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ANTIFUNGAL ALKALOIDS AND LIMONOID DERIVATIVES FROM *DICTAMNUS DASYCARPUS*

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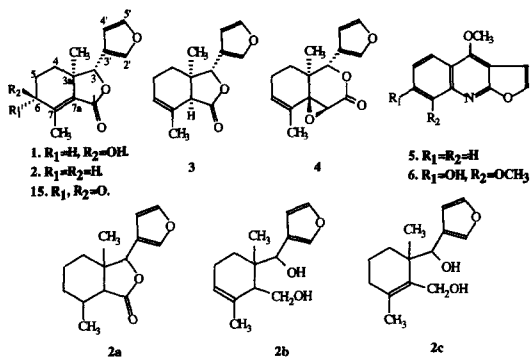
Key Word Index—*Dictamnus dasycarpus*; Rutaceae; antifungal activity; limonoids; limonoid derivatives; furoquinoline alkaloids; sesquiterpenes; steroids; dasycarpol; 6β-hydroxyfraxinellone.

Abstract—From the root bark of *Dictamnus dasycarpus* (Rutaceae), four limonoid derivatives, two furoquinoline alkaloids, five limonoids, two sesquiterpenes and three steroids were isolated and their structures elucidated on the basis of various spectroscopic methods. Among the identified compounds, one was determined to be a new natural product, 6β-hydroxyfraxinellone, while six compounds were found to be active against the plant pathogenic fungus *Cladosporium cucumerinum*. The relationship between the structures of limonoid derivatives and their inhibitory activity against fungal growth was investigated. © 1997 Elsevier Science Ltd

INTRODUCTION

The root bark of *Dictamnus dasycarpus* Turcz. (Bai-Xian-Pi) is a traditional Chinese medicine used for treatment of jaundice, cough and rheumatism. It has also been widely used to treat some skin diseases. The water extract of the plant was reported to inhibit the growth of many kinds of human pathogenic fungi *in vitro* [1]. To our best knowledge, no chemical work has been done on antifungal components of *Dictamnus* species. In continuing our work on antifungal compounds from plant origin, we found that the dichloromethane extract of the root bark of *Dictamnus dasycarpus* exhibited growth inhibition effect against the plant pathogenic fungus *Cladosporium cucumerinum*. Further bioassay-guided isolation led to the purification of six active components (**1**–**6**). Their structures were identified on the basis of spectroscopic methods as 6β-hydroxyfraxinellone (**1**), fraxinellone (**2**) [2], isofraxinellone (**3**) [3], calodendrolide (**4**) [4], dictamnine (**5**) [5] and haplopine (**6**) [6]. Among them, compound **1** was a new natural product, and has been named dasycarpol. Dictamnine (**5**) was the most active compound against *C. cucumerinum* (0.6 μg on TLC plate), while fraxinellone (**2**) was the most abundant active component isolated from the plant (>0.2%). Besides the above six active compounds, five limonoid compounds, obacunone (**7**) [7], 7α-acetylobacunol (**8**) [8], 7α-acetyldihydronomilin (**9**) [9], limonin (**10**) [10], limonin diosphenol (**11**) [11], two sesquiterpenes, β-elemol (**12**) [12], dictamnol (**13**) [13] and three steroids, pregnelolone (**14**) [13], 7α-hydroxysitosterol [14] and sitosterol were also identified from the plant. Compounds **3**, **4**, **8**, **9**, **12** and 7α-hydroxylsitosterol are reported for the first time from the genus *Dictamnus*.



Scheme 1.

RESULTS AND DISCUSSION

The root bark of *Dictamnus dasycarpus* was extracted with dichloromethane and methanol, successively. The dichloromethane extract exhibited several components active against the growth of the plant pathogenic fungus *Cladosporium cucumerinum* in bioassay tests on TLC plates. Bioactivity-guided isolation yielded six active compounds (**1**–**6**), compounds

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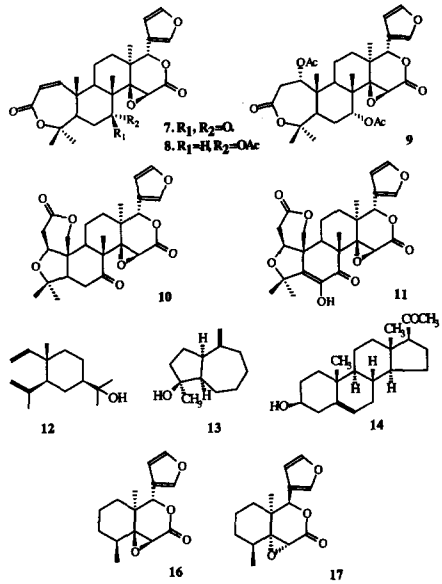

Scheme 2.

Table 1. ^1H (500 MHz) and ^{13}C (125 MHz) NMR data of compound **1** (DMSO-d_6)

No.	^1H	^{13}C
1		170.0 *s*
3	5.00 *s*	82.2 *d*
3a		42.9 *s*
4α	1.46 *ddd* 14.4, 4.2, 4.0	26.1 *t*
4β	1.73 *m*	
5α	1.80 *m*	27.6 *t*
5β	1.76 *m*	
6	3.90 *m*	65.4 *d*
6-OH	5.25 *d* 6.8	
7		147.0 *s*
7a		128.0 *s*
2′	7.72 *m*	140.3 *d*
3′		120.0 *s*
4′	6.52 *m*	109.1 *d*
5′	7.72 *m*	144.0 *d*
3a-CH_3	0.76 *s*	19.0 *q*
7-CH_3	2.11 *br s*	15.4 *q*

1–4 were limonoid derivatives, while compounds **5** and **6** were furoquinoline alkaloids.

Compound **1** was obtained as a colourless oil. The maximum of the absorption band at 218 nm in its UV spectrum and the three aromatic proton signals at δ 7.72 (2H, *m*) and 6.52 (1H, *m*) in its ^1H NMR spectrum indicated the existence of a furan ring in its structure. These characteristic signals were also found in several limonoids (**7–11**) isolated from the same plant. In the ^{13}C NMR spectrum ($CDCl_3$) of compound **1**, 14 carbon signals belonging to two methyls, two methylenes, five methines and five quaternary carbons were observed. The above data suggested that **1** might be a limonoid derivative similar to fraxinellone (**2**) previously identified from this plant [2]. Further comparison of the ^{13}C NMR data of **1** and **2** indicated that a methylene group in the structure of **2** was oxidized into an oxymethine group in the case of **1**, which was also confirmed by an additional proton signal appearing at δ 4.13 in the ^1H NMR spectrum of **1**. The above information revealed that compound **1** should be a hydroxyl-substituted fraxinellone. In order to determine the position and the relative configuration of the hydroxyl group, the NMR spectra of **1** were further measured in deuterated DMSO. In the ^1H NMR spectrum of **1**, one doublet (exchangeable by addition of D_2O) was present at δ 5.25 (1H, *d*, $J = 6.8$ Hz). This doublet was coupled with a multiplet at δ 3.90 (1H, *m*) according to the ^1H-^1H DQF-COSY spectrum. The latter multiplet further coupled with an allylic methyl at δ 2.11 (3H, *br s*). The hydroxyl group was therefore located at the C-6 position. Further analysis of the ^1H-^1H DQF-COSY, TOCSY, ROESY and HSQC spectra led to the assignment of all other proton and carbon signals (Table 1). In the HMBC spectrum of **1**, a series of ^{13}C-^1H long range correlation signals were observed, which also confirmed the skeleton of **1** (Fig. 1). In the ROESY spectrum of **1**,

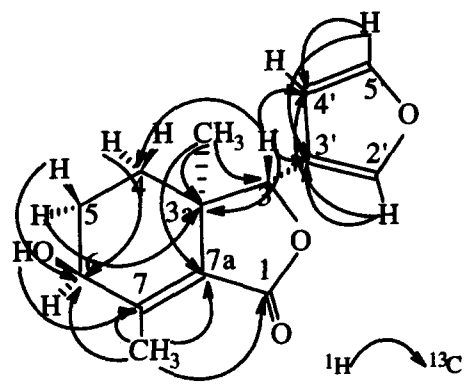

Fig. 1. Main ^1H-^{13}C long range correlation signals in the HMBC spectrum of compound **1**.

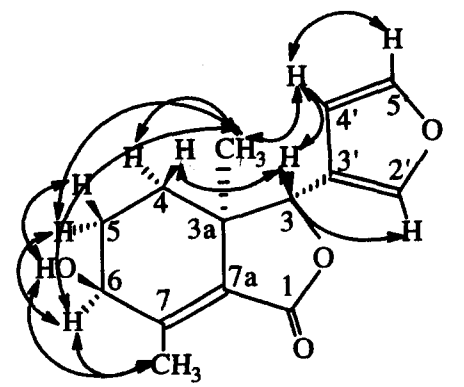

Fig. 2. Main ROE signals observed in the ROESY spectrum of compound **1**.

correlations were found between the signals of CH_3-3a and H-4α, H-5α, H-6 and H-4′. According to the above results, H-6 and CH_3-3a were both in the α-configuration (Fig. 2). Further analyses of the ROESY spectrum and a molecular model of **1** revealed that the furan group was also in the α-configuration as

Table 2. Minimum amounts needed to inhibit the growth of *C. cucumerinum* on TLC plates

Compound	1	2	2a	2b	2c	3	4	5	6	Propiconazole
Amount (μg)	5	5	10	5	5	2	5	0.6	10	0.1

found for several other limonoid derivatives (**2–4**). Therefore, the structure of **1** was determined to be 6β-hydroxyfraxinellone. A 6-hydroxy substituted fraxinellone was obtained before by Joël *et al.* [15], by reduction of fraxinellonone (**15**) isolated from another Rutaceae plant, *Fagaropsis glabra*, but the configuration of the hydroxyl group was not determined. Comparison of ^{1}H NMR data of **1** with those of the reduced product of **15** revealed significant differences between their chemical shifts. Therefore, the 6-hydroxyl group should be in different configurations in the two compounds. Compound **1** was thus determined to be a new natural product, and has been named dasycarpol.

Compounds **2–4** were also identified as limonoid derivatives. Fraxinellone (**2**) was assumed to arise from limonoids by the loss of the A, B rings and C-16, and calodendrolide (**4**) and isofraxinellone (**3**) were probably the intermediate biodegraded products [2–4]. The isolation of compounds **2–4** in the same plant in our work supported the above assumption.

The activities of the four limonoid derivatives (**1–4**) against *C. cucumerinum* are not very strong. For example, there was some growth inhibition effect at 5 μg in the TLC bioassay test but the minimum inhibitory concentration (MIC) of **2** was greater than 500 μg ml^{-1}. It seemed that these limonoid derivatives act as fungistatic agents to protect the plant against fungal attacks. However, their diminished activities can be compensated by the prodigious concentration (e.g. $>0.2\%$ for **2**). The minimum amounts of samples needed for the observable growth inhibition against *C. cucumerinum* are given in Table 2.

Compared to the four limonoid derivatives (**1–4**), the furoquinoline alkaloid dictamnine (**5**) exhibited strong antifungal activity against *C. cucumerinum*. Haplopine (**6**) was not very stable on a TLC plate and, therefore, exhibited a relative low activity in the bioassay on a TLC plate (Table 2). Dictamnine (**5**) was also subjected to an agar-dilution assay against *C. cucumerinum*, and the MIC (minimum inhibitory concentration) was determined to be 25 μg ml^{-1}. Compound **5** was reported before to be an antimicrobial substance against several human pathogenic bacteria and fungi [16]. It was suggested by Grayer and Harborne [17] that furoquinoline alkaloids may also play a role in the defence of plants against potentially pathogenic fungi. This assumption was confirmed by our results.

From the dichloromethane extract five limonoids, obacunone (**7**) [7], 7α-acetylobacunol (**8**) [8], 7α-acetyldihydronomilin (**9**) [9], limonin (**10**) [10] and limonin diosphenol (**11**) [11], were also identified. Limonoids, because of their economic significance as insect antifeedants, have attracted much attention. The conclusions about insect herbivory as a driving force in the evolutionary diversification of limonoids will still have to await structure-activity studies with ecologically relevant insects [18]. Two model compounds (**18** and **19**) based on the C and D rings and the associated furan ring were reported to be slightly more active than limonin (**10**) as antifeedant, and the presence of the furan and epoxide functions was regarded to be critical for the activity [19]. According to our results, despite the structural similarities between limonoids (**7–11**) and their derivatives (**1–4**), the five limonoids showed no growth inhibition activity against *C. cucumerinum* even when 100 μg were spotted on TLC plates. Therefore, the region of the C and D rings and the associated furan ring seemed to be critical, not only for the antifeedant activity, but also for the growth inhibitory activity against *C. cucumerinum*. In an effort to find the important structural elements of limonoid derivatives for the activity against *C. cucumerinum*, fraxinellone (**2**) was reduced with $NaBH_4$ to give three major products **2a–2c**. It was interesting to find that **2a–2c** also inhibited the growth of *C. cucumerinum* on TLC plates, and **2b** and **2c** were more active toward *C. cucumerinum* than **2a** (Table 2). From the above results, it seemed that the lactone groups in these compounds were not critical for the growth inhibitory effect, while the furan ring and the surrounding oxygen-containing groups might be important for such an activity. Therefore, it seemed that the existence of the A and B rings in limonoids might inhibit their activity against fungal growth. Biodegradation of limonoids into their derivatives may thus produce a positive response of the plants against potentially pathogenic fungal attacks.

EXPERIMENTAL

General. $[\alpha]_D$ were measured with a Perkin–Elmer 241 MC polarimeter. NMR spectra were obtained on Varian VXR 200 MHz and Bruker DMX 500 MHz instruments. Chemical shifts were reported in δ (ppm), with TMS as int. standard. MS spectra were recorded using EI, DCI or TSP loop modes on a Finnigan TSQ 700 instrument.

Plant material. The root bark of *Dictamnus dasycarpus* Turcz. was purchased from Shanghai Medicine Materia Corporation, and was identified by Prof. Jixian Guo of the School of Pharmacy, Shanghai Medical University. Voucher specimens are deposited in the Herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences and at the Insti-

tute of Pharmacognosy and Phytochemistry, University of Lausanne (no. 95143).

Extraction and isolation. The root bark of *Dictamnus dasycarpus* Turcz (3 kg) was powdered and then percolated with CH_2Cl_2 and MeOH successively ×3 at room temp. (10 l×3). The filtrate was evapd to dryness and gave a CH_2Cl_2 extract (150 g) and a MeOH extract (90 g). The CH_2Cl_2 extract (149 g) was subjected to chromatography on a silica gel (230–400 mesh) column, with a petrol–EtOAc gradient (8:1 → 0:1) as eluent to give 14 frs (frs. 1–14).

Compound **2** was obtained from frs 2–4 as white needle crystals (3.5 g). The residue of frs 2–4 was chromatographed on silica gel (230–400 mesh) column, eluted with a petrol–EtOAc gradient (8:1 → 5:1) to give **2** (2.8 g) and sitosterol (60 mg). Compound **3** (20 mg) as obtained as a light yellow oil by filtration on Sephadex LH-20 with MeOH as eluent. Compounds **4** (15 mg) and **12** (15 mg) were purified as an amorphous powder and colourless oil, respectively, through chromatographies on silica gel columns, with petrol–*iso*-Pr_2O (3:2) and toluene–Et_2O (3:1) as eluent.

Compound **1** was obtained as a colourless oil from frs 7–9 by chromatography on silica gel (230–400 mesh) column, with petrol–EtOAc (1:1) as eluent. The frs containing compounds **5** and **13** were applied to a Sephadex LH-20 column, eluted with MeOH and $CDCl_3$–MeOH (1:1), and were further chromatographed on a silica gel column with petrol–Me_2CO (3:2) and petrol–EtOAc (4:1) as eluents. Pure compound **5** (300 mg), positive to Dragendorff reagent, was obtained as colourless crystals from petrol–EtOAc (4:1). Compound **13** (50 mg) was obtained as a colourless oil.

Compound **7** (2.0 g) crystallised from frs 11 and 12. Compound **6** (15 mg) and **14** (17 mg) were obtained as amorphous powder from frs 10 and 11 through chromatographies on silica gel column, eluted with mixts of petrol–Me_2CO (2:1) and a $CHCl_3$–MeOH (30:1 → 20:1) gradient and also by filtration on Sephadex LH-20 with MeOH and $CHCl_3$–MeOH (1:1) as eluents.

Frs 13 and 14 were subjected to repeated chromatographies on silica gel column, with a mixt. of petrol and Me_2CO (3:2) and a $CHCl_3$–Me_2CO (10:1 → 7:1) gradient as eluents, and also to filtration on Sephadex LH-20 column, with MeOH and $CHCl_3$–MeOH (1:3). Purified compounds **8** (20 mg), **9** (25 mg), **10** (150 mg), **11** (120 mg) and 7α-hydroxysitosterol (21 mg) were obtained as amorphous powders.

Bioassay. Bioautography with *C. cucumerinum* for evaluation of antifungal activity of the samples was performed by silica gel TLC bioautography [20, 21].

Compound **1**. Colourless oil. $[\alpha]_D^{25} - 128.2°$ ($CHCl_3$, *c* 0.12). UV λ_{max}^{MeOH}: 218 nm. EI-MS *m/z*: 248 $[M]^+$. TSP-MS loop injection mode *m/z*: 249 $[M+H]^+$. ^1H NMR (200 MHz, $CDCl_3$): δ 7.45–7.47 (2H, *m*, H-1′, H-4′), 6.35 (1H, *br s*, H-2′), 4.94 (1H, *s*, H-3), 4.13 (1H, *dd*, 3.0, 2.0, H-6), 2.25 (3H, *s*, 7-CH_3), 1.66–1.99 (4H, *m*, H-4α, β and H-5α, β), 0.85 (3H, *s*, 3a-CH_3). ^{13}C NMR (50 MHz, $CDCl_3$): δ 169.7 (*s*), 146.1 (*s*), 143.5 (*d*), 139.8 (*d*), 130.5 (*s*), 120.1 (*s*), 108.4 (*d*), 83.1 (*d*), 67.3 (*d*), 43.5 (*s*), 27.8 (*t*), 26.6 (*t*), 18.9 (*q*), 15.7 (*q*). ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6): Table 1.

Reduction of **2**. Compound **2** (200 mg) was dissolved in 20 ml of absolute EtOH and 120 mg $NaBH_4$ were added. After keeping the reaction mixt. at 40° for 30 hr, the EtOH was evapd, and then 50 ml H_2O were added to the residue. The mixt. was extracted with $CHCl_3$ (50 ml×4). The $CHCl_3$ soln was then washed with H_2O until neutral, and then evapd to dryness to give a light yellow oil, which was subjected to CC on silica gel, eluted with a petrol–EtOAc gradient (4:1 → 2:1) to give three major products **2a** (140 mg), **2b** (15 mg) and **2c** (35 mg).

Compound **2a**. Colourless needle crystals. MP: 95–97°. EI MS *m/z*: 234 $[M]^+$, 206 $[M\text{-}CO]^+$. ^1H NMR (200 MHz, $CDCl_3$): δ 7.43 (1H, *m*), 7.37 (1H, *m*), 6.30 (1H, *m*), 4.89 (1H, *s*), 2.39 (1H, *d*, 4.4), 1.40–1.80 (6H, *m*), 1.31 (3H, *d*, 7.0), 1.20 (1H, *m*), 0.96 (3H, *s*). ^{13}C NMR (50 MHz, $CDCl_3$): δ 176.5 (*s*), 143.6 (*d*), 139.9 (*d*), 121.7 (*s*), 109.0 (*d*), 81.7 (*d*), 47.5 (*d*), 42.8 (*s*), 34.5 (*d*), 30.6 (*t*), 28.4 (*t*), 22.0 (*t*), 19.3 (*q*), 18.4 (*q*).

Compound **2b**. Colourless oil. ^1H NMR (200 MHz, $CDCl_3$): δ 7.38 (2H, *m*), 6.42 (1H, *m*), 5.43 (1H, *m*), 4.66 (1H, *s*), 3.72 (2H, *m*), 1.78 (3H, *br s*), 0.90 (3H, *s*). ^{13}C NMR (50 MHz, $CDCl_3$): δ 142.3 (*d*), 140.3 (*d*), 131.6 (*s*), 125.3 (*s*), 123.3 (*d*), 110.6 (*d*), 72.1 (*d*), 62.1 (*t*), 49.3 (*d*), 39.4 (*s*), 25.6 (*t*), 23.1 (*q*), 22.1 (*t*), 16.9 (*q*).

Compound **2c**. Colourless oil. ^1H NMR (200 MHz, $CDCl_3$): δ 7.38 (2H, *m*), 6.40 (1H, *m*), 4.74 (1H, *s*), 4.18 (2H, *m*), 1.78 (3H, *br s*), 1.03 (3H, *s*). ^{13}C NMR (50 MHz, $CDCl_3$): δ 142.3 (*d*), 140.0 (*d*), 136.0 (*s*), 135.9 (*s*), 126.0 (*s*), 110.1 (*d*), 73.4 (*d*), 58.0 (*t*), 41.1 (*s*), 35.1 (*t*), 32.5 (*t*), 20.0 (*q*), 19.8 (*q*), 18.0 (*t*).

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