BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **19**: 498–505 (2005)
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/bmc.470

ORIGINAL RESEARCH

Structure determination of oligomeric alkannin and shikonin derivatives

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Received 14 September 2004; accepted 3 November 2004

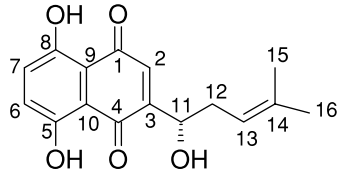
ABSTRACT: Monomeric alkannin and shikonin (A/S) are potent pharmaceutical substances with a wide spectrum of biological activity and comprise the active ingredients for several pharmaceutical preparations. Therefore, the determination of the impurities, degradation products or byproducts in alkannin and shikonin samples is of great importance. Oligomeric alkannin and shikonin are formed during biosynthesis of these bioactive secondary metabolites in Boraginaceaous root plants, during tissue culture production of A/S, during alkaline hydrolysis of A/S esters and also thermal treatment of A/S. In the present study, a dimeric alkannin/shikonin compound was isolated by size exclusion chromatography from alkannin and shikonin commercial samples and its structure was determined by one- and two-dimensional NMR spectroscopy. The structure of the most abundant oligomeric species in these samples, a dimeric naphthoquinone, was established for the first time, indicating that coupling of the side chain of one naphthoquinone unit with the aromatic ring of a second naphthoquinone leads to dimer formation. This type of coupling allows further oligomerization by leaving one isohexenyl side chain available at the second monomer unit. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: alkannin; shikonin; SEC; NMR; oligomeric naphthoquinones; isohexenylnaphthazarins; polymerization; pigments

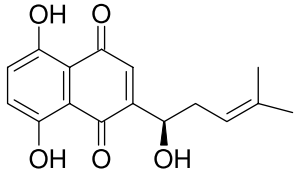
INTRODUCTION

The enantiomeric naphthoquinone natural products alkannin **1** and shikonin **2** (A/S, Scheme 1) exhibit a wide range of pharmaceutical, including anti-inflammatory, antibacterial, wound healing and antitumor activity (Papageorgiou *et al.*, 1999). Alkannin (*S*-enantiomer) and shikonin (*R*-enantiomer) are optical antipodes of plant origin, being mainly found in the roots of the pharmaceutical plants *Alkanna tinctoria* and *Lithospermum erythrorhizon*. A/S are included in pharmaceutical preparations in several forms (Papageorgiou *et al.*, 1999).

The use of alkannin and shikonin in pharmaceutical preparations, as ingredients of cosmetic formulations or as food colorants and additives is affected by oligomerization, because oligomers decrease the concentration of the active monomeric compounds, and possess inferior coloration and reduced solubility. In a recent study (Papageorgiou *et al.*, 2002) we have introduced qualitative and quantitative analysis of monomeric and oligomeric/polymeric A/S derivatives with the aid of size exclusion chromatography (SEC). Root extracts and commercial samples of alkannin and shikonin contain significant amounts of oligomeric isohexenylnaphthazarins (IHN; Papageorgiou *et al.*, 2002; Assimopoulou and Papageorgiou, 2004a). The mechanism of oligomerization during biosynthesis and the structure of these IHN oligomers remains hitherto unknown, while a mechanism of oligomerization of **1** and **2** in alkaline media has been recently proposed (Assimopoulou and Papageorgiou, 2004b) and the



Alkannin **1**



Shikonin **2**

Scheme 1.

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Abbreviations used: A, alkannin; IHN, isohexenylnaphthazarins; S, shikonin.

Published online 14 January 2005

influence of several factors on oligomerization has been studied (Assimopoulou and Papageorgiou, 2004a). Several shikonin dimer metabolites have been isolated and structurally characterized during biotransformation of shikonin by human intestinal bacteria (Meselhy *et al.*, 1994).

Since biological activity of A/S is affected by oligomerization, it is crucial to determine the degradation products of A/S and specifically oligomeric isohexenylnaphthazarins. Therefore, our research focuses on the isolation and structural characterization of IHN oligomers in both commercial samples of alkannin and shikonin that are prepared either by alkaline hydrolysis of A/S esters from *Alkanna tinctoria* roots or biotechnologically by tissue cultures, respectively. In the present work, dimeric A/S compound was isolated by SEC from alkannin and shikonin commercial samples and its structure was elucidated by a detailed one- and two-dimensional NMR investigation of the dimeric A/S fraction and also of the commercial alkannin and shikonin samples in different solvents. The naphthoquinone ring connectivity of the main dimers present in these important natural products is also established.

The aim of this study was to determine the oligomeric impurities or degradation products or byproducts of the pharmaceutical substances alkannin and shikonin that may affect their biological activity towards a quality control of pharmaceuticals containing A/S as active ingredients.

EXPERIMENTAL

Commercial alkannin and shikonin samples were purchased from Roth (Karlsruhe, Germany) and Ikeda (Tokyo, Japan), respectively, and used without further purification. According to information supplied by the manufacturer, the alkannin commercial sample was obtained by alkaline hydrolysis of alkannin esters extracted from the roots of *A. tinctoria*, while the shikonin sample was produced by a plant tissue culture manufacturing process. Chiral HPLC analysis showed that the enantiomeric ratio A:S of the samples was 90.6:9.4 (A:S) for alkannin, and 9.8:90.2 (A:S) for shikonin (Assimopoulou and Papageorgiou, 2004c). The oligomeric alkannin fraction and specifically dimeric A/S was isolated by size exclusion chromatography (Papageorgiou *et al.*, 2002; Assimopoulou and Papageorgiou, 2004a) in order for the structure of the dimeric A/S to be elucidated.

SEC experiments. Organic SEC was used for qualitative and quantitative determination of oligomeric/polymeric and monomeric A/S, and isolation of oligomeric and specifically dimeric A/S. Measurements were performed by means of a liquid chromatograph, equipped with a UV–vis detector (Fasma 500), and a Marathon III HPLC pump (all from Rigas Laboratories, Thessaloniki, Greece), and software for process control and data handling (Chrom & Spec for Windows, Ampersand Ltd, Multi-Channel Chromatography Data Station, version 1.44a). The column used was GPC 300 × 8 mm i.d. MZ-Gel SD plus column containing 10 μm particles with 100 Å pores (MZ Analysentechnik, Mainz, Germany); separations were performed at ambient temperature. The mobile phase, Tetrahydrofuran (THF) (Merck, Lichrospher®, HPLC grade), was degassed before use; the mobile phase flow rate was 0.5 mL min^{-1} and the injection volume 250 μL. UV–visible spectrophotometry was performed with a Shimadzu type UV-160A. The detector wavelength was set at 520 nm. Dimeric and monomeric A/S were isolated from the output of the column. ESI-MS spectra were obtained using a ThermoFinnigan LCQ Advantage.

NMR experiments. ^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker AMX-500 spectrometer operating at 500.1 and 125.4 MHz for the two nuclei, respectively, at ambient temperature. Chemical shifts in $CDCl_3$ and CD_3OD solvents are reported relative to internal Tetramethylsilane (TMS). ^{1}H-^{1}H homonuclear gradient COSY two-dimensional NMR spectra were obtained using 256 increments of 1 K data points, 16 scans and four dummy scans with a recycle delay of 1 s. ^{1}H-^{13}C heteronuclear gradient multiple quantum correlation [gHMQC] and multiple bond correlation [gHMBC] two-dimensional NMR spectra were obtained using 128 increments of 1 K data points, 16 scans and four dummy scans with a recycle delay of 1 s. The HMQC experiment was optimized for one bond ^{1}H-^{13}C couplings of 140 Hz by setting the evolution delay to 3 ms. The HMBC experiment used an evolution delay of 60 ms optimized for long-range ^{1}H-^{13}C *J*-couplings of ~8 Hz. Before Fourier transformation all two-dimensional data sets were zero-filled to a 1 K × 1 K matrix, and a square-sinusoidal window function was used for processing (Braun and Kalinowski, 1998).

RESULTS AND DISCUSSION

Polymerization of isohexenylnaphthazarins crucially affects their use in pharmaceuticals, cosmetics and as food additives, because it reduces the concentration of the active monomeric isohexenylnaphthazarins. Hence, the structure of oligomeric A/S has to be elucidated. Therefore, in the present study an attempt was made to determine the structures of A/S oligomers. Thus, A/S oligomers and also monomeric alkannin and shikonin were isolated by SEC, as described in our previous papers (Papageorgiou *et al.*, 2002; Assimopoulou and Papageorgiou, 2004a) and submitted to NMR investigation in order for the structure of the dimeric A/S to be elucidated. SEC chromatographs of the alkannin and shikonin commercial samples that show the monomeric and oligomeric fractions of alkannin and shikonin samples that were isolated are depicted in Fig. 1.

Structural characterization of isohexenylnaphthazarin dimers that were isolated by SEC from alkannin and shikonin commercial samples was performed by a detailed ^{1}H and ^{13}C one- and two-dimensional NMR

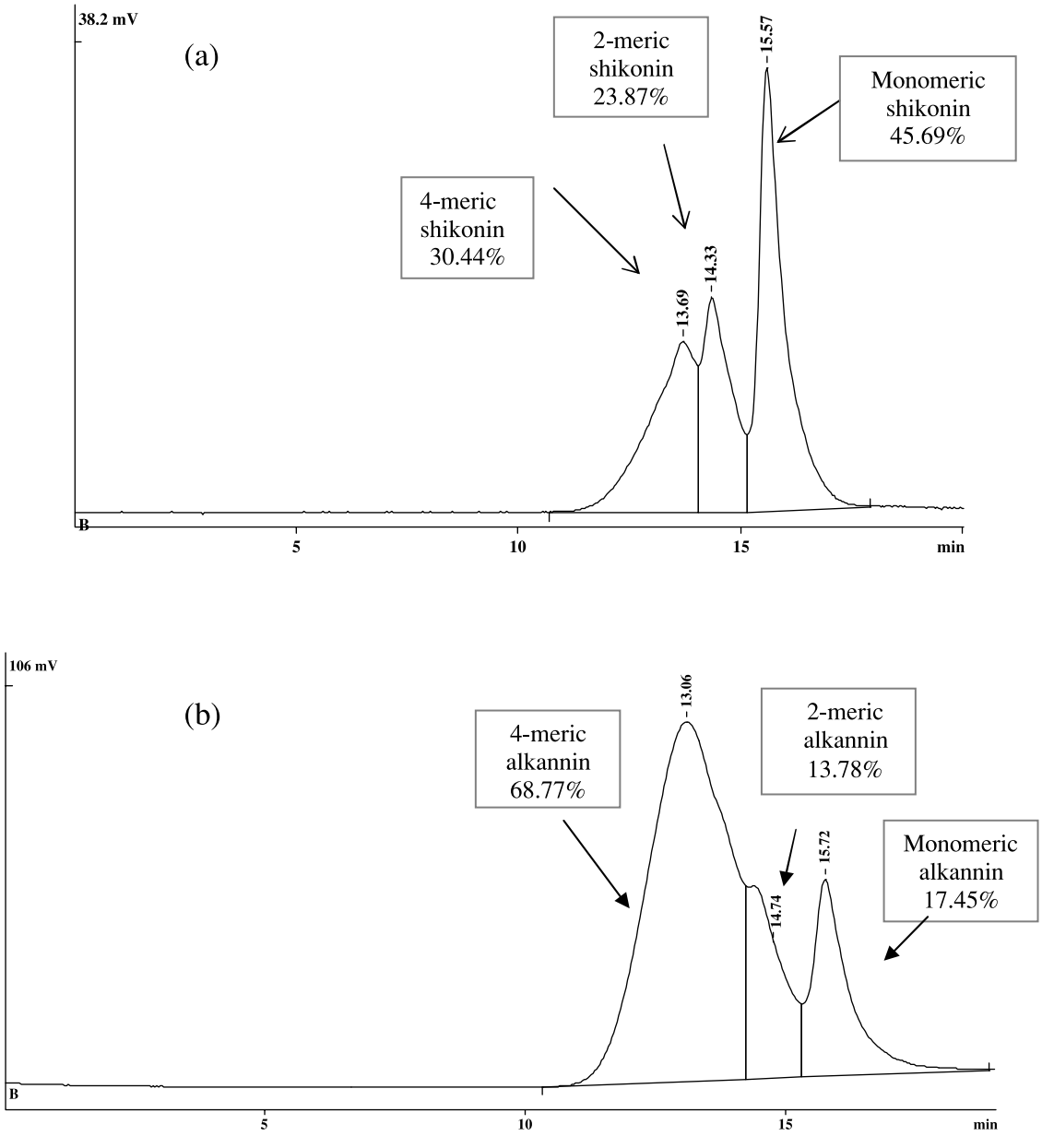


Figure 1. SEC analysis of the commercial sample of (a) shikonin (Ikeda Corp.) and (b) alkannin (Roth).

investigation of monomeric and oligomeric fractions of the commercial alkannin and shikonin samples in different solvents.

First, ^{1}H and ^{13}C NMR spectra of commercial **1** and **2** were acquired in $CDCl_3$ and CD_3OD solvents. Figure 2 compares the ^{1}H NMR spectra of a commercial sample of **1** (a), isolated alkannin monomer (b) and isolated by SEC alkannin dimers (c) in $CDCl_3$ solvent. The alkannin commercial sample was provided by Carl Roth Co. (Karlsruhe, Germany) and was prepared by alkaline hydrolysis of A/S esters from *Alkanna tinctoria* roots (see Experimental). The ^{1}H NMR spectrum in Fig. 2(a) is dominated by peaks that also appear in Fig. 2(b) and originate from monomeric **1**, in agreement with ^{1}H NMR chemical shift data reported in the literature (Inoue *et al.*, 1985; Nicolaou and Hepworth 1998). However, several minor proton peaks appear in Fig. 2(a) that cannot be attributed to monomeric alkannin, other minor naphthoquinones reported in *Alkanna tinctoria* root extracts, such as deoxyalkannin (Mallavadhani *et al.*, 1998), or esterified IHNs, that could be present in the alkannin sample in traces due to incomplete hydrolysis or purification procedure (Papageorgiou *et al.*, 1999, 2002; Assimopoulou and Papageorgiou, 2004b). These protons also appear in the spectrum of the alkannin dimeric fraction in Fig. 2(c), alongside with peaks similar to those of monomeric **1**. Especially prominent is the appearance

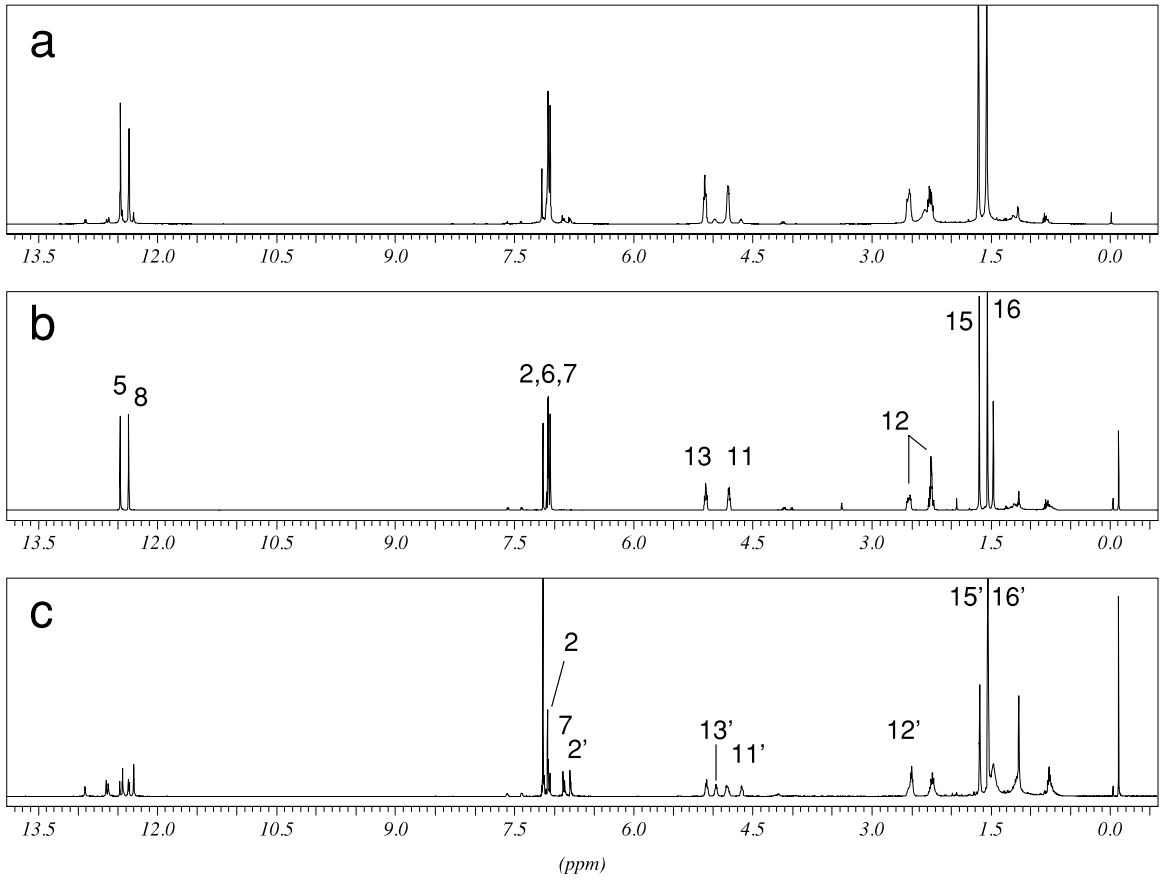


Figure 2. ^{1}H NMR spectra, 500 MHz, of: (a) a commercial alkannin sample; (b) isolated alkannin monomer; and (c) isolated alkannin dimer in $CDCl_3$. Labelling in (b) according to Scheme 1; that in (c) according to structure B in Scheme 2.

of four new peaks in the spectral regions δ 4.5–5.5 (H-11′, H-13′) and δ 6.9 and 7.0 (H-2′, H-7′), and the observation of several new phenolic -OH protons at low fields, besides the two -OH peaks expected from monomeric **1**. Peaks due to H-11′ and H-13′ indicate that the oligomers contain a modified isohexenyl side chain structure. However the observation of signals H-11 and H-13 in the NMR spectrum of oligomeric material [Fig. 2(c)] indicates that some of the isohexenyl substituents remain unmodified during oligomerization. The ^{13}C NMR spectra of commercial alkannin and shikonin samples were found to contain a number of carbon signals not attributable to monomeric A/S (Inoue *et al.*, 1985; Nicolaou and Hepworth, 1998). Table 1 summarizes the ^{1}H and ^{13}C NMR chemical shifts of the signals attributed to the monomeric and oligomeric fractions of the alkannin and shikonin commercial samples. The NMR spectroscopic data of Fig. 2 and Table 1 verify that a considerable amount of oligomers is present in commercial A/S samples, in agreement with recent SEC studies (Papageorgiou *et al.*, 2002; Assimopoulou and Papageorgiou, 2004a). These data also show that the basic structure of the most abundant type of oligomers is similar in both commercial alkannin and shikonin samples, although the alkannin sample is formed during A/S esters alkaline hydrolysis from root extracts and the shikonin sample originates from a tissue culture process.

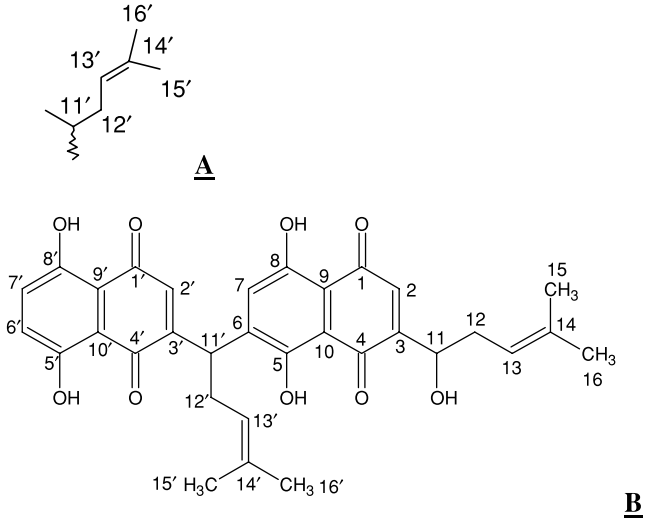
The ESI-MS spectrum of the isolated oligomeric fraction that produced the NMR spectrum of Fig. 2(c) showed that it contained almost exclusively an organic compound with a molecular weight of 558, which clearly represents a naphthoquinone dimer.

Further NMR experiments were conducted in order to elucidate the structure of this dimer found in commercial A/S samples, namely ^{13}C DEPT NMR, two-dimensional ^{1}H-^{1}H (gCOSY) and ^{1}H-^{13}C (gHMQC, gHMBC) correlation experiments in $CDCl_3$ solvent. The cross-peak proton signals observed in the ^{1}H-^{1}H 2D gCOSY spectrum of the dimer in $CDCl_3$ solvent established the connectivity of protons as 11′–12′–13′–15′. ^{13}C DEPT experiments showed that: (a) C-11′ is a methine carbon connected to two aromatic quartenary carbons, and one methylene (C-12′); (b) C-13′ is a vinyl carbon and C-15′ is a methyl carbon. Thus the presence of a modified isohexenyl side chain fragment A (Scheme 2) in the molecular structure of the naphthoquinone dimer was established. The observation

Table 1. ^{1}H and ^{13}C NMR chemical shifts (δ) of monomeric and oligomeric alkannin and shikonin in commercial samples, assigned by two-dimensional NMR experiments.

Sample	Position					
	1 ($CDCl_3$)		**2** ($CDCl_3$)		**2** (CD_3OD)	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
			Monomeric			
1	—	180.5	—	180.5	—	182.5
2	7.1–7.2	132.35	7.1–7.2	132.35	7.24	133.45
3	—	151.4	—	151.4	—	154.8
4	—	179.7	—	179.7	—	181.5
5	—	165.5	—	165.5	—	166.5
6	7.1–7.2	131.85*	7.1–7.2	131.85*	7.33	133.26*
7	7.1–7.2	132.28*	7.1–7.2	132.28*	7.33	133.03*
8	—	164.9	—	164.9	—	165.9
9	—	112.0	—	112.0	—	113.5
10	—	111.5	—	111.5	—	113.1
11	4.9	68.3	4.9	68.3	5.05	68.8
12	2.3/2.7	35.6	2.3/2.7	35.6	2.4/2.65	36.9
13	5.2	118.5	5.2	118.5	5.4	121.1
14	—	137.3	—	137.3	—	135.8
15	1.75	25.9†	1.7	25.9†	1.8	26.3†
16	1.65	18.1†	1.6	18.1†	1.6	18.3†
			Oligomeric			
2	7.24	129.9	7.24	129.9	7.3	131.2
2′	6.9	134.3	6.9	134.3	7.1	135.8
3′	—	150.7	—	NO[a]	—	152.6
6	—	147.1	—	147.3	—	NO[a]
7	7.0	131.7	7.0	131.7	7.15	133.4
9′	—	111.9	—	NO[a]	—	NO[a]
10′	—	111.6	—	NO[a]	—	NO[a]
11′	4.65	37.5	4.75	37.5	4.8	38.7
12′	2.6	30.8	2.6	30.8	2.75	32.1
13	5.2	118.6	5.2	118.6	5.4	121.1
13′	5.1	119.9	5.1	119.9	5.25	122.2
14′	—	135.3	—	135.3	—	135.7
15′	1.65	25.7	1.75	25.7	1.75	26.1
16′	1.65	18.1	1.65	18.1	1.68	18.3

[a] Peak not observed due to low S/N of quarternary carbons.
*,† Peaks with the same symbol may be interchanged. See Schemes 1 and 2 for numbering.



Scheme 2.

of ^{1}H-^{13}C long-range J couplings by acquiring a two-dimensional gHMBC NMR spectrum of the naphthoquinone dimer was crucial in elucidating its structure. The experimentally observed long range couplings are depicted in Fig. 4. The fact that H-11′ appears at very low fields is consistent with C-11′ being attached to two different naphthoquinone rings, and this was verified by the ^{1}H-^{13}C ^{3}J long-range coupling observed between C-11′ and H-2′/H-7/H-12′. The structure of fragment A and its connectivity to two adjacent naphthoquinone rings in the molecular structure of the oligomers was further confirmed by the observed signals of H-12′ with C-11′, C-13′, C-14′, C-3′ and C-6; of H-7 with C-6, C-11′; and of H-13′ with C-12′, C-15′, C-16′ in the two-dimensional gHMBC spectrum of **2** in CD_3OD (Fig. 3). Furthermore, it was found that H-2′ and H-2 show long-range connectivity with carbons in the carbonyl spectral region, while H-7 does not.

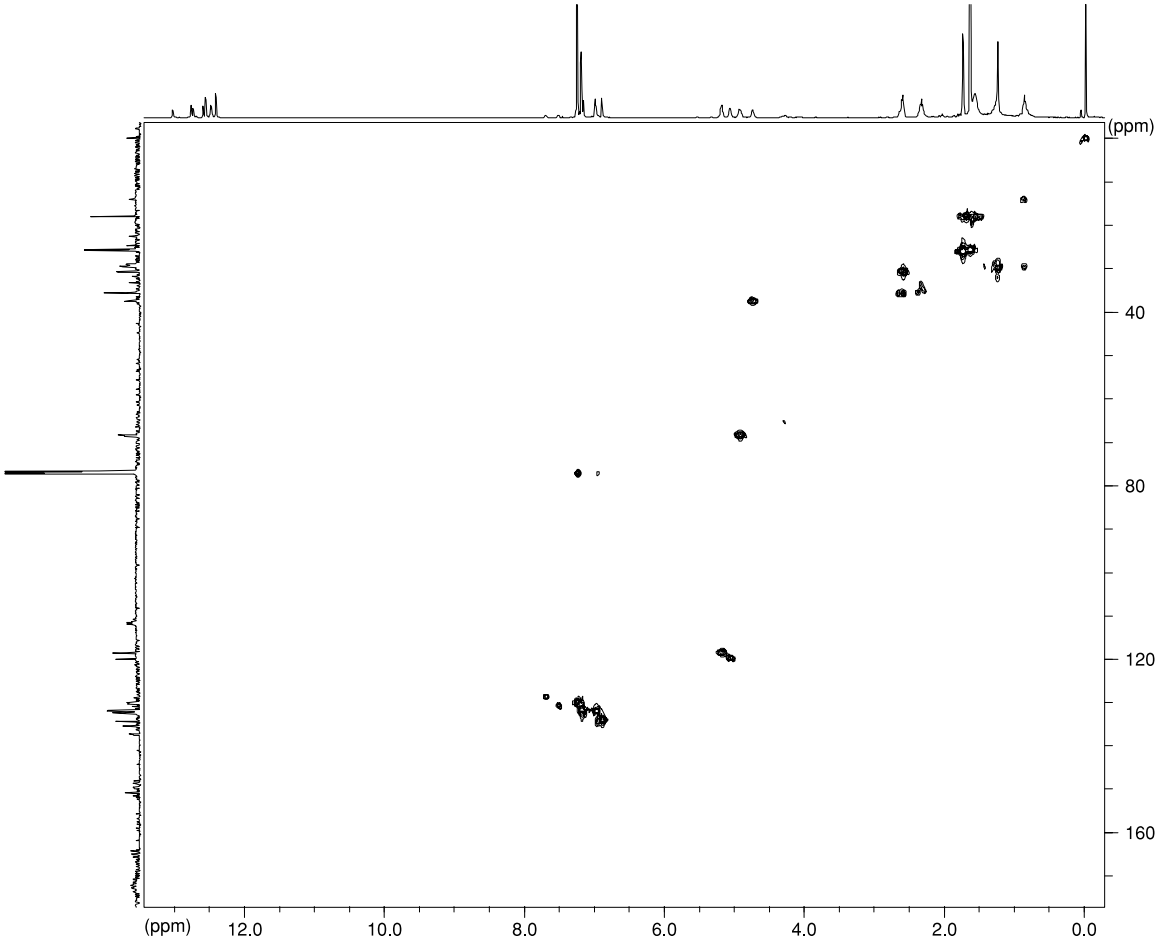


Figure 3. gHMQC two-dimensional NMR spectrum, 500 MHz, of alkannin oligomers in $CDCl_3$. One-bond ^{13}C-^{1}H correlations are shown.

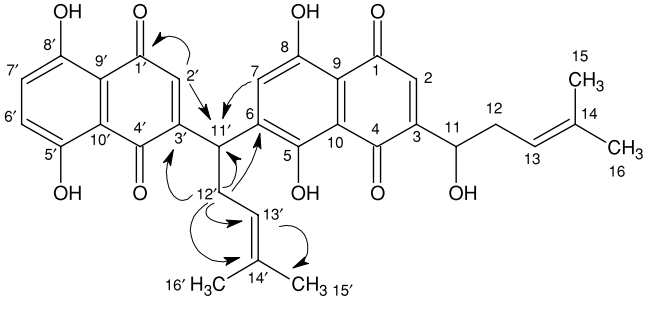


Figure 4. Selected gHMBC long range ^{13}C-^{1}H correlations observed for structure B in the gHMBC two-dimensional NMR spectrum of alkannin oligomers in $CDCl_3$.

Based on the analysis of the NMR and MS spectroscopic data, structure B in Scheme 2 is proposed for the major dimer isolated by SEC from alkannin and shikonin commercial samples, produced by hydrolysis of A/S esters and tissue cultures, respectively. In structure B dimerization occurs between the hydroxyl-bearing carbon C-11 and the phenolic ring of a second A/S molecule. In fact, there are two different possible isomeric structures for B, since coupling may also occur at carbon position 7; however these cannot be differentiated with the data currently available. The presence of oligomers originating from coupling of C-11 with C-2 is not supported by the present data, since in this case the bridge carbon C-11′ would show coupling with only one aromatic/quinoid proton (H-2′). This type of coupling has been observed in dimerization of **1**, **2** during biotransformation by human intestinal bacteria (Meselhy *et al.*, 1994). It is worth noting that none of the several types of metabolite dimers reported (Meselhy *et al.*, 1994) was observed in the commercial samples of the present study. Dimers produced through direct coupling of naphthoquinone rings, similar to those observed for other hydroxynaphthoquinones (Mallavadhani *et al.*, 1998) do not appear to be present in alkannin and shikonin commercial samples. Finally, the present NMR data exclude the presence of photooxidized IHN products (Cheng *et al.*, 1995) in the commercial samples. It is important that in structure B of Scheme 2 the dimers retain an intact isohexenyl side chain, which is available for further polymerization,

thus explaining the observation of higher A/S oligomers (Papageorgiou *et al.*, 2002). Furthermore, the presence of several separate -OH signals appearing in the low-field region of the ^{1}H NMR spectra of Fig. 2(a) indicates, in accordance with another paper (Assimopoulou and Papageorgiou, 2004a), that the commercial samples studied in fact contain several types of oligomers in smaller quantities, structure B being only the predominant one.

The mechanism through which oligomers of structure B are produced can only be postulated at this early stage. It is crucial to identify all the oligomeric structures contained in A/S commercial samples in smaller quantities and to examine whether oligomers are formed in plant roots during biosynthesis. It is possible that the predominant A/S oligomers characterized in this study are biosynthetic byproducts, since they have the same basic structure in both alkannin (obtained by A/S esters alkaline hydrolysis from root extracts) and shikonin samples (obtained by a plant tissue culture process). A bioreductive side chain alkylation mechanism has been proposed to account for the cytotoxic behaviour of **1** and **2** (Moore, 1977), while very few ring functionalization reactions of **1**, **2**, or their derivatives have been reported (Papageorgiou *et al.*, 1999). Anhydroalkannin, a compound that has been isolated from the roots of Nanshikon *Macrotomia euchroma* Pauls (Kyogoku *et al.*, 1973), and is produced during shikonin biotransformation (Meselhy *et al.*, 1994), might play a role as an indermediate in the formation of oligomers. The study of A/S oligomers using electron spray ionization-ion trap–mass spectroscopy in conjuction with two-dimensional NMR spectroscopy is currently underway, in an effort to fully elucidate the molecular structure of each of the A/S oligomers formed during biosynthesis, A/S ester hydrolysis and plant tissue culture, and establish the mechanism of oligomerization.

It is crucial that with this study we have isolated by SEC and determined the structures of an oligomeric A/S (dimer) from alkannin and shikonin commercial samples. Oligomeric A/S is a byproduct of A/S produced either through hydrolysis of A/S esters from *Alkanna tinctoria* roots or tissue culture production of these secondary metabolites, or biosynthesis of A/S in Boraginaceaous roots or thermal treatment of A/S. Purification of the bioactive monomeric A/S can be achieved from oligomeric A/S, and their structures have now been elucidated. Also, the biological activity of the dimeric A/S can now be tested.

CONCLUSION

Polymerization of isohexenylnaphthazarins crucially affects their use in pharmaceuticals, cosmetics and as food additives, because it reduces the concentration of the active monomeric isohexenylnaphthazarins. Hence, the structure elucidation of A/S byproducts, impurities or degradation products formed during biosynthesis, hydrolysis of naturally occurring isohexenylnaphthazarin esters or tissue culture production, is crucial for A/S use in pharmaceuticals. The present research is a continuation of the works recently presented (Papageorgiou *et al.*, 2002; Assimopoulou and Papageorgiou, 2004a,b), which introduce the determination of monomeric, oligomeric and polymeric A/S by SEC, investigate several parameters affecting the polymerization of isohexenylnaphthazarins and examine polymerization of A/S in alkaline media.

In the present paper, oligomeric A/S was isolated from alkannin and shikonin commercial samples by the use of SEC and finally its structure was elucidated by one- and two-dimensional NMR spectroscopy. As shown, dimers are formed by coupling of the side chain of one monomeric A/S unit with the aromatic ring of a second unit.

With that study the purification of the bioactive monomeric A/S can be achieved from impurities, byproducts and polymerization products. It is the first time that an oligomeric A/S is isolated and its structure is determined. This study is classified on the quality control of active ingredients of pharmaceuticals that are natural products, on purity control of active ingredients, determination of byproducts or degradation products and stability control of biologically important compounds.

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