search for active principles.

Depending on the mode of preparation, the composition of an extract may vary considerably. This, in turn, may determine to a large extent the outcome of pharmacological profiling. For a systematic assessment of the bioactivity profile of *Isatis*, we, therefore, prepared lipophilic and polar extracts from fresh and dried root and leaves. These extracts were then submitted to a panel of cell-based and mechanism-based *in vitro* assays targetting leucotriene B_4 (LTB_4), thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) and rabbit platelet activating factor (PAF) receptors, COX-1 and COX-2, 5-LOX, 15-LOX, phospholipase A_2 (PLA_2), phospholipase C (PLC), and human leucocytic elastase. Inhibition of adhesion factor expression was assayed for intercellular adhesion molecule 1 (ICAM-1), endothelial adhesion molecule (ELAM-1) and vascular adhesion molecule 1 (VCAM-1). Cell-based assays were carried out for COX-2 (in HUV-EC cells) and iNOS activity (in RAW264,9 cells). Mixed lymphocyte reaction, immune stimulation as well as immune suppression, was assessed with murine lymphocytes, and inhibition of histamine and serotonin release was separately measured in stimulated rat mast cells (Danz, 2000).

Of the six extracts tested in the assay panel, a DCM extract from dry leaves displayed the most promising *in vitro* pharmacological profile (Figure 2). Particular features were a marked COX inhibitory activity, with a preferential inhibition of COX-2 over COX-1 in assays with isolated enzymes, inhibition of 5-LOX, iNOS, serotonin and histamine release, and of human leucocytic elastase. COX and 5-LOX are key enzymes in the formation of pro-inflammatory prostaglandins and leucotrienes, and hence, the targets of clinically used NSAID's and antiasthmatic drugs (Mutschler, 2001). iNOS contributes to oedema formation, hyperalgesia and pain (Moncada et al., 1991). Serotonin and histamine are also important mediators of inflammation and pain (Mutschler, 2001). Leucocytic elastase, a major proteinase of neutrophils released in connection with inflammation, cleaves important extracellular matrix proteins (Bieth, 1998). Given the potency and the unique activity profile combining effects on several key targets in inflammatory processes, the DCM extract was deemed of sufficient promise for further investigations.

Search for active compounds – The COX-2 inhibitory principle in woad

For the discovery of new plant-derived natural products leads and for the development of science-based phytopharmaceuticals, the identification of active principles in an extract is a prerequisite. The classical approach of preparative activity-directed fractionation (Hamburger and Hostettmann, 1991) is tedious and not always successful. With the advances in on-line spectroscopy and bioassay technology, the discovery process can be miniaturized to analytical scale, and, hence, streamlined for increased efficiency (Danz et al., 2001).

The principle of HPLC-based activity profiling implemented in our laboratory is shown in Figure 3. An extract is separated by analytical gradient HPLC, and the effluent analyzed by on-line DAD and ESI MS. Via a T-split, a portion of the effluent is simultaneously fractionated in the 96-well microtitre format for bioassay. Bioactivity can thus be linked to specific peaks in the chromatogram, and to their UV-vis and MS data. Database searches based on structural information derived from the on-line spectra may provide tentative structural assignments. Preparative purification of an active principle is only carried out if the compound is deemed of sufficient interest.

Activity profiling of the extract for COX-2 activity is shown in Figure 4. In a first round, COX-2 inhibition was localized in a single time window, which was assayed in a second round at higher resolution. The COX-2 inhibitory principle finally was a single minor compound. It was readily identified on the basis of its UV-vis and ESI-MS spectra as tryptanthrin (Figure 5). The major peak in the extract was indirubin, which, however, had only marginal COX activity. The identity of tryptanthrin was subsequently confirmed by preparative isolation and by synthesis (Danz et al., 2000, 2001).

COX-2 in HUV-EC cells	IC_{50} 8.1 µg/ml
COX-2 human enzyme	IC_{50} 1.2 µg/ml
COX-1 human enzyme	IC_{50} > 40 µg/ml
5-LOX human enzyme	IC_{50} 5.1 µg/ml
Inducible NO synthase in RAW 264.7 cells	IC_{50} 10 µg/ml
Serotonin release from rat mast cells	IC_{50} 2.5 µg/ml
Histamine release from rat mast cells	IC_{50} 2.3 µg/ml
Human leukocytic elastase	IC_{50} 1.0 µg/ml

Figure 2. *In vitro* activity profile of the dichloromethane extract.

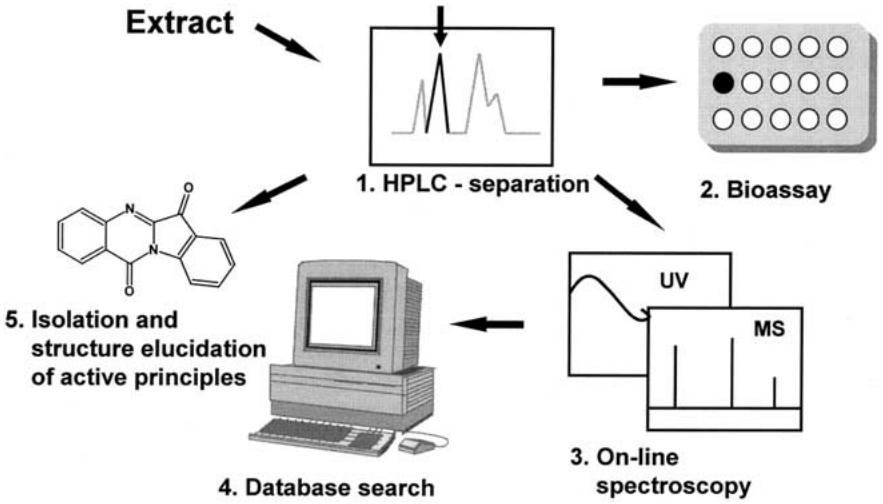

Figure 3. Principle of HPLC-based activity profiling of extracts.

Tryptanthrin had been isolated from *Isatis tinctoria* as an antifungal in 1980 already (Honda et al., 1980), and was later investigated by several other groups (Tang and Eisenbrand, 1992; Seifert and Unger, 1994; Mitscher and Baker, 1998). Its potential as an anti-inflammatory, however, was not discovered at that time. The potency of tryptanthrin in the activity profiling and its structural uniqueness as a COX-2 inhibitor warranted a more detailed *in vitro* pharmacological study in cell- and mechanism-based assays (Figure 6).

Tryptanthrin strongly inhibited COX-2 catalyzed eicosanoid synthesis in LPS-stimulated Mono Mac 6 cells. Dose-inhibition curves for tryptanthrin and a number of known COX-inhibitors are shown in Figure 6A. 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 acetylsalicylic acid was lower by two orders of magnitude (IC_{50} 3.8 μM). 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 was comparable to those in Mono Mac 6 cells, although all IC_{50} values were shifted by about one order of magnitude (IC_{50} values of 0.25 μM, 0.21 μM and 0.013 μM, respectively) (Danz et al., 2002b).

The inhibition of COX-1 catalysed TXB_2 formation in HEL cells by tryptanthrin, acetylsalicylic acid, and the two non-selective COX inhibitors diclofenac and indometacin was compared (Figure 6B). The potency of tryptanthrin (IC_{50} 0.36 μM) was about one hundred times lower than that of the two non-selective inhibitors. Taken together, the data in these three cell lines suggested a certain degree of selectivity towards the COX-2 isoenzyme. The COX-2 selectivity of tryptanthrin was substantiated in primary cells. Its effects on COX-2 activity in BAEC and COX-1 catalysed eicosanoid synthesis in bovine thrombocytes

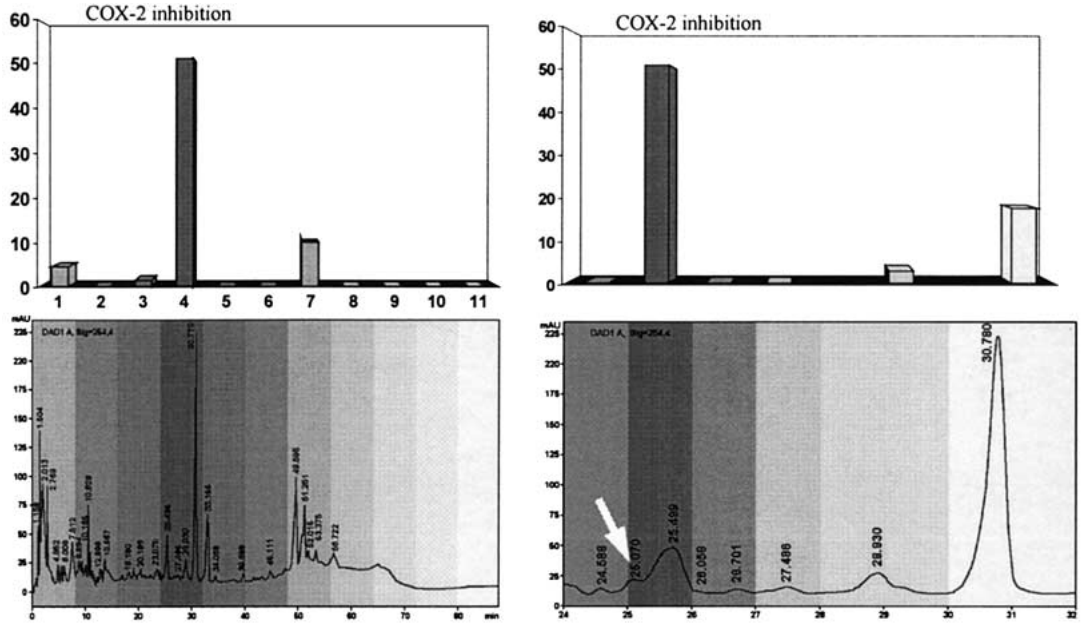

Figure 4. Activity profiling of the SFE extract. (A) HPLC profile obtained by gradient elution. The grey shades indicate time windows for individual fractions. Above: Activity profile (COX-2 inhibition in Mono Mac-6 cells) of fractions. (B) Expanded time window 24–32 min and (above) activity in bioassay. The arrow indicates the tryptanthrin peak. Sample amount injected: 200 μg; one quarter of each fraction was used for bioassay (Reprinted from Danz et al., 2001).

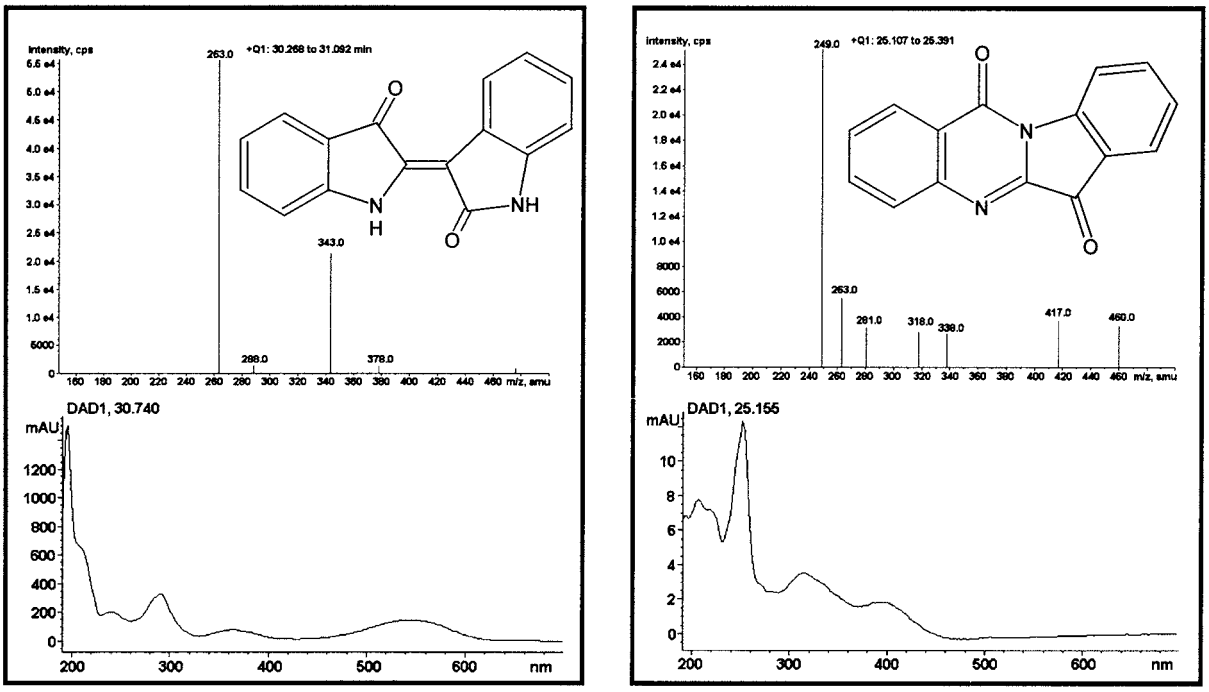

Figure 5. DAD and ESI MS of indirubin (left) and tryptanthrin (right) recorded on-line.

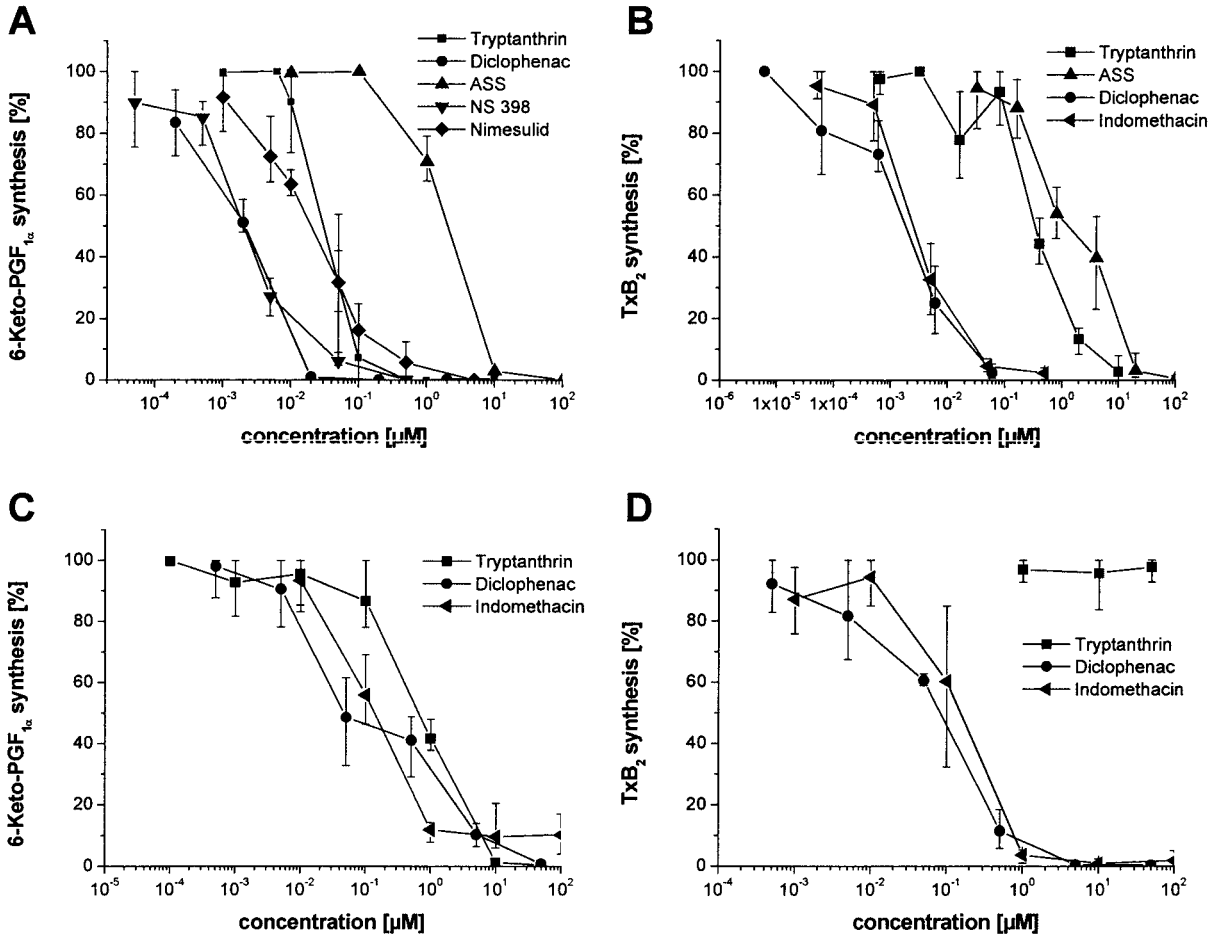

Figure 6. In vitro activity of tryptanthrin and selected NSAID's on eicosanoid synthesis in LPS stimulated Mono Mac 6 (A); HEL cells (B); and on purified COX-2 (C) and COX-1 (D) (Reprinted from Danz et al., 2002b).

was compared with indometacin as a non-selective COX inhibitor and DFU as a selective COX-2 inhibitor as reference compounds (Danz et al., 2002b).

Assays with purified COX-2 and COX-1, in comparison to the non-selective inhibitors diclofenac and indometacin, confirmed tryptanthrin as a COX-2 selective inhibitor (Figures 6C and D). COX-2 was inhibited with an IC_{50} of 0.83 μM, whereas COX-1 activity remained unaffected at concentrations up to 50 μM. In contrast, the non-selective reference compounds displayed comparable inhibitory potencies towards the two isoenzymes.

The pronounced activity of the *Isatis* extract on purified 5-LOX prompted us to test the extract and tryptanthrin in a cell based assay with human neutrophils. The A23187-induced LTB_4 synthesis was taken as an indirect measure for 5-LOX activity (Thomet et al., 2001). The extract stongly inhibited LTB_4 release (IC_{50} value 1.0 μg/ml), and the potency of tryptanthrin was comparable to that of the clinically used 5-LOX inhibitor zileuton (IC_{50} values of 0.15 and 0.35 μM, respectively). Tryptanthrin was thus, in part responsible, for the activity of the extract. Taking the low tryptanthrin concentration into account, however, it is evident that other constituents contribute to the activity of the extract (Danz et al., 2002b).

The inhibitory properties of tryptanthrin on nitric oxide synthesis was reported by another group in research parallel to our investigations (Ishihara et al., 2000). Taken together, tryptanthrin inhibits the formation of three major groups of pro-inflammatory substances in cell based assays at nM to μM concentrations. The compound has no obvious structural resemblance with any of the selective COX-2 inhibitors of synthetic origin in clinical use or in development (Dannhard and Kiefer, 2001) (Figure 7). The

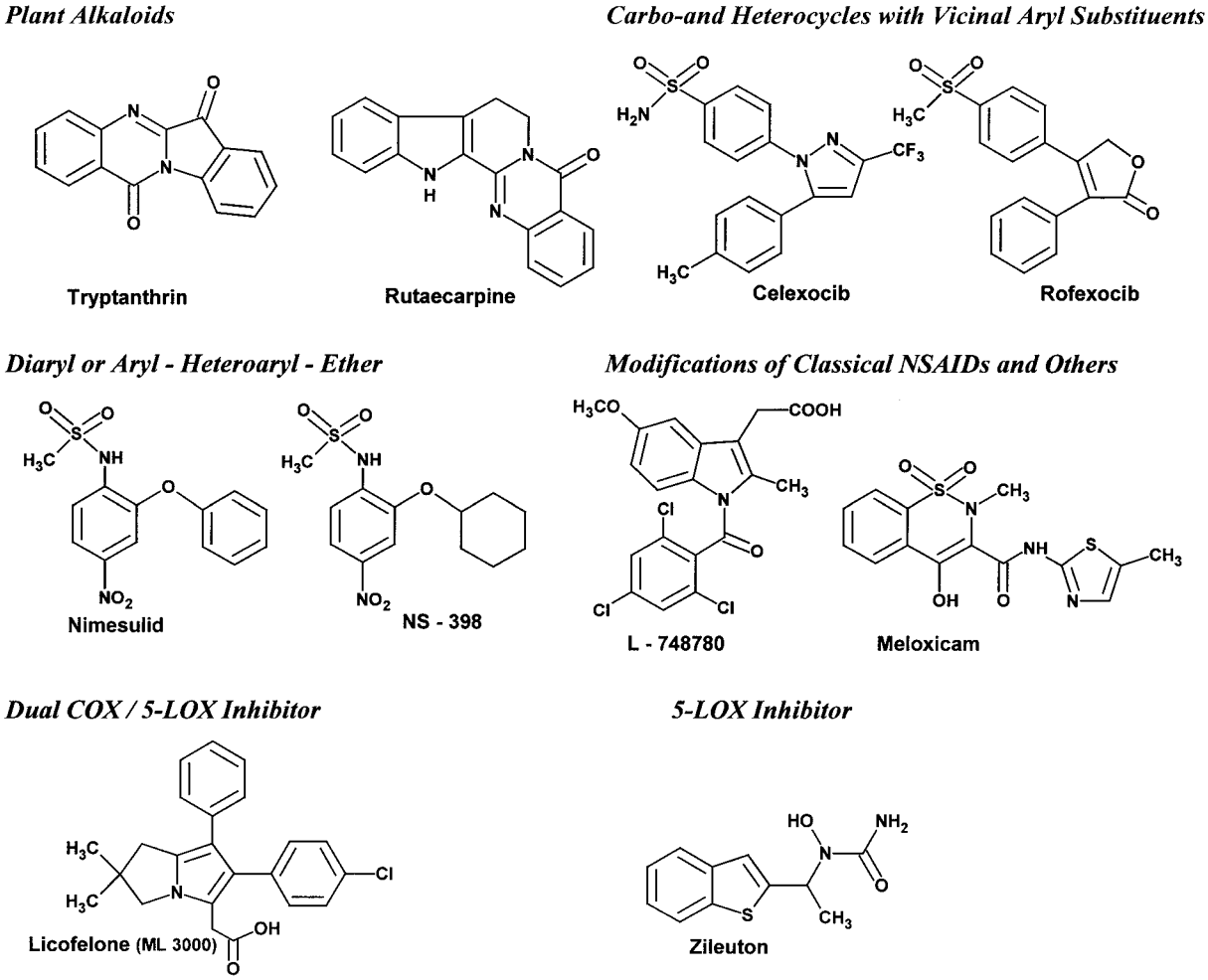

Figure 7. Structures of tryptanthrin, rutaecarpine, and of synthetic compounds representing major classes of COX-2, 5-LOX (zileuton) and dual COX/5-LOX (ML 3000) inhibitors.

closest structural similarity was found with the alkaloid rutaecarpine, a potent COX-2 inhibitor from *Evodia rutaecarpa* (Moon et al., 1999).

The simultaneous inhibition of 5-LOX-mediated leucotriene formation by tryptanthrin was of particular interest. It is believed that simultaneous inhibition of COX and 5-LOX should prevent redirection of the substate to other key enzymes in the arachidonic acid cascade and, hence, lead to improved anti-inflammatory drugs (Fiorucci et al., 2001). Numerous dual inhibitors have been synthesized on the basis of this concept, and one of them, ML3000 (licofelone), is currently in Phase III clinical trials (Boileau et al., 2002). Again, tryptanthrin has no structural similarity with these dual inhibitors, nor with 5-LOX inhibitors such as the antiasthmatic drug zileuton (Steinhilber, 1999).

Tryptanthrin is thus a molecule that fullfils most requirements for a promising lead compound. It combines a unique spectrum of activities and considerable potency *in vitro* with structural features that are considered as typical of orally available drugs in terms of molecular mass, lipophilicity, number of proton acceptor and donor sites (Lipinski et al., 1997). The compound is easily amenable to synthesis, and its scaffold offers numerous possibilities of structural modifications for lead optimization.

In vivo pharmacology

In vivo activities of a DCM extract, a supercritical CO_2 (SFE) extract and tryptanthrin were assessed in the TPA-induced mouse ear oedema and in carrageenan-induced mouse paw oedema as models for acute inflammation. Topical administration of extracts (0.5 mg/ear) significantly inhibited the ear oedema (32% for SFE extract, 62% for DCM extract). Oral administration (100 mg/kg for SFE extract, 125 mg/kg for DCM extract) inhibited the ear oedema by 37% and 33%, respectively, whereas effects of indometacin (10 mg/kg) and tryptanthrin (70 mg/kg) were not significant by this route of administration (Recio et al., 2002).

In the mouse paw oedema model, the extracts showed dose dependent inhibition which was strongest between 1 and 3 h after administration. The ED_{50}'s were 78 mg/kg (SFE extract) and 165 mg/kg (DCM extract). Indometacin (10 mg/kg) showed maximal inhibition (65%) after 3 h. No clear dose-effect relationship was found for tryptanthrin at doses up to 70 mg/kg. The weak activity of the purified compound was possibly due to its poor solubility, resulting in low cutaneous penetration and oral bioavailability. Given the extract's multiple pharmacological activities, the synergistic effect of several active principles is probably responsible for the pronounced anti-inflammatory properties of the extract.

The low solubility of pure tryptanthrin and its weak *in vivo* activity prompted us to consider bioavailability issues in more detail. First, we decided to shed some light on the intriguing findings from the topical application in the mouse ear model. For that, suitable analytical tools were needed for accurate monitoring of the skin penetration of tryptanthrin. After the careful review of current methodologies, we opted for skin microdialysis, an emerging technique that allows for a time-resolved *in situ* measurement of local drug concentrations (Schnetz and Fartasch, 2001). An attractive feature in view of clinical studies was that the method is minimally invasive and thus can be used in human volunteers. In skin microdialysis, the concentration of drug substances penetrating the skin upon topical application is determined in the dialysis fluid of a semipermeable capillary placed below the skin surface. The dialysis fluid collected at the capillary outlet is analyzed. We have recently established and validated a microdialysis method for tryptanthrin using the pig foreleg as a model (Oberthür et al., 2002b). A hollow fibre (i.d. 200 μm, exclusion limit 5000 amu) was placed in the dermis at approx. 1 mm below the skin surface. Defined solutions of tryptanthrin and woad extracts were applied onto the skin area above the fibre. Tryptanthrin concentrations in the dialysis fluid (flow rate of 2 μl/min) were determined by ESI LC-MS, using d_8-tryptanthrin (Oberthür et al., 2002a) as internal standard. A short, narrow-bore HPLC column was used without eluent split and with detection in the SIM mode to achieve the highest sensitivity. In the pig foreleg, measurable tryptanthrin concentrations were found in the dialysate 30 min after the topical application of test compound or extract. Tryptanthrin penetration from extracts was proportionally higher than when a solution of pure compound was applied. These data support the notion that extract substances may enhance the penetration of the otherwise poorly soluble tryptanthrin. Following these findings, a pilot study in volunteers evaluating the effects of *Isatis* extract and tryptanthrin in clinically relevant skin irritation models is underway.

Plant selection and extraction

The *Isatis* project is currently being pursued primarily from the perspective of the possible development of a new phytopharmaceutical. For such products, the quality and efficacy is, to a large extent, determined by the quality of the herbal raw material. Hence, plant selection and breeding, optimal agricultural practices and post-harvest processing are of major importance. With knowledge of the active principles in a medicinal plant, objective and quantifiable measures for quality can be defined.

We recently initiated a selection programme for woad with optimal pharmaceutical qualities, assuming that the considerable genetic diversity of woad would probably translate into significant differerences of the secondary metabolite pattern (Fiehn, 2002). As a first step, a total of 67 *Isatis* samples were screened for tryptanthrin content with the aid of a validated method for automated extraction by PLE (Benthin et al., 1999) and subsequent dosage by LC-MS. The geographic origin of specimens ranged from Morocco to Western Europe and East Asia and was probably fairly representative of the genetic diversity of woad. The tryptanthrin content in the leaf samples was between 0.56 and 16.74×10^{-3}%, corresponding to an almost 30-fold difference (Danz et al., 2002). The fact that the COX-2 inhibitory compound in *Isatis* may vary to such an extent highlights the importance of using the

active principles as objective quality traits in plant selection and breeding. Obviously, a similar screening is needed for the assessment of the other active principles in woad.

If *Isatis* is to be considered under the perspective of a phytopharmaceutical, the issue of extraction has to be resolved in an adequate manner. Apart from a few exceptions, phytopharmaceuticals are prepared by extraction of the crude drug with aqueous ethanol of varying proportions (Hänsel et al., 1999). The most potent anti-inflammatory activity of woad, however, was in a DCM extract. Considering the extraction step from a developmental viewpoint, an alternative extraction was needed to replace the environmentally unacceptable chlorinated solvent. Extraction with supercritical CO_2 appeared to be the most suitable technology. Numerous SFE applications for natural products have been reported (Kaiser et al., 2001), and several large-scale processes for foods, spices and fragrances have been successfully implemented in the industry over the past two decades (Taylor, 1996). We recently optimized a SFE process to produce an extract which was comparable to the DCM extract with respect to the chromatographic fingerprint and the pharmacological profile (Danz et al., 2001).

Conclusions and perspectives

The example of *Isatis tinctoria* highlights a number of issues of general interest to natural product scientists. The investigation of plants from temperate climatic zones has been somewhat neglected in the past years in favor of more intensive research on tropical plants. The example of *I. tinctoria*, and numerous other cases from the literature, demonstrate that equally promising plants are to be found in temperate areas. Secondly, there is a widely held belief among pharmacognosists that the potential for interesting discoveries would be primarily in the 'uncharted territories' of the plant kingdom. Again, the example shows that even extensively studied plants may be the source of exciting and unexpected discoveries, such as novel bioactivities of considerable promise. We believe that *Isatis* is not an isolated case in that respect, and we strongly advocate the re-investigation of such plants. Given the complexity of a plant's metabolome, any species can only be considered as 'investigated' under the specific angle of these earlier studies, and must be regarded as 'unknown' in all other aspects. This is particularly true with respect to bioactivities. Recent examples from our own laboratory support this viewpoint (Dittmann et al., 2002; Hamburger et al., 2002).

Following our initial discovery of the anti-inflammatory potential of woad (Danz et al., 1999, 2000), other groups have also investigated the anti-inflammatory principles in *Isatis*. The iNOS activity of tryptanthrin was found as a consequence of such efforts (Ishihara et al., 2000), and the effects of isaindigotone from *Isatis indigotica* on leucocytic functions reported shortly after (Molina et al., 2001).

A rapidly growing body of evidence confirms that woad is a long-neglected medicinal plant of considerable pharmaceutical promise. A continued systematic investigation is needed to further substantiate its anti-inflammatory potential, so that this ancient herb may possibly reintegrate into the repertory of European phytotherapy and novel, rational phytopharmaceuticals be developed.

References

Benthin B, Danz H & Hamburger M (1999) Pressurized liquid extraction (PLE) of medicinal plants. J. Chromatogr. A 837: 211–219.

Bieth JG (1998) Leucocytic elastase. In: Barett AJ, Rawlings ND (eds) Handbook of proteolytic enzymes. Academic Press, London, pp. 54–60.

Blumenthal M (1998) The complete German Commission E monographs. American Botanical Council, Austin, Texas.

Boileau C, Martel-Pelletier J, Jouzeau JY, Netter P, Moldovan F, Laufer S, Tries S & Pelletier JP (2002) Licofelone (ML-3000), a dual inhibitor of 5-lipoxygenase and cyclooxygenase, reduces the level of cartilage chondrocyte death *in vivo* in experimental dog osteoarthritis: Inhibition of pro-apoptotic factors. J. Rheumatol. 29: 1446–1453.

Bugge G & Heiner C (1995) Untersuchungen zur Wirksamkeit von Färberwaid (*Isatis tinctoria* L.) gegenüber holzzerstörenden Pilzen. Forst Holz 50: 353–354.

Dannhard G & Kiefer W (2001) Cyclooxygenase inhibitors – current status and future prospects. Eur. J. Med. Chem. 36: 109–126.

Danz H (2000) Untersuchungen zur antiinflammatorischen Wirkung und zur Analytik von Tryptanthrin in *Isatis tinctoria* L. PhD Thesis, University of Jena.

Danz H, Stoyanova S, Hamburger M & Brattström A (1999) Preferential inhibition of cyclooxygenase-2 (COX-2) by a plant extract (ZE 550). In: Luijendijk T, De Graaf P, Remmelzwaal A & Verpoorte R (eds) Abstracts of the Joint Meeting 2000 years of natural products research – past, present, and future (p. 769). Leiden Amsterdam Centre for Drug Research, Leiden.

Danz H, Stoyanova S, Wippich P, Brattström A & Hamburger M (2000) Identification and isolation of the cyclooxygenase-2 inhibitory principle in *Isatis tinctoria*. Arch. Pharm. 333 (Suppl. 1): 11.

Danz H, Stoyanova S, Wippich P, Brattström A & Hamburger M (2001) Identification and isolation of the cyclooxygenase-2 inhibitory principle in *Isatis tinctoria*. Planta Med. 67: 411–416.

Danz H, Baumann D & Hamburger M (2002a) Quantitative determination of the dual COX-2/5-LOX inhibitor tryptanthrin in *Isatis tinctoria* L. by ESI-LC-MS. Planta Med. 68: 152-157.

Danz H, Stoyanova S, Thomet OAR, Simon HU, Dannhardt G, Ulrich H & Hamburger M (2002b) Inhibitory activity of tryptanthrin on prostaglandin and leucotriene synthesis. Planta Med. 68: 875–880.

Diel F, Well S, Ficht F & Paetzold M (1992) Tea of *Isatis tinctoria* (woad) responses by allergic patients *in vivo* and *in vitro*. Akt. Ernähr. Med. 17: 34–36.

Dittmann K, Gerhäuser C, Klimo K & Hamburger M (2002) Monoamine oxidase (MAO) and inducible nitric oxide synthase (iNOS) inhibitory activity of *Salvia miltiorrhiza*. In: Canigueral S. (ed) Book of Abstracts of the 50^{th} Annual Congress of the Society for Medicinal Plant Research (p. 158). CITA Publicaciones y Documentacion, Valencia.

Dornberger K & Lich H (1982) Screening nach antimikrobiell sowie potentiell cancerostatisch wirksamen Pflanzeninhaltsstoffen. Pharmazie 37: 215–221.

Epstein E, Nabors MW & Stowe BB (1967) Origin of indigo of woad. Nature 216: 547–549.

Ernst E & Chrubasik S (2000) Phyto-anti-inflammatories. A systematic review of randomized, placebo-controlled, double-blind trials. Rheum. Dis. Clin. N. Am. 26: 13–27.

Fiehn O (2002) Metabolomics – the link between genotypes and phenotypes. Plant Mol. Biol. 48: 155–171.

Fiorucci S, Meli R, Bucci M & Cirino G (2001) Dual inhibitors of cyclooxygenase and 5-lipoxygenase. A new avenue in anti-inflammatory therapy? Biochem. Pharmacol. 62: 1433–1438.

Fréchard A, Fabre N, Pean C, Montaut S, Fauvel MT, Rollin P & Fouraste I (2001). Novel indole-type glucosinolates from woad (*Isatis tinctoria* L.). Tetrahedron Lett. 42: 9015–9017.

Gilbert KG, Garton S, Karam MA, Arnold GM, Karp A, Edwards KJ, Cooke DT & Barker JHA (2002) A high degree of genetic diversity is revealed in *Isatis* sp. (dyer's woad) by amplified fragment length polymorphism (AFLP). Theor. Appl. Genet. 104: 1150–1156.

Goetz JK & Schraudolf H (1983) Two natural indole glucosinolates from Brassicaceae. Phytochemistry 22: 905–907.

Hamburger M & Hostettmann K (1991) Bioactivity in plants: The link between phytochemistry and medicine. Phytochemistry 30: 3864–3874.

Hamburger M, Riese U, Graf H, Melzig M, Ciesielski S, Baumann D, Dittmann K & Wegner C (2002). Constituents in Evening Primrose oil with radical scavenging, cyclooxygenase and neutrophil elastase inhibitory activities. J. Agric. Food. Chem. 50: 5533–5538.

Hänsel R, Sticher O & Steinegger E (1999) Pharmakognosie – Phytopharmazie. 6th ed. Springer, Berlin.

Hartleb I & Seifert K (1994) A novel anthranilic acid derivative from *Isatis tinctoria*. Planta Med. 60: 578–579.

Hartleb I & Seifert K (1995) Acid constituents from *Isatis tinctoria*. Planta Med. 61: 95–96.

Hoessel R, Leclerc S, Endicott JA, Nobel MEM, Laurie A, Tunnah P, Loest M, Damiens E, Marie D, Marko D, Niederberger E, Tang W, Eisenbrand G & Meijer L (1999) Indirubin, the active constituent of a Chinese antileukemia medicine, inhibits cyclin-dependent kinases. Nature Cell Biol. 1: 60–67.

Honda G, Tosirisuk V & Tabata M (1980) Isolation of an antidermatophytic, tryptanthrin, from indigo plants, *Polygonum tinctorium* and *Isatis tinctoria*. Planta Med. 38: 275–276.

Hurry JB (1930) The woad plant and its dye. Oxford University Press, London.

Index Kewensis (1997) Oxford University Press, London.

Ishihara T, Kohno K, Ushio S, Kanso I, Ikeda M & Kurimoto M (2000) Tryptanthrin inhibits nitric oxide and prostaglandin E2 synthesis by murine macrophages. Eur. J. Pharmacol. 407: 197–204.

Kaiser CS, Römpp H & Schmidt PC (2001) Pharmaceutical applications of supercritical carbon dioxide. Pharmazie 56: 907–926.

Kimoto T, Yamamoto Y, Hino K, Koay S, Aga H, Hashimoto T & Hanaya T (1999) Cytotoxic effects of substances in indigo plant (*Polygonum tinctorium* Lour.) on maligant tumour cells. Natural Med. 53: 72–79.

Kokobun T, Edmonds J & John P (1997) Indoxyl derivatives in woad in relation to medieval indigo production. Phytochemistry 49: 79–87.

Lipinski CA, Lombardo F, Dominy BW & Feeney PJ (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug Deliv. Rev. 23: 3–25.

Lockwood GB & Belkhiri A (1991) Glucosinolate spectrum of some Algerian Cruciferae. Plant Syst. Evol. 176: 11–20.

Lyss G, Schmidt TJ, Merfort I & Pahl HL (1997) Helenalin, an anti-inflammatory sesquiterpene lactone from *Arnica* selectively inhibits transcription factor NF-κB. Biol. Chem. 378: 951–961.

Mitscher LA, Baker WR (1998) A search for novel chemotherapy against tuberculosis amongst natural products. Pure Appl. Chem. 70: 365–371.

Molina P, Tarraga A, Gonzalez-Tejero A, Rioja I, Ubeda A, Terencio MC, Alcaraz MJ (2001) Inhibition of leucocyte functions by the alkaloid isaindigotone from *Isatis indigotica* and some new synthetic derivatives. J. Nat. Prod. 64: 1297–1300.

Moncada S, Palmer RMJ & Higgs EA (1991) Nitric oxide: Physiology, pathophysiology and pharmacology. Pharmacol. Rev. 43: 109–142.

Moon C, Murakami M, Kudo I, Son KH, Kim HP, Kang SS & Chang HW (1999) A new class of COX-2 inhibitor, rutaecarpine from *Evodia rutaecarpa*. Inflammat. Res. 48: 621–625.

Mutschler E, Geisslinger G, Kroemer HK & Schäfer-Korting M (2001) Arzneimittelwirkungen. 8th ed. Wissenschaftliche Verlagsgesellschaft, Stuttgart.

Oberthür C, Hoffmann B & Hamburger M (2002a) Synthesis of *d*-tryptanthrin. Pharmazie 57: 586–587.

Oberthür C, Heinemann C, Elsner P, Benfeldt E & Hamburger M (2002b) A subcutaneous microdialysis method combined with ESI LC–MS analysis for studying the skin penetration of tryptanthrin in *Isatis* extracts. In: Canigueral S. (ed) Book of Abstracts of the 50^{th} Annual Congress of the Society for Medicinal Plant Research (p. 70). CITA Publicaciones y Documentacion, Valencia.

Recio MC, Hamburger M & Rios JL (2002) *In vivo* anti-inflammatory activity of *Isatis tinctoria* extracts and tryptanthrin. In: Canigueral S (ed) Book of Abstracts of the 50th Annual Congress of the Society for Medicinal Plant Research (p. 94). CITA Publicaciones y Documentacion, Valencia.

Richter BE, Jones BA, Ezzell JL, Porter NL, Avdalovic N & Pohl C (1996) Accelerated solvent extraction: A technique for sample preparation. Anal. Chem. 68: 1033–1039.

Schnetz E & Fartasch M (2001) Microdialysis for the evaluation of penetration through the human skin barrier – a promising tool for future research? Eur. J. Pharm. Sci. 12: 165–174.

Seifert K & Unger W (1994) Insecticidal and fungicidal compounds from *Isatis tinctoria*. Z. Naturforsch. C 49: 44–48.

Song Z, Johanson HK, Moser C & Hoiby N (1996) Effects of Chinese medicinal herbs on rat model of chronic *Pseudomonas aeruginosa* lung infection. APMIS 104: 350–354.

Steinhilber D (1999) 5-Lipoxygenase: a target for antiinflammatory drugs revisited. Curr. Med. Chem. 6: 71–85.

Strobel J & Gröger D (1989) Presence of indigo precursors in *Isatis*. Biochem. Physiol. Pflanz. 184: 321–327.

Tang W & Eisenbrandt G (1992) Chinese drugs of plant origin. Springer, Berlin.

Taylor LT (1996). Supercritical fluid extraction. Wiley, New York.

Thomet OAR, Wiesmann UR, Schapowal A, Bizer C & Simon HU (2001). Role of petasin in the potential anti-inflammatory activity of a plant extract of *Petasites hybridus*. Biochem. Pharmacol. 61: 1041–1047.

Vane JR, Bakhle YS & Botting RM (1998) Cyclooxygenases 1 and 2. Annu. Rev. Pharmacol. Toxicol. 38: 97–120.

Vogel HG & Vogel WH (eds.) (1997). Drug discovery and evaluation – Pharmacological assays. Springer, Berlin.

Wu XY Liu Y, Sheng W, Sun J & Qin G (1997a) Chemical constituents of *Isatis indigotica*. Planta Med. 63: 55–57.

Wu XY, Qin GW, Cheung KK & Cheng KF (1997b) New alkaloids from *Isatis indigotica*. Tetrahedron 53: 13323–13328.

Zhang F, Altorki NK, Mestre JR, Subbaramaiah K & Dannenberg AJ (1999) Curcumin inhibits cyclooxygenase-2 transcription in bile acid- and phorbol ester-treated human gastrointestinal epithelial cells. Carcinogenesis 20: 445–451.

Zhu YP (1998) Chinese Materia Medica. Harwood Academic, Amsterdam.