Flavonoids from *Ulex* Species

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Nine flavonoids have been isolated from *Ulex jussiaei* and *U. minor* (Leguminosae). From both species the isoflavonoids ulexin A and the new naturally occurring ulexin B have been identified, together with isoderrone, the pterocarpan (-)-maackiain and (-)-4-methoxymaackiain, and the chalcone isobavachromene. The pterocarpan (-)-2-methoxymaackiain was only present in the first species and the isoflavones isolupalbigenin and ulexone A have been identified in the second one. ¹³C NMR data of isobavachromene, isolupalbigenin and ulexone A are also included. The antifungal activity of the isolated compounds was tested by the bioautographic method against *Cladosporium cucumerinum*. The most active compounds were the pterocarpan, the chalcone and the isoflavones with non-hydroxylated open chain prenyl substituents.

Introduction

Leguminosae species are particularly rich in flavonoids and in the Papilionoidea subfamily the occurrence of isoflavonoids is a characteristic feature. Isoflavones and pterocarpan are the two major groups of the isoflavonoid compounds known in nature. Most of them have biological relevance as phytoalexins, insecticide or antitumoral agents.

The *Ulex* genus (Leguminosae, subfamily Papilionoidea) is widespread in Portugal. Different species grow through out the country and some of them are endemic (Santo *et al.*, 1997). This genus has proven to be a source of new isoflavonoid structures (Harborne, 1962; Sirat and Russell, 1989; Russell *et al.*, 1990; Rodriguez *et al.*, 1990; Máximo and Lourenço, 1998) and in continuation of our search for bioactive compounds in *Ulex* species, we report the results of the study of the flavonoid fraction of *Ulex jussiaei* and *Ulex minor*.

The structures of the isoflavonoids and the chalcone were established by analysis of their spectroscopic data, by comparison with literature data for known compounds, and also with authentic samples (compounds **3–5** and **7**).

All the compounds were tested against the fungus *Cladosporium cucumerinum* by the bioauto-

graphic TLC bioassay. The pterocarpan (**4**, **5** and **7**), the chalcone (**6**) and the isoflavones with non-hydroxylated open chain prenyl substituents (**8** and **9**) exhibited antifungal activity. For the isoflavones tested this substitution seems to be an important requirement for antifungal activity.

Materials and Methods

Plant material

Plant material of *Ulex jussiaei* was collected at Quinta da Capela/Sintra (Portugal) and of *U. minor* at Peninha/Sintra (Portugal), both in April 1994. Voucher specimens are deposited in the herbarium of Museu, Laboratório, Jardim Botânico da Faculdade de Ciências da Universidade de Lisboa [ASCE 2591] and [ASCE 2592] respectively.

Flavonoid extraction

Dried and finely powdered aerial parts of *U. jussiaei* (2 kg) were extracted successively with petroleum ether (26 l) and dichloromethane (30 l) at room temperature. The dried dichloromethane extract (40.5 g) was chromatographed on a silica gel 60 column (Merck 7734) eluted with *n*-hexane-EtOAc mixtures (9:1), (8:2) and (7:3) (v/v) to collect fractions a, b and c respectively. Fraction a was

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separated on a silica gel column and eluted with *n*-hexane-EtOAc (97:3). After silica gel 60 F₂₅₄ TLC (Merck 5554) eluted with *n*-hexane-EtOAc (9:1), pure compound (**2**) (5.5 mg) was obtained. Fraction c was successively fractionated on silica gel 60 columns and on silica gel 60 TLC plates using *n*-hexane-EtOAc, *n*-hexane-Et₂O or CHCl₃-MeOH mixtures as eluents to isolate pure compounds (**7**) (3.4 mg), (**5**) (12.0 mg), (**1**) (3.9 mg), (**6**) (0.1 mg), (**4**) (23.2 mg) and (**3**) (13.3 mg) in order of increasing chromatographic polarity.

Dried and finely powdered aerial parts of *U. minor* (1.2 kg) were treated in the same way as the preceding plant, to obtain 13.6 g of dichloromethane extract. Using the same chromatographic procedure as described above, pure compounds (**2**) (1.1 mg), (**9**) (19.0 mg), (**5**) (35.3 mg), (**1**) (1.7 mg), (**6**) (0.1 mg), (**4**) (0.6 mg), (**3**) (21.2 mg) and (**8**) (21.2 mg) were obtained in order of increasing chromatographic polarity.

The previously known compounds, ulexin B (**2**) (Singhal *et al.*, 1980), isoderrone (**3**) (Máximo and Lourenço, 1998; Tahara *et al.*, 1989), (-)-maackiain (**4**) (Soby, *et al.*, 1996), (-)-4-methoxymaackiain (**5**) (Máximo and Lourenço, 1998; Cook *et al.*, 1978), isobavachromene (**6**) (Filho *et al.*, 1975; Miyase *et al.*, 1980), (-)-2-methoxymaackiain (**7**) (Máximo and Lourenço, 1998; Mizuno *et al.*, 1990), isolupalbigenin (**8**) (Tahara *et al.*, 1994) and ulexone A (**9**) (Russell *et al.*, 1990), were identified by their physical (mp) and spectroscopic data (IR, UV, ¹H RMN, ¹³C RMN, HMBC, EIMS).

Physical and spectroscopic measurements

Mps: uncorr. The specific optical rotation [α]_D^t was calculated from the values measured on a Perkin Elmer 241MC polarimeter (conc. in g/100 ml). NMR spectra were recorded on Bruker ARX 400, Bruker AM 200 and Varian Inova 400 appa-

ratus. The ^1H RMN and ^{13}C RMN spectra were recorded in CDCl_3 and referenced to the signal of residual CHCl_3 (δ 7.26 and 77.0). The EIMS were recorded on a Kratos MS25RF apparatus. The FTIR spectra were recorded on a Perkin Elmer Spectrum 1000 apparatus. The UV spectra were recorded on a Perkin Elmer Lambda 2 and a Milton Roy Spectronic 1201. Silica gel 10% deactivated with water (Merck 7734) was used for the column chromatography separations.

Ulexin A (1)

White crystals (3.9 mg), mp 167–169° (EtOAc-*n*-hexane). $[\alpha]_{\text{D}}^{25} -9.8^\circ$ (CHCl_3 ; c 0.107). IR $\nu_{\text{max}}^{\text{KBR}} \text{ cm}^{-1}$ 3392, 2921, 2850, 1645, 1628, 1491, 1463, 1374, 1265, 1185, 1075. UV $\lambda_{\text{max}}^{\text{MeOH}} \text{ nm}$ ($\epsilon \text{ cm}^{-1} \text{ M}^{-1}$) 226 (114 316), 238 (110 000), 269 (119 737), 326 (39 895), +NaOMe 226, 238, 271, 328, +NaOAc 237sh, 270, 326, + AlCl_3 230, 274, 310, 380. ^1H NMR: Table I. ^{13}C NMR: Table II. EIMS (70 eV) m/z (rel. int.): $[\text{M}]^+$ 420 (9), 405 (6), 402 (6), 387 (29), 371(3), 349 (100), 335 (11), 321(5), 186 (6), 169 (8), 167 (8), 165 (6).

Ulexin B (2)

Yellow crystals (5.5 mg), mp 175–177° (EtOAc-*n*-hexane). IR $\nu_{\text{max}}^{\text{KBR}} \text{ cm}^{-1}$ 3040, 2980, 2930, 1655, 1620, 1580, 1495, 1450, 1360, 1130, 1060. UV $\lambda_{\text{max}}^{\text{MeOH}} \text{ nm}$ ($\epsilon \text{ cm}^{-1} \text{ M}^{-1}$) 228 (33 572), 242 sh, 281(42 436), 321 sh, 348 sh, +NaOMe 228, 282, 320, 340, + AlCl_3 232, 295, 342 sh, 378 sh. ^1H NMR: Table I. ^{13}C NMR: Table II. EIMS (70 eV) m/z (rel. int.): $[\text{M}]^+$ 402 (20), 387 (100), 371 (5), 203 (5), 186 (30), 169 (5).

Cell suspension for thin-layer chromatography (TLC) bioassay

Cladosporium cucumerinum was purchased from the culture collection of industrial microorganisms (CCMI) INETI-LMI. *C. cucumerinum* CCMI 206 was grown on malt extract agar at 25 °C in pyrex Petri dishes for ten days. The mycelium was harvested from the agar plates in a small volume of fresh Homans and Fuchs nutrient broth (Homans and Fuchs, 1970), filtered through four layers of sterilized gauze and diluted in nutrient broth in order to obtain 10^6 cuf ml^{-1} .

Bioautographic TLC bioassay

Aliquots of the test compounds (0.02 ml of solutions 5 mg ml^{-1}) were spotted, in quadruplicate, on silica gel 60 F₂₅₄ TLC plates (Merck 5554), which were eluted with the appropriate eluent for each sample (CHCl_3 -MeOH 0.6% for compounds **1**, **2**, **4**–**7**, **9** and CHCl_3 -MeOH 1.25% for compounds **3** and **8**). Developed chromatograms were dried and the spots of each compound were marked under 254 nm UV light. In a glove box a 20 ml sample of the cell suspension of *C. cucumerinum* was sprayed evenly over each plate. Plates were incubated in closed pyrex trays lined with moist paper at 25 °C for two to three days, protected from light. Bioautograms were evaluated by clear spots, indicating zones of inhibition.

Results and Discussion

Ulexin A (**1**) isolated from both *U. jussiaei* and *U. minor*, $\text{C}_{25}\text{H}_{24}\text{O}_6$ (m/z 420 $[\text{M}]^+$ in EIMS), was obtained as white crystals. The ^1H RMN and ^{13}C RMN spectra (Tables I and II, respectively) showed characteristic signals of an isoflavone structure (proton signals at $\delta_{\text{C-5-OH}}$ 13.25 s, $\delta_{\text{H-2}}$ 7.82 s and carbon signals at $\delta_{\text{C-2}}$ 152.6 d, $\delta_{\text{C-3}}$ 123.3 s, $\delta_{\text{C-4}}$ 180.9 d). The IR spectrum showed the cor-

Table . ^1H NMR spectral data for compounds **1**, **2**, and **3** (400 MHz, CDCl_3 , coupling constants (*J*) in Hz).

H	1	2	3
2	7.82 s	7.81 s	7.83 s
5-OH	13.25 s	13.15 s	12.87 s
6	—	—	6.27 s
8	6.46 s	6.33 d (0.6)	6.32 s
2'	7.16 sl	7.17 d (2.3)	7.15 s
5'	6.83 d (8.2)	6.83 d (8.2)	6.82 d (8.2)
6'	7.23 dl (8.2)	7.23 dd (2.2,8.3)	7.20 d (8.2)
1''A, 1''B	—	—	—
2''	—	—	—
3''	5.63 d (9.8)	5.64 d (9.8)	5.62 d (9.8)
4''	6.35 d (9.8)	6.35 d (10.1)	6.33 d (9.2)
5''-Me	1.44 s	1.45 s	1.43 s
6''-Me	1.44 s	1.45 s	1.43 s
1'''A	3.18 dl (14.6)	—	—
1'''B	2.92 dd (15.0,8.0)	—	—
2'''	4.42 d (7.5)	—	—
3'''	—	5.62 d (10.1)	—
4'''A	4.99 s	6.73 d (10.0)	—
4'''B	4.87 s	—	—
5'''-Me	1.87 s	1.47 s	—
6'''-Me	—	1.47 s	—

δ values for compounds **1**, **2** and **3** are referenced to the signal of residual CHCl_3 (δ 7.26 ppm).

Table II. ^{13}C NMR spectral data of compounds **1**, **3**, **6** (100 MHz) and **2**, **8** (50 MHz) (CDCl_3).

C	1	2	3	6	8
C = O	–	–	–	191.7 s	–
α	–	–	–	118.0 d	–
β	–	–	–	144.0 d	–
1	–	–	–	127.5 s	–
2	152.6 d	152.4 d	152.8 d	130.5 d	152.7 d
3	123.3 ^a s	123.6 s	123.7 s	116.0 d	123.6 s
4	180.9 s	180.6 s	180.9 s	157.4 s	181.3 s
5	160.3 s	157.0 s	162.8 s	116.0 d	160.7 s
6	109.3 s	105.6 s	99.6 d	130.5 d	99.7 d
7	163.1 s	159.5 s	162.4 s	–	160.7 s
8	95.1 d	94.8 d	94.1 d	–	105.2 s
9	156.7 s	157.3 s	158.1 s	–	155.1 s
10	105.1 s	105.6 s	106.1 s	–	106.7 s
1'	123.4 ^a s	123.0 s	122.8 s	113.5 s	123.1 s
2'	127.0 d	126.9 d	127.0 d	160.6 s	130.6 d
3'	121.3 s	121.3 s	121.4 d	109.0 s	127.2 s
4'	153.3 s	153.4 s	153.3 s	159.4 s	154.7 s
5'	116.5 d	116.5 d	116.5 q	108.2 d	116.0 d
6'	129.6 d	129.5 d	129.5 q	130.5 d	128.2 d
1''	–	–	–	–	29.8 t
2''	76.6 s	76.6 s	76.6 s	77.6 s	121.6 d
3''	131.0 d	131.1 d	131.1 d	128.1 d	135.0 s
4''	122.1 d	122.1 d	122.0 d	115.9 d	17.9 ^b q
5''	28.1 q	28.1 q	28.1 q	28.4 q	25.7 ^b q
6''	28.1 q	28.1 q	28.1 q	28.4 q	–
1'''	28.2 t	–	–	–	21.6 t
2'''	77.5 d	78.8 s	–	–	121.2 d
3'''	146.6 s	128.1 d	–	–	135.0 s
4'''	110.5 t	115.5 d	–	–	17.9 ^b t
5'''	18.6 q	28.3 q	–	–	25.7 ^b q
6'''	–	28.3 q	–	–	–

δ values are referenced to the signals of the solvent (δ 77.0 ppm). ^{a,b} Interchangeable signals.

responding carbonyl absorption ($\nu_{\text{C=O}}$ 1645 cm^{-1}) of a conjugated ketone on ring C of the isoflavone. The ^1H RMN spectrum of (**1**) also exhibited the presence of a dimethylchromene system ($\delta_{\text{H-2'}}$ 7.16 s, $\delta_{\text{H-5'}}$ 6.83 d, $J_{5',6'} = 8.2$ Hz, $\delta_{\text{H-6'}}$ 7.23 d, $J_{6',5'} = 8.2$ Hz, $\delta_{\text{H-3''}}$ 5.63 d, $J_{3'',4''} = 9.8$ Hz, $\delta_{\text{H-4''}}$ 6.35 d, $J_{4'',3''} = 9.8$ Hz, $\delta_{5''\text{-Me}}$ 1.44 s, $\delta_{6''\text{-Me}}$ 1.44 s). These signals form an identical pattern for rings B and D when compared with those observed for isoderone (**3**) (Tahara *et al.*, 1989). The ^{13}C NMR of both compounds (**1**) and (**3**) are also superimposable for carbon shifts of the dimethylchromene system (C-2' – C-6' and C-2'' – C-4''; see Table II). These data suggested that only ring A substitution is different in both compounds. Compound (**1**) possessed one aromatic proton and an additional 2-hydroxy-3-methyl-3-butenyl substituent ($\delta_{\text{H-1''A}}$ 3.18 d, $J_{1''\text{A},1''\text{B}} = 14.6$ Hz, $\delta_{\text{H-1''B}}$ 2.92 dd, $J_{1''\text{B},1''\text{A}} = 15.0$ Hz, $J_{1''\text{B},2''} = 8.0$ Hz, $\delta_{\text{H-2''}}$ 4.42 d, $J_{2''\text{A},1''\text{B}} = 7.5$ Hz, $\delta_{\text{H-4''A}}$ 4.99 s, $\delta_{\text{H-4''B}}$ 4.87 s and $\delta_{\text{H-5''}}$

δ_{Me} 1.87 s. It is possible to assume that the substituent 2-hydroxy-3-methyl-3-butenyl of (**1**) should be located at C-6 comparing the chemical shifts of the aromatic H-8 proton of isoderone (**3**) (δ 6.32 s) and compound (**1**) (δ 6.46 s) (see Table I). The $\delta_{\text{C-5-OH}}$ 13.25 ppm is also characteristic for isoflavones with prenyl substituents at C-6. Moreover the chemical shifts of ^1H NMR spectrum of ring A protons of compound (**1**) are also in agreement with those of lupinisol A (**10**), isolated from *Lupinus* species (Soby *et al.*, 1996) (see Table I).

All the ^{13}C RMN spectrum signals of compound (**1**) were assigned from the HMQC and HMBC data. The chemical shifts of the protonated carbon atoms were established from the HMQC spectrum that gave the following assignments: δ 18.6 q (C-5'''), 28.1 q (C-5'', C-6''), 28.2 t (C-1'''), 95.1 d (C-8), 110.5 t (C-4'''), 116.5 d (C-5'), 122.1 d (C-4''), 127.0 d (C-2'), 129.6 d (C-6'), 131.0 d (C-3''), 152.6 d (C-2). The only protonated carbon atom that could not be assigned from HMQC spectrum was C-2''' as it is overlapped with the chloroform signal. It was assigned from the HMBC spectrum (δ 77.5 d) because this signal shows ^3J -coupling with δ 4.99 s (H-4''A), δ 4.87 s (H-4''B) and 1.87 s (CH_3 -5'''). From the HMBC data it was possible to assign all the quaternary carbon atoms.

From the above data we can establish that ulexin A has structure (**1**).

Ulexin B (**2**) found also in both plants, $\text{C}_{25}\text{H}_{22}\text{O}_5$ (m/z 402 $[\text{M}]^+$ in EIMS) was isolated as yellow crystals. It has already been reported (Singhal *et al.*, 1980) as obtained synthetically from the isoflavone lupalbigenin. The ^1H RMN spectrum of (**2**) (Table I) and the spectrum of the hemisynthetic compound (Singhal *et al.*, 1980) are identical. Now we report the EIMS and the ^{13}C RMN unequivocal assignment (Table II) and also the IV and UV spectra of this new naturally occurring metabolite. The assignment of the ^{13}C NMR signals was achieved from HMQC and HMBC experiments, in the same way as described for compound (**1**).

Here we include some corrections to the ^{13}C NMR spectrum of ulexone A (**9**) (Russell *et al.*, 1990). From our assignment, by two dimension NMR experiments (HMQC and HMBC), the following resonances were corrected: C-5' (116.6 d), C-4'' (122.2 d), C-6' (129.6 d), C-3'' (131.2 d). The assignment correspond to the interchange between C-5'/C-4'' and C-6'/C-3'' resonances.

The ^{13}C NMR spectral data for isobavachromene (**6**) and isolupalbigenin (**8**), not previously reported, are now presented in Table II.

The results of the bioautographic assay of the described flavonoids against *Cladosporium cucumerinum* are summarized in Table III. The three tested pterocarpan, (-)-maackiain **4**, (-)-4-methoxymaackiain **5** and (-)-2-methoxymaackiain **7**, the chalcone isobavachromene **6** and the isoflavones isolupalbigenin **8** and ulexone A **9**, clearly inhibited the fungus growth. Less active were the isoflavones ulexin B **2** and isoderrone **3**, and the isoflavone ulexin A **1** showed no activity.

From the bioassay of the isoflavones (**1-3**, **8** and **9**) some comments can be drawn on structure/ac-

tivity basis (Tahara and Ibrahim, 1995). The results suggest that: a) the antifungal activity increases with prenyl substitution (compounds **3** and **9**); b) hydroxylation of the prenyl side chains is a detoxification feature of the metabolite (compounds **1** and **8**); and c) compounds with open prenyl side chains are in general more active than those with cyclic prenyl groups (compounds **2** and **8,9**). In general and according to Laks and Pruner (1989) a certain degree of lipophilicity of the flavonoid is relevante. The relative acidity and number of hydroxyl groups in each structure appear to be an important factor affecting the antifungal activity.

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Table III. Bioautographic TLC assay of compounds **1-9** with *Cladosporium cucumerinum*.

Concentration	Flavonoid								
	1	2	3	4	5	6	7	8	9
5 mg ml ⁻¹	-	±	±	+	+	+	+	+	+

+, Inhibition of growth. ±, Partial inhibition of growth. -, No inhibition of growth.

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