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Antibacterial and antiviral naphthazarins from *Maharanga bicolor*

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Maharanga bicolor, Boraginaceae, is used in the Nepalese ethnomedicine for the treatment of several diseases. In the course of screening investigations the dichloromethane extract of the roots of *Maharanga bicolor* was found to inhibit the growth of gram positive bacteria. Bio-assay directed fractionation led to the isolation of five active naphthazarins, deoxyalkannin (**1**), alkannin (**2**), acetylalkannin (**3**), alkannin β-hydroxyisovalerate (**4**) and alkannin β-acetoxyisovalerate (**5**). Compounds **2**–**5** showed antibacterial activity against multi resistant human pathogenic *Staphylococcus* and *Enterococcus* species and **1**, **4** and **5** showed antiviral activity against herpes simplex virus type-1.

1. Introduction

Despite tremendous progress in the development of human medicines, infectious diseases are still a major threat to public health due to the rapid emergence of drug resistance and the high risk of nosocomial infections (Moellering 1998; Okeke et al. 2005).

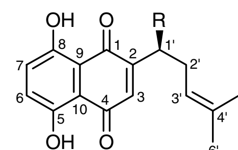
In the course of a search for new anti-infective compounds from Nepalese medicinal plants, a series of extracts were screened for antibacterial activity. Among the plant extracts screened, the dichloromethane extract of the roots of *Maharanga bicolor* (Wall. ex G. Don) A. DC. (Boraginaceae) showed high antibacterial potential with a minimal inhibitory concentration (MIC) of 0.25 μg/disc against some gram positive bacteria (Rajbhandari and Schöpke 1999).

M. bicolor commonly known as Maharangi in Nepal is a perennial herb distributed in the alpine region of Nepal at an altitude of 3000–4000 m. In Nepalese traditional medicine, the leaves are used as a purgative, flowers as cordial agents and roots are used in the treatment of burns and wounds (Suwal 1970). In this work we describe the structure of antibacterial compounds of this plant, first structure-activity relationships and further biological activities.

2. Investigations, results and discussion

The dichloromethane extract of *M. bicolor* inhibited the growth of gram positive bacteria. Bio-assay directed fractionation led to the isolation of five naphthazarins **1**–**5**.

The bio-active compounds were identified by means of spectroscopic methods including intense 2D NMR experiments. Comparison with literature data showed that all compounds are naphthazarins known from other sources. Compound **5** has been isolated from the roots of *Alkanna tinctoria* Tausch and its structure was established only on the basis of ^{1}H NMR spectra (Papageorgiou 1977). However no C-13 and 2D NMR data were available to date. Compound **4** has been isolated from *Arnebia hispidissima* DC. Borg. (Boraginaceae, Khan et al. 1983), compound **3** from *Arnebia euchroma* I. M. Johnst. (Boraginaceae, Shen et al. 2002), *Onosma leptantha* (Boraginaceae, Kundakovic et al. 2006) and *A. hispidissima* (Khan et al. 1983) and compound **2** from *Onosma echiodes* (Boraginaceae, Papageorgiou et al. 1979). Compound **1** has been obtained from the callus culture of *Lithospermum erythrorhizon* Siebold & Zucc. (Boraginaceae, Tabata et al. 1974).



1. R=H

2. R=OH

3. R= $OCOCH_3$

4. R=$OCOCH_2C(CH_3)_2OH$

5. R=$OCOCH_2C(CH_3)_2OCOCH_3$

Compounds **1**–**5** were evaluated for their antibacterial and antiviral potential. All five compounds showed potent antibacterial activity against *Bacillus subtilis* (MICs of **1**–**5**: 1.0, 0.25, 0.125, 0.25, 0.5 μg/disc respectively). Because of this strong activity the efficiency against other sensitive and several multi resistant bacterial strains was investigated (Table 1). Compounds **2**–**5** exhibited potent activity also against multi drug resistant human pathogenic bacteria of *Staphylococcus* and *Enterococcus* species. Compounds **4** and **5** displayed a broad range of activity at the tested concentrations of 2.5 and 5 μg/disc respectively whereas compound **3** inhibited the growth of all tested organisms except *E. faecium* at a concentration of

Table 1: Antibacterial activities of compounds 2–5

Organisms	Strain	Inhibition diameter (mm, without diameter of the disc)								
		2		3		4		5		Ampicillin
		2.5#	25#	1.25#	12.5#	2.5#	25#	5#	50#	10#
S. aureus	ATTC 6538	10	12	12	14	14	16	10	14	26
S. aureus	NGS*	10	14	10	12	12	14	8	10	–
S. aureus	34289*	18	22	10	12	10	14	8	10	–
S. aureus	38418*	10	16	10	12	10	14	–	10	15
S. epidermidis	473*	–	12	10	10	12	14	10	14	–
S. epidermidis	847*	–	10	12	14	10	14	8	10	–
S. haemolyticus	148*	–	10	10	12	12	14	8	12	–
Enterococcus faecium	945*	–	–	–	–	10	12	8	10	–
Enteroccous faecalis	573*	–	–	10	10	11	13	8	10	–
Pseudomonas aeruginosa	396*	–	–	–	–	–	–	–	–	–

amount of compound in μg/disc
* multiresistant strains
–: no activity at the tested concentration

1.25 μg/disc. Compound **2** was found to be less effective. None of the compounds showed activity against *Pseudomonas aeruginosa* at the tested concentration.

It was reported that the antimicrobial properties of these pigments are caused by the presence of the naphthoquinone moiety and that the esterification at the aliphatic hydroxyl group enhances the activity (Papageorgiou et al. 1979). This is in agreement with our results summarized in Table 1. According to the MIC values against *B. subtilis* the greatest activity also against multi resistant strains was shown by compound **3**. The activity was found to depend on the nature of substituents at the hydroxyl group of the ester side chain. Comparison of the antibacterial activities of the compounds indicated that esterification of the C-1′ hydroxyl group of alkannin with acetic acid enhances the activity while substitution with a more spacious residue as in compounds **4** and **5** causes a decrease in comparison to compound **3**.

Antiviral analysis was performed including the antiviral system herpes simplex virus type-1 (HSV-1)/Vero cells and influenza/MDCK cells following tests for cytotoxicity. The results are summarized in Table 2.

All the tested compounds showed cytotoxicity to Vero and MDCK cells. The toxicity also depends on the substitution pattern of the C-1′ hydroxyl group. The toxicity was found to decrease with the increase in chain length, the lowest cytotoxicity was shown by **4** and **5**.

Compounds **1, 4** and **5** inhibited the replication of HSV-1. The highest activity was exhibited by compound **5** and **1** with IC_{50} values of 1.6 and 1.8 μg/ml respectively followed by **4** with IC_{50} of 3.2 μg/ml. Thus, it can be assumed that the presence of an acetoxy group at C-9′ position in **5** enhanced the antiviral activity in comparison to **4**. Similarly, the absence of a substituent in C-1′ position of **1** could be the reason for the antiviral activity. None of the tested compounds inhibited the replication of influenza A virus.

The results of this study indicate that esters of alkannin could be potential candidates for the development of new anti-infective agents.

Table 2: Cytotoxicity and antiviral activities of compounds 1–5

Compd.	Cytotoxicity CC_{50} (μg/mL)		Antiviral activity IC_{50} (μg/mL)	
	VERO cells	MDCK Cells	HSV-1	Influenza A
1	4.5	2.5	1.8	–
2	3.5	<2.5	–	–
3	4.0	4.0	–	–
4	17.0	9.5	3.2	–
5	14.0	4.0	1.6	–
Amantadin sulphate			–	15.0
Aciclovir			0.1	–

–: no activity

3. Experimental

3.1. General procedure

Nuclear magnetic resonance spectra were measured in CD_3OD on Bruker DPX 300 and Bruker ARX 400 spectrometers with TMS as the internal standard. MS were obtained on Finnigan TSQ 700 equipped with a Finnigan electro-spray source (ESI-MS). CD spectra were recorded on circular-dichrograph Jasco-710. Column chromatography was conducted using silica gel 60 (70–230 mesh) Merck, Sephadex LH-20, Pharmacia Biotech. Precoated Silicagel 60 GF_{254}, (0.2 mm) plates (Merck) were used for thin layer chromatography and spots were visualised using UV light. HPLC separation was carried out on Merck-Hitachi system consisting of L-4000 UV detector using Merck Lichrocart RP-18 analytical and preparative columns (125 mm × 4 mm ID, RP Select B, 250 mm × 10 mm ID, RP 18) respectively.

Roots of *M. bicolor* were collected from Dolpa region of Karnali zone in 1996 and authenticated by Mrs T. M. Shrestha. A voucher specimen (no. 9022) is deposited at herbarium section of the Ministry of Forest and Soil Conservation, His Majesty's Government of Nepal.

3.2. Extraction and isolation

Air dried roots of *M. bicolor* (125 g) were finely ground and extracted with dichloromethane (1.5 L) by soxhlet extraction to get 11.4 g of the crude extract.

A portion of the dichloromethane extract (1.4 g) was chromatographed on 140 g Sephadex LH-20 using methanol. The fractions (fraction size 20 ml) were monitored by TLC using hexane-ethylacetate-ethanol (20 : 1 : 1). The fractions having the same TLC character were pooled together to afford six major fractions, S1 (obtained from fractions 1–12), S2 (obtained from fractions 13–17), S3 (obtained from fractions 18–20), S4 (obtained from fractions 21–23), S5 (obtained from fractions 24–28) and S6 (obtained from fractions 29–42). The activity was found to be highest in fractions S2, S3 and S4. The active fraction S2 (1.25 g) was further subjected to silicagel column chromatography (250 g) using hexane with an increasing amount of ethylacetate and ethanol (hexane, 2% AcOEt in hexane, 5% AcOEt in hexane, 10% AcOEt in hexane, 15% AcOEt in hexane, 20% AcOEt in hexane, 50% AcOEt in hexane, AcOEt, 50% EtOH in AcOEt, and EtOH). Altogether nine major fractions (S2-1 to S2-9) were pooled after TLC examination. The highest activity was found in fractions S2-3, S2-4, S2-5 and S2-6. The fractions S2-3 and S2-5 were purified using semi-preparative HPLC with an isocratic solvent system of 75% CH_3CN in H_2O λ^{max} 218 nm to collect compound **1** (13 mg, Rt 15 min) and com-

pound **5** (55 mg, Rt 13 min). Similarly fraction S2-6 was purified in HPLC with an isocratic solvent system of 60% CH_3CN in H_2O, λ^{max} 218 nm to collect compound **4** (27 mg, Rt 16 min).
Fraction S3 (1.25 mg) was further subjected to CC on silicagel using hexane with an increasing amount of ethylacetate and ethanol (hexane, 2% AcOEt in hexane, 5% AcOEt in hexane, 10% AcOEt in hexane, 15% AcOEt in hexane, 20% AcOEt in hexane, 50% AcOEt in hexane, AcOEt, 50% EtOH in AcOEt, and EtOH) to afford nine major fractions (S3-1 to S3-9). The fractions S3-5, S3-6 and S3-7 were found to be highly active. The fractions S3-6 and S3-7 were further purified in HPLC using an isocratic solvent of 80% CH_3CN in H_2O and 60% CH_3CN in H_2O to get compound **3** (66 mg, Rt 24 min) and compound **2** (8 mg, Rt 11 min) respectively.

3.3. Characterization of compounds

*3.3.1. Compound **1** (dexoxyalkannin)*

ESI-MS (negative ion mode): m/z 272 $[M-H]^-$, 254 $[M\text{-}18\text{-}H]^-$, 204 $[M\text{-}68\text{-}H]^-$; UV-VIS: 214, 281 and 512 nm; ^{1}H NMR: δ 7.27, s (H-6, H-7), 6.19, s (H-3), 2.67, m (H-1′), 5.22, m (H-3′), 2.36, m (H-2′a, H-2′b), 1.73, d, J = 0.7 Hz (H-6′), 1.63, d, J = 0.7 Hz (H-5′); ^{13}C NMR: see Table 3.

*3.3.2. Compound **2** (alkannin)*

ESI-MS (negative ion mode): m/z 286.9 $[M-H]^-$, 269 $[M\text{-}18\text{-}H]^-$, 241 $[M\text{-}45\text{-}H]^-$, 218 $[M\text{-}69\text{-}H]^-$, 190 $[M\text{-}97\text{-}H]^-$, 173 $[M\text{-}114\text{-}H]^-$; UV-VIS: 214, 278, 480 (s); 515, 560 (s) nm; CD spectroscopy: 310 nm (positive maximum), 360 nm (negative maximum); ^{1}H NMR: δ 7.4, s (H-6, H-7), 7.2, s (H-3), 5.4, m (H-1′), 5.0, m (H-3′), 2.6, m (H-2′a), 2.4, m (H-2′b), 1.8, d, J = 0.7 Hz (H-6′), 1.6, d, J = 0.7 Hz (H-5′); ^{13}C NMR: see Table 3.

*3.3.3. Compound **3** (alkannin-1′-acetate)*

ESI-MS (negative ion mode): m/z 330 $[M-H]^-$, 270 $[M\text{-}60\text{-}H]^-$; UV-VIS: 214, 289, 490 (s), 518, 555 (s) nm; CD spectroscopy: 308 nm (positive maximum), 360 nm (negative maximum); ^{1}H NMR: δ 7.62, s (H-6, H-7), 7.05, s (H-3), 6.03, m (H-1′), 5.22, m (H-3′), 2.17, s (H_3-8′), 2.64, m (H-2′a), 2.54, m (H-2′b), 1.72, d, J = 0.7 Hz (H-6′), 1.62, d, J = 0.7 Hz (H-5′); ^{13}C NMR: see Table 3.

*3.3.4. Compound **4** (alkannin-1′-β-hydroxy-isovalerate)*

ESI-MS (negative ion mode): m/z 387 $[M-H]^-$, 270 $[M\text{-}117\text{-}H]^-$; UV-VIS: 214, 490 (s); 519, 555 (s) nm; CD spectroscopy: 310 nm (positive maximum), 360 nm (negative maximum); ^{1}H NMR: δ 7.24, s (H-6, H-7), 7.1, s (H-3), 6.1 m (H-1′), 5.21, m (H-3′), 3.0, d, J = 14.5 Hz (H-8′a), 3.1, d, J = 14.5 Hz, (H-8′b), 2.63, m (H-2′a), 2.54, m (H-2′b), 1.7, d, J = 0.7 Hz (H-6′), 1.6, d, J = 0.7 Hz (H-5′), 1.4, s (H_3-10’), 1.4, s, H_3-11′); ^{13}C NMR: see Table 3.

Table 3: ^{13}C NMR data of compounds 1–5 (chemical shifts δ)

Carbon atom	1	2	3	4	5
C-1	184.2	181.2	177.2	177.5	176.8
C-2	152.5	154.5	148.9	148.9	148.7
C-3	131.8	132.3	132.2	132.6	132.1
C-4	184.2	182.2	178.8	179.0	178.3
C-5	164.1	165.6	168.6	168.9	169.0
C-6	132.3	132.9	134.0	134.0	134.2
C-7	132.1	132.7	133.8	133.7	134.0
C-8	164.1	166.2	169.1	169.2	169.6
C-9	113.1	113.2	113.1	113.1	112.7
C-10	113.1	112.8	112.8	112.8	112.5
C-1′	27.8	68.5	70.7	70.8	71.0
C-2′	30.6	36.5	33.8	33.9	33.8
C-3′	123.9	120.8	119.2	119.4	119.3
C-4′	135.6	135.4	136.9	136.8	137.0
C-5′	25.8	25.9	25.8	25.9	25.9
C-6′	17.7	18.0	17.9	18.0	18.0
C-7′	–	–	171.5	171.8	170.6
C-8′	–	–	20.7	40.1	45.0
C-9′	–	–	–	70.3	80.7
C-10′	–	–	–	29.3	26.9
C-11′	–	–	–	29.8	27.0
C-12′	–	–	–	–	172.2
C-13′	–	–	–	–	22.4

*3.3.5. Compound **5** (alkannin-1′-β-acetoxy-isovalerate)*

ESI-MS (negative ion mode): m/z 429 $[M-H]^-$, 285 $[M\text{-}144\text{-}H]^-$, 270 $[M\text{-}159\text{-}H]^-$, 255 $[M\text{-}174\text{-}H]^-$; ESI-MS (positive ion mode): m/z 453 $[M + Na]^+$, 381 $[M + Na\text{-}49]^+$, 293 $[M + Na\text{-}160]^+$, 273 $[M + Na\text{-}180]^+$; EI-MS: m/z 430, 270 (base peak); UV-VIS: 214 nm, 279 nm, 490 (s), 520 and 560; CD spectroscopy: 301 nm (positive maximum), 355 nm (negative maximum); ^{1}H NMR: δ 7.24, s (H-6, H-7), 7.07 s (H-3), 6.05, m (H-1′), 5.21, m (H-3′), 3.0, d, J = 14.5 Hz (H-8′a), 3.1, d, J = 14.5 Hz, (H-8′b), 2.68, m (H-2′a), 2.53, m (H-2′b), 1.73, d, J = 0.7 Hz (H-6′), 1.69, d, J = 0.7 Hz (H-5′), 1.59, s (H_3-11′), 1.56, s, H_3-10′); ^{13}C NMR: see Table 3.

3.4 Biological analysis

3.4.1. Antibacterial assay

The following test organisms were used: *Bacillus subtilis* SBUG 14, *Staphylococcus aureus* ATCC 6538; as multi drug resistant human pathogenic bacteria: *Enterococcus faecium* 945, *Enterococcus faecalis* 573, *Staphylococcus aureus* 34289, *Staphylococcus aureus* 38418; *Staphylococcus aureus* north German reference strain (NGS, obtained from Smith Kline Beecham), *Staphylococcus epidermidis* 473, *Staphylococcus epidermidis* 847, *Staphylococcus haemolyticus* 148 and *Pseudomonas aeruginosa* 396. The strain SBUG 14 was provided from the strain collection of the Institute of Microbiology, Ernst Moritz Arndt University Greifswald (SBUG), the strains 945, 573, 473, 847, 148 and 396 were clinical isolates obtained from the Institute of Hygiene, Greifswald and the strains 34289 and 38418 were received from the Institute of Medical Microbiology, Ernst Moritz Arndt University Greifswald.
A modified disc diffusion method was used to determine the antibacterial activity as described before (Rajbhandari and Schöpke 1999). Ampicillin was used as positive control and the paper disc loaded with equal volume of solvent was used as negative control. MICs were determined only against *B. subtilis*. On the basis of the results of MICs obtained for *B. subtilis*, the antibacterial tests against multi-drug resistant bacteria were carried out at two different concentrations, ten fold and hundred fold the MICs required for *B. subtilis*.

3.4.2. Antiviral assay

The compounds were tested for antiviral activity against herpes simplex virus type 1 and influenza virus A as described before (Rajbhandari et al. 2001). Each 1 mg of test compound was dissolved in 100 µl DMSO prior to dissolve in tissue culture medium and stocked at a concentration of 1 mg/ml. The cytotoxicity and antiviral activity were determined by treating the confluent monolayers with twofold serial dilutions (10, 5.0, 2.5, 1.25, 0.625 µg/mL) of **1**–**5** in medium for 72 h. Amantadin sulphate and aciclovir were used as standard compounds.

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