Planta medica

Journal of Medicinal Plant Research

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Hippokrates Verlag
Stuttgart

Vol. 38
March 1980

No. **3**

Review Article

Naturally Occurring Isohexenylnaphthazarin Pigments: A New Class of Drugs

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Key Word Intex: Boraginaceae; Alkannin; Shikonin; Naphthazarin; Biosynthesis;
Antibacterial Activity; Antitumor Activity; Cytotoxicity; Wound Healing.

Abstract

The chemistry, biosynthesis, biological properties and the wound healing activity of isohexenylnaphthazarin pigments are being reviewed. Dermatological studies indicate that these long known plant products may be regarded as a new class of drugs.

Introduction

The isohexenylnaphthazarins, commonly known as alkannins, are lipophilic red pigments [1, 2, 3]. They were found in the outer surface of the roots of at least a hundred and fifty species that belong to the genus *Lithospermum*, *Echium*, *Onosma*, *Anchusa* and *Cynoglossum* of

the family *Boraginaceae* [1]. Their occurrence in *Jatropha glandulifera*, a member of the *Euphorbiaceae*, should be considered as an exception [4, 5].

The use of alkannins as dyes was known to ancient Greeks and Romans who employed the roots of *Anchusa tinctoria* or *Alkanna tinctoria* for this purpose. The wound healing properties of these roots were described by DIOSCORIDES.

The healing properties of the extracts of *Lithospermum radix* (Shikon) attracted the attention of the Chinese who used them as folk remedies, even though the coloring properties were known to them and to other nations in the Far East. Even today the inhabitants of India and Pakistan employ these dyes [6, 7]. Among the plants used for this purpose is *Arnebia nobilis*. A detailed study on the pigments in this plant has shown that these are mixtures of alkannin and its esters [8, 9].

Although the wound healing activity of alkannin was known all over the world, such use has faded away with time. Ironically the use as a food colorant [10, 11] survived and is still used as such in our days. At least twelve European countries allow its use as colorant in food and wine.

Alkannin attracted the attention of chemists in the forties when it was used for a short period as an analytical reagent with limited success [12, 13].

Chemistry

All presently known naturally occurring isohexenylnaphthazarins are listed in Table I. The structural features common to all these pigments are the naphthazarin moiety and the isohexenyl side chain. Whereas they differ in the follow-

ing respects: (1) their manner of rotating polarised light (chirality), (2) the position of the alcoholic group in the side chain, and (3) the acid moiety which esterifies the alcoholic group.

Alkannin, the first identified member of the naphthazarins, was isolated from two European varieties, *Alkanna tinctoria* [14, 28, 29] and *Onosma echinoides* [30, 31]. Its structure was elucidated by BROCKMANN [14] and found to be the *laevo*-rotatory 5,8-dihydroxy-2-(1'-hydroxy-4'-methylpent-3'-enyl)-1,4-naphthaquinone (Table I, Nr. IV). The antipode of alkannin, shikonin, was isolated by MAJIMA and KURODA [32] from the roots of *Lithospermum erythrorhizon* (Shikon in Japanese) but was correctly identified only by BROCKMANN [14] who established its close identity with alkannin. The Japanese pigment was not only *dextrorotatory* but its specific rotation was less than that of the *laevorotatory* alkannin; hence it was considered to be a mixture of ($\pm$) alkannin (named shikalkin) and (+) alkannin (called shikonin) in the ratio 1:4.

The absolute configuration of shikonin has been studied by ARAKAWA and NAKAZAKI [33]. Shikonin (XVII) was subjected to ozonolysis in acetic acid solution and subsequently treated with hydrogen peroxide. The resulting dimethyl malic acid ester (XVIII) was converted into the amide and found to be identical with D(+) malamide (XIX). Hence shikonin was found to have (R) - and alkannin the (S) - configuration (Fig. 1).

During the isolation from the plant material some of the pigments shown in Table I formed artifact naphthaquinones. Thus, the cyclic ether XX was formed [9] during the alkaline hydrolysis of arnebin-2 (Table I, Nr. XIV).

Table I

The up-to-day reported naturally occurring isohexenylnaphthazarin pigments

Nr.	R	Name [Reference]
I	$-\text{CH}_2\text{CH}_2\text{CH}_2\text{CHMe}_2$	Alkannan [14]
II	$-\text{CH}_2\text{CH}_2\text{CH} = \text{CMe}_2$	Deoxyalkannin [15, 16] or Deoxyshikonin [17] or Arnebin-7 [18]
III	$-\text{CH} = \text{CHCH} = \text{CMe}_2$	Anhydroalkannin [17]
IV	$-\text{CH}(\text{OH})\text{CH}_2\text{CH} = \text{CMe}_2$	Alkannin [7, 8, 14, 19, 20, 25] or Shikonin [21, 22]
V	$-\text{CHCH}_2\text{CH} = \text{CMe}_2$	Acetylalkannin [16] or Acetylshikonin [4, 23]
VI	$\begin{array}{c} \\ \text{OCOCH}_3 \\ \\ -\text{CHCH}_2\text{CH} = \text{CMe}_2 \end{array}$	Isobutylshikonin [23]
VII	$\begin{array}{c} \\ \text{OCOCHMe}_2 \\ \\ -\text{CHCH}_2\text{CH} = \text{CMe}_2 \end{array}$	Isovalerylalkannin [16, 24] or Isovalerylshikonin [17]
VIII	$\begin{array}{c} \\ \text{OCOCH}_2\text{CHMe}_2 \\ \\ -\text{CHCH}_2\text{CH} = \text{CMe}_2 \end{array}$	α -Methyl-n-butyl-alkannin [16] or α -Methyl-n-butyl-shikonin [17]
IX	$\begin{array}{c} \\ \text{OCOCHMeCH}_2\text{Me} \\ \\ -\text{CHCH}_2\text{CH} = \text{CMe}_2 \end{array}$	β,β -Dimethylacryl-alkannin [8, 16, 25] or β,β -Dimethylacryl-shikonin [4, 23]
X	$\begin{array}{c} \\ \text{OCOCH} = \text{CMe}_2 \\ \\ -\text{CHCH}_2\text{CH} = \text{CMe}_2 \end{array}$	Teracrylshikonin [26]
XI	$\begin{array}{c} \\ \text{OCOC}(\text{Me}) = \text{CMe}_2 \\ \\ -\text{CHCH}_2\text{CH} = \text{CMe}_2 \end{array}$	Angelicalkannin [24] or Angelicshikonin [15]
XII	$\begin{array}{c} \\ \text{OCOC}(\text{Me}) = \text{CHMe} \\ \\ -\text{CHCH}_2\text{CH} = \text{CMe}_2 \end{array}$	β -Hydroxy-isovaleryl-shikonin [26]
XIII	$\begin{array}{c} \\ \text{OCOCH}_2\text{C}(\text{OH})\text{Me}_2 \\ \\ -\text{CHCH}_2\text{CH} = \text{CMe}_2 \end{array}$	β -Acetoxy-isovaleryl-alkannin [27]
XIV	$\begin{array}{c} \\ \text{OCOCH}_2\text{C}(\text{OCOCH}_3)\text{Me}_2 \\ \\ -\text{CHCH}_2\text{CH}_2\text{C}(\text{OH})\text{Me}_2 \end{array}$	β,β -Dimethylacryl-hydroxy-alkannin or Arnebin-2 [9]
XV	$\begin{array}{c} \\ \text{OCOCH} = \text{CMe}_2 \\ \\ -\text{CHCH}_2\text{CH}_2\text{C}(\text{OH})\text{Me}_2 \end{array}$	Acetyl-hydroxy-alkannin or Arnebin-6 [9]
XVI	$\begin{array}{c} \\ \text{OCOCH}_3 \\ \\ -\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{OH})\text{Me}_2 \end{array}$	Hydroxy-alkannan or Arnebin-5 [9]

Fig. 1: Determination of absolute configuration of shikonin [33].

ring the formation of the tautomer structure XXIV_b is favoured

¹³C-NMR data for naphthazarin and their methyl ethers and acetates have also been published [37].

Biosynthesis

It has also been reported [34] that during isolation of shikonin angelate (Table I, Nr. XI), the side chain cyclised to give the unusual four-membered ring system XXI.

Some shikonin derivatives, such as dihydroshikonin (XXII) and 8-hydroxy-2-isohexyl-4-oxo-tetralone (XXIII), which had not been reported previously, were prepared by catalytic reduction of shikonin [35].

A study on ¹H-NMR spectra of some of the discussed naphthaquinones has also been reported [36]. In case of alkannin an alternative structure was thus postulated. Because of the disturbance of the electronic equilibrium of the quinonoid

Alkannin is considered as the representative naphthazarin pigment for a study of the biosynthesis of these pigments. At first NEELAKANTAN and SESHADRI suggested that the probable biosynthetic sequence involved lengthening of the side chain of 2-methyl-naphthazarin (XXVI) by a C₅-isoprene unit [38]. The formation of 2-methyl-naphthazarin itself was postulated to arise from 3,5-dihydroxyphthalic acid (XXV) or phthalaldehyde involving stages of nuclear dehydroxylation and nuclear hydroxylation as shown in Figure 2.

SHUKLA ET AL. [9], although tracer studies were not carried out, suggested that the alkannin molecule might be derived

Fig. 2: Proposal for alkannin biosynthesis according to NEELAKANTAN and SESHADRI [38].

from a phenol and two C_5 units joined head to tail (XXVII). The isolation of 5-geranyl-2-methyl-1,4-benzoquinone (XXVIII) from *Pyrola media* [39] provides support for this view.

In tracer studies with alkannin, formed on the leaf surface of *Plagiobothrys arizonicus*, SCHMID and ZENK [40] postulated (Fig. 3) a pathway involving prenylation

of 4-hydroxy-benzoate (XXIX) either successively with two molecules of isopentenyl pyrophosphate or with geranyl pyrophosphate (XXX).

Recent data seem to contradict the results obtained by ZENK, as labeling studies indicated that the plants were unable to utilize exogenously administered mevalonic acid [41].

Fig. 3: Proposal biosynthetic sequence for alkannin by SCHMID and ZENK [40].

During the last few years TABATA et al. [42, 43] studied the various physiological and physicochemical factors which influence the formation of naphthazarin pigments in *Lithospermum erythrorhizon* callus cultures.

Biological Effects

All isohexenylnaphthazarins are biologically extremely potent compounds, because they have pronounced antibacterial, antitumor and wound healing activities. The wound healing activity of all members has probably the most important clinical significance. It is of interest that extracts of *Boraginaceous* roots have been used for centuries as folk remedies for a variety of disorders such as eczema, keratoderma, dermatophytosis, corns callus, acne vulgaris, burns and even hemorrhoids [44, 45]. However, a scien-

tific study to support this efficacy has only recently been started.

Antibacterial activity

A few years ago, a systematic study began on the antibacterial activity of these pigments [46]. In *vitro*, shikonin and its derivatives, except for anhydroalkannin, inhibited at a dose range of 10–160 µg/ml the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Sarcina lutea*, *Bacillus subtilis*, but not that of *Escherichia coli* and *Pseudomonas aeruginosa*; some shikonin derivatives slightly inhibited the growth of *Saccharomyces cerevisiae* [16, 47, 48, 49].

Pigments (shikonin derivatives) from *Lithospermum erythrorhizon* callus cultures showed similar properties as those from plant roots [42, 43]. Thus, the chloroform extracts of the callus cultures which contain large amounts of the pigments showed antibacterial action similar

to that of the chloroform extract of the roots against Gram-positive bacteria [50]. Cell cultures are therefore a possible source of the naphthaquinones due to the decreasing availability and unsuccessful cultivation of this plant.

SHUKLA ET AL. [8], studied the antibiotic activity of arnebins (alkannin derivatives from the roots of *Arnebia nobilis*) and found that β,β -dimethylacryl-hydroxy-alkannin (Table I, Nr. XIV) exhibited the greatest antibacterial potency, inhibiting the growth of Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and fungi (*Candida albicans* and *Cryptococcus neoformans*) at a concentration 6.25 $\mu\text{g/ml}$.

Studies [51] on the relationship of structure to antimicrobial properties of naphthaquinone pigments and other constituents of *Alkanna tinctoria* indicated that:

1. Polymerization of naphthaquinones results in a complete loss of their antimicrobial activity. This is particularly important because naphthaquinones are subject to polymerization in the presence of air, light and heat [52]. Thus, it is possible that during the isolation of these substances considerable pharmacological activity is lost due to polymerization.
2. The naphthaquinone moiety of alkannin and its esters appears to be necessary for antimicrobial activity. Alkylation of the phenolic groups leads to complete loss of activity, while acylation does not appear to influence the antibacterial properties.
3. Since alkannin and its esters are active, it appears that the aliphatic side chain acts as a delivery system for the naphthaquinone. Thus, it is suggested that

esterification at the aliphatic hydroxyl group might lead to products with enhanced antimicrobial activity.

Antitumor activity

The National Cancer Institute (NCI, Maryland, USA) has reported the cancer screening data for 1599 quinones [53]. Approximately 15% of them show activity in one or more of the screens employed, that is, with L1210 leukemia, W-256 system cells, CA-755, S-180, and KB cells. Among the tested naphthaquinones are alkannin, β,β -dimethylacrylate and alkannin acetate [54]. From a structural point of view alkannin and their esters are suitable to function as bio-reductive alkylating agents and show cytotoxic properties [55]. In fact, alkannin and naphthazarin (the parent ring system of alkannin) exhibited marked cytotoxic properties at 1×10^{-7} M when tested on cultured hamster cells [56]. According to this function as bio-reductive alkylating agents, alkannin and its derivatives (which are functionalized with a leaving group X at the α -position on the side chain, Fig. 4, XXXI) should be reduced *in vivo* to the hydroquinone XXXII. Subsequent loss of HX would give the hydroquinone XXXIII, which would then function as a potent alkylating agent via a Michael addition reaction (Fig. 4).

The above mentioned results point to a serious problem since alkannin is used for the artificial coloring of certain foods [7]. The cytotoxic properties already investigated *in vitro* [56] should be studied *in vivo* as well. However, alkannin was fed to mice for fifteen weeks at 1% of the diet with no evidence of toxicity [57].

It should be also noted that 2,3-bis (substituted methyl)naphthazarins and related compounds were synthesized [58].

Fig. 4: Bioreductive alkylating function of isohexenylnaphthazarins.

X = leaving group (-OH or -OCOR).

N = nucleophilic center on a biomolecule (DNA, reductase, or the like).

These compounds were prepared as potential biological alkylating agents. Screening results indicated that 5,8-bis(benzoyloxy)-2,3-dimethyl-1,4-naphthazarin possessed borderline activity against leukemia P388 and that naphthazarin diacetate possessed confirmed cytotoxicity against the cell culture of human epidermoid carcinoma of the nasopharynx.

Wound healing activity

Though *Anchusa tinctoria* and *Alkanna tinctoria* have been mentioned in the "Greek Herbal of Dioscorides" [45] for the healing of wounds, it was only 1978 that this medicinal property has been confirmed and that the active components of the roots were determined [59]. Recently

the wound healing properties of alkannin esters were studied in the Dermatological Clinic of Heidelberg Hospital (Hamburg, W. Germany) by the double blind method and were further assessed on another series of patients in Andreas Syngrou Hospital for Skin Diseases (Athen, Hellas).

The clinical studies in the Heidelberg Hospital lasted for 3 years and involved 72 patients suffering from indolent ulcers (ulcus cruris), unsuccessfully treated over long periods with other known therapeutic agents [60]. Topical application of this speciality for 5 to 6 weeks resulted in complete healing or at least marked improvement. Furthermore, no skin inflammation was observed during therapy. The percentage of success was 80%.

Fig. 5: A 62 old woman. Indolent ulcer of the right leg, ten years' duration.

Before treatment.

After 4 weeks' treatment with alkannin esters.

The dramatic improvement in a particularly severe case of *ulcus cruris* is shown in Figure 5.

Other biological and pharmacological activities

During the past thirty years various biological and pharmacological activities of the discussed naphthaquinones have been reported. It has been found that: (1) shikonin inhibits yeast carboxylase [61]; (2) a 10% aquatic tincture and 1:1 extract of the *Lithospermum purpurocaeruleum* was bactericidal for pyogenic bacteria and *Escherichia coli*. The preparations accelerated epithelization and healing of wounds and burns [62]; (3) alkannin oily solutions possess protective properties against solar and uv-radiation [63]; (4) shikonin in concentrations of 20–30 µg/

ml had a bactericidal effect on lactic acid and acetic acid bacteria [64, 65]; (5) shikonin stimulated peroxidase from horseradish [66]; (6) shikonin had a significant antiamoebic action on *Entamoeba histolytica* when added at 0.5–10 µg/ml to the medium. However, when administered orally, shikonin showed weak therapeutic effects [67]; (7) shikon (*Lithospermum officinale* L. var. *erythrorhizon*) has anti-inflammatory action and a slight antipyretic effect [68]. Furthermore, shiunko (a main prescription of shikon) is an effective ointment for cutaneous injuries [69, 70].

Conclusion

Alkannin pigments possess potent anti-inflammatory, antibacterial and even anti-

cancer action. However, their strong wound healing properties in combination with lack of side effects, consecrate them as a new class of drugs, which is coming to fill a serious gap in the therapeutic armamentarium. Present research is dealing with the effect of these pigments on collagen synthesis.

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