# Isolation of Alkaloidal Constituents of *Zanthoxylum usambarense* and *Zanthoxylum chalybeum* Using Ion-Pair HPLC

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Quaternary alkaloids of two Kenyan medicinal plants belonging to the genus *Zanthoxylum*, *Z. usambarense* and *Z. chalybeum*, were examined using ion-pair HPLC. Both plants contained similar alkaloids, but colored protoberberines were found only in *Z. chalybeum*. From the stems of *Z. usambarense* a new alkaloid named usambanoline (**1**) was isolated and characterized.

*Zanthoxylum* species (Rutaceae) have worldwide importance as natural medicines, and a few of these are used in Kenya. Thus, the bark and leaves of *Z. usambarense* and *Z. chalybeum* have been used in the treatment of severe colds and to alleviate stomachache and toothache. The former species has also been used in the highlands of central Kenya as a folk remedy by the Kikuyu and Kamba tribes. The latter species has similar uses in the Masai, Digo, and Shambaa tribes in the hills of the East Coast District of Kenya. Much of the pharmacological activity of these species may be attributed to their alkaloidal constituents because many pharmacologically active quaternary alkaloids have been found in various *Zanthoxylum* species. Several reports on the alkaloids of *Z. chalybeum* have already been published, and the presence of chelerythrine, skimmianine, nitidine,[1] *N*-methylflindersine, arnottianamide, and dihydrochelerythrine[2] has been reported. However, when we examined HPLC profiles of the quaternary alkaloidal fraction of *Z. chalybeum*, many peaks were found that could not be attributed to alkaloids already reported. It is probable that polar and water-soluble quaternary alkaloids have tended to evade previous researchers.

In the course of isolating alkaloids from plant materials, we have found that quaternary alkaloid-containing fractions may be efficiently concentrated by means of ion-pair extraction using sodium perchlorate.[3,4] The resulting quaternary alkaloidal fraction may then be separated by ion-pair HPLC, in which sodium perchlorate is also utilized. We have applied this purification method to *Z. usambarense*, and from the stems we previously isolated eight quaternary alkaloids, which were identified as (+)-tembetarine, (+)-magnoflorine, (+)-*N*-methylplatydesmine, (−)-oblongine, (−)-*cis*-*N*-methylcanadine, nitidine, chelerythrine, and (−)-usambarine, with the last compound being a new alkaloid.[4] Because the chromatographic profiles of *Z. chalybeum* and *Z. usambarense* resembled each other, we compared the alkaloids of these two *Zanthoxylum* species. Preparative HPLC of both these plants was also carried out, and the alkaloids were purified.

From fractions 1, 2, and 4 of the stems of *Z. usambarense*, (+)-magnoflorine, chelerythrine, and nitidine were obtained, respectively. From fraction 3, (+)-*N*-methylplatydesmine, (−)-oblongine, (−)-usambarine, (−)-*cis*-*N*-methylcanadine, and a new alkaloid (**1**) were purified. (+)-Tembetarine and (−)-edulinine were purified from their mixture by recycle-HPLC. With similar procedures, from the roots of *Z. chalybeum*, these alkaloids, with the exception of (−)-edulinine, were obtained, and in addition, jatrorrhizine and palmatine were purified.

Compound **1**, an orange substance ($\lambda_{max}$ 395 nm), was optically inactive, which suggested that this compound has a conjugated aromatic ring system. From the IR spectrum the presence of a hydroxyl group was apparent. The molecular formula of **1** was determined as $C_{21}H_{22}NO_5^+$ by SIMS. The structure of this compound was determined by measuring various 2D NMR spectra including $^1H-^1H$ COSY, $^{13}C-^1H$ COSY, HMBC, and NOESY. Four singlet aromatic protons and two *o*-substituted doublet protons were evident. In addition, the presence of two methoxyl groups, a methylenedioxy group, and an *N*-methyl group were also apparent. The presence of a $CH_2CH_2$ moiety was suggested from the COSY NMR spectrum. It was determined that this moiety was linked to an aromatic ring because an HMBC correlation from a singlet proton (7.11 ppm) to an aliphatic carbon (36.67 ppm) was clearly observed. Close examination of the $^1H-^1H$ COSY, $^{13}C-^1H$ COSY, and HMBC spectra revealed the presence of the partial structure **A**. Using similar procedures, we also identified the presence of an isoquinoline moiety **B**. From the index of hydrogen deficiency (=12), no additional ring

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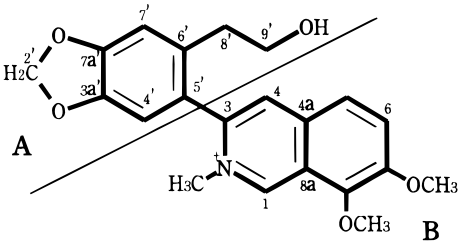
**Table 1.** Yields of Alkaloids Isolated from Two *Zanthoxylum* Species[a]

| compound | *Z. usambarense*[b] (%) | *Z. chalybeum*[c] (%) |
|---|---|---|
| (+)-tembetarine | 0.003 75 | 0.216 |
| (+)-magnoflorine | 0.0793 | 0.0041 |
| (−)-edulinine | 0.000 62 | |
| (+)-*N*-methylplatydesmine | 0.0011 | 0.0017 |
| (−)-oblongine | 0.0043 | 0.0027 |
| (−)-usambarine | 0.0011 | 0.0013 |
| usambanoline (**1**) | 0.000 39 | 0.000 17 |
| jatrorrhizine | | 0.0336 |
| (−)-*cis*-*N*-methylcanadine | 0.0333 | 0.0242 |
| palmatine | | 0.0029 |
| nitidine | 0.000 83 | 0.0123 |
| chelerythrine | 0.0048 | 0.0064 |

[a] Each compound was purified as the perchlorate salt. [b] Compounds were isolated from 1.22 kg of stems. [c] Compounds were isolated from 824 g of roots.

or double bond existed in the molecule. Consequently, we inferred that the $CH_2CH_2$ unit does not form a ring but exists as an open chain structure. The rather low-field chemical shifts of the H-9′ (3.70 ppm) and C-9′ (63.02 ppm) signals, compared with those of H-8′ (2.53 and 2.68 ppm) and C-8′ (36.67 ppm), suggested that a heteroatom, presumably a hydroxyl atom, has attached to C-9′. Summarizing these data, the structure shown is suggested for compound **1**.




**1**

The presence of this type of isoquinoline alkaloid seems to be rare in the plant kingdom. In cell cultures of *Corydalis incisa* the formation of the 4-methyl-7,8-(dimethylenedioxy) derivative of **1** has been reported.[5] In *C. incisa* the biosynthetic pathways of benzo[*c*]-phenanthridines from the *N*-methyltetrahydroberberines via the protopines[6] were established. Large amounts of protopine and several other related alkaloids have been found in *Corydalis* species. In the case of *Zanthoxylum* species, however, no protopine or other possible intermediate was found. When the C−N bond of (+)-tembetarine is cleaved hydrolytically and aromatized, compound **1** is produced. Furthermore, if compound **1** is dehydrated and cyclized, chelerythrine is formed. Therefore, the biosynthetic pathways of benzo-[*c*]phenanthridines in *Zanthoxylum* plants are of interest.

The yields of the alkaloids obtained using preparative HPLC are summarized in Table 1 for the two plants in this investigation. Accordingly, it was found that *Z. usambarense* and *Z. chalybeum* contain similar alkaloids, but the colored protoberberines, jatrorrhizine and palmatine, were found only in *Z. chalybeum*. The main alkaloids in *Z. usambarense* were (+)-magnoflorine and (−)-*cis*-*N*-methylcanadine, while those of *Z. chalybeum* were (+)-tembetarine, jatrorrhizine, (−)-*cis*-*N*-methylcanadine, and nitidine. Both *Zanthoxylum* species contained a considerable proportion of the benzo[*c*]-

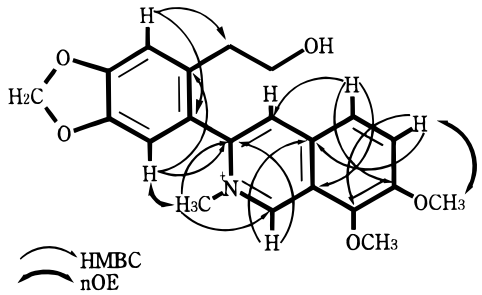



**Figure 1.** HMBC and NOE interactions observed for usambanoline (**1**).

phenanthridine alkaloids chelerythrine and nitidine. The content of nitidine, which possesses antitumor activity,[7,8] was higher in *Z. chalybeum* than in *Z. usambarense*.

## Experimental Section

**General Experimental Procedures.** $^1$H-, $^{13}$C-, and various 2D-NMR spectra, including $^1$H−$^1$H COSY, $^{13}$C−$^1$H COSY, HMBC, and NOESY, were obtained using a VXR-500 instrument. LRSIMS and HRSIMS were measured with a Hitachi M-4100 instrument using glycerol as matrix. The primary ion was $Cs^+$, and the accelerating voltages of the primary and secondary ions were 15 and 6 kV, respectively. The IR spectrum and the optical rotation were measured with a Shimadzu FT IR-8200 spectrometer and JASCO DIP-181 polarimeter, respectively. The HPLC apparatus included a dual-pump system (Model 510, Waters), a gradient controller (Model 680, Waters), and a photodiode-array detector (Model 990, Waters). HPLC conditions for analytical ion-pair HPLC were as follows: column, Cosmosil 5C18-AR (5 $\mu$m, ODS-type), 150 × 6 mm i.d. (Nacalai Tesque); mobile phase (**A**) 0.2 M sodium perchlorate, 60% perchloric acid (1000:0.2) and (**B**) acetonitrile [(**A**)/(**B**) = 80/20 to 60/40, 40 min, 2 mL/min]. In the case of the preparative scale HPLC work, a larger column of Cosmosil 5C18-AR (250 × 20 mm i.d.) was utilized.

**Plant Material.** The roots of *Z. chalybeum* were collected at Shimba Hills National Reserve in Kenya. The stems and a small amount of roots of *Z. usambarense* were collected at Aberdare in central Kenya near Mount Kenya. Both plants were identified and authenticated by Mr. M. G. Mungai (Eastern African Herbarium, P.O. Box 45166, Nairobi, Kenya), and voucher specimens representing these two species are deposited at Kobe Pharmaceutical University and at the Eastern African Herbarium. The extraction method, which was a combination of conventional partition-extraction procedures and ion-pair extraction of the quaternary alkaloids, was similar to those reported previously.[4,5] After being dried, fine-cut plant materials (824 g of the roots of *Z. chalybeum* and 1220 g of the stems of *Z. usambarense*) were extracted with 2 L of hot MeOH for 5 h. The extraction was repeated four times. After the MeOH was evaporated a 500-mL portion of 2% aqueous tartaric acid was added, and the alkaloids were dissolved. Fat-soluble substances were removed with two 500-mL portions of $Et_2O$. The aqueous layer was basified by $Na_2CO_3$ and extracted first with three 500-mL portions of $Et_2O$ (fraction 1) and then with three 500-mL portions of $CHCl_3$ (fraction 2). To the residual

aqueous layer was added sodium perchlorate so that the concentration of perchlorate was about 0.5 M. The aqueous layer was extracted with three 500-mL portions of 1,2-dichloroethane. Thus, quaternary alkaloids extracted under weakly alkaline conditions were obtained (fraction 3). The aqueous layer was then acidified by perchloric acid and extracted with three 500-mL portions of 1,2-dichloroethane (fraction 4). The organic solvent from each fraction was removed by evaporation. The residue was dissolved in a mixture of DMSO and 0.5 M sodium perchlorate (1:1) and then submitted to ion-pair HPLC in both analytical and preparative scale.

**Usambanoline Perchlorate (1).** Usambanoline perchlorate, 7,8-dimethoxy-3-[6-(2-hydroxyethyl)-1,3-benzodioxolo-5-yl]-2-methylisoquinolinium perchlorate, was obtained as an orange crystalline solid [MeOH−$(C_2H_5)_2O$]: mp 191 °C; IR (KBr) $\nu_{max}$ 3456 (OH), 1508, 1488, 1380 $cm^{-1}$; $^1H$ NMR [$(CD_3)_2CO$] $\delta$ 2.53, 2.68 (1 H each, m, H-8′), 3.70 (2 H, m, H-9′), 4.18 (3 H, s, $OCH_3$-7), 4.20 (3 H, s, $OCH_3$-8), 4.39 (3 H, s, $NCH_3$), 6.15, 6.16 (1 H each, d, $J$ = 1.0 Hz, H-2′), 7.04 (1 H, s, H-4′), 7.11 (1 H, s, H-7′), 8.11 (1 H, d, $J$ = 9.0 Hz, H-5), 8.28 (1 H, d, $J$ = 9.0 Hz, H-6), 8.39 (1 H, s, H-4), 10.06 (1 H, s, H-1); $^{13}C$ NMR [$(CD_3)_2CO$] $\delta$ 36.67 (C-8′), 47.87 ($NCH_3$), 57.61 ($OCH_3$-7), 62.48 ($OCH_3$-8), 63.02 (C-9′), 102.93 (C-2′), 110.34 (C-4′), 110.80 (C-7′), 124.11 (C-8a), 124.14 (C-5), 125.75 (C-5′), 127.83 (C-6), 128.51 (C-4), 133.33 (C-4a), 135.02 (C-6′), 143.95 (C-3), 145.36 (C-8), 147.44 (C-3a′), 147.99 (C-1), 150.75 (C-7a′), 152.22 (C-7); LRSIMS $m/z$ [$M^+$] 386 (55), 167 (15), 149 (100), 119 (25); HRSIMS [$M^+$] 368.1492 (368.1496 calcd for $C_{21}H_{22}NO_5^+$).

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