Flavonoids from *Ulex* Species

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Ulex Species, Isoflavones, Pterocarpans

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 pterocarpans (-)-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. ^{13}C 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 pterocarpans, 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 pterocarpans are the two major groups of the isoflavonoid compounds known in nature. Most of them have biological relevance as phytoallexins, 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 bioautographic TLC bioassay. The pterocarpans (**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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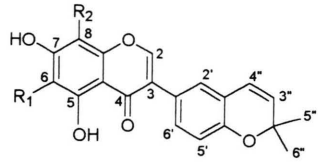

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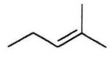
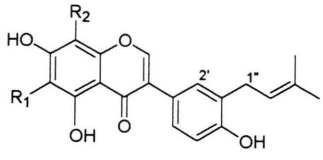
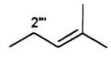
9 R_1 = H R_2 = 



8 R_1 = H R_2 = 

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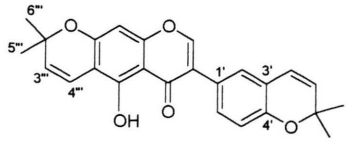

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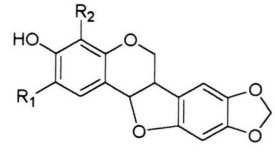
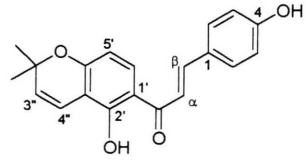


4 $R_1 = R_2$ = H

5 R_1 = H R_2 = OCH_3

7 R_1 = OCH_3 R_2 = H



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separated on a silica gel column and eluted with *n*-hexane-EtOAc (97:3). After silica gel 60 F_{254} 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-Et_2O or $CHCl_3$-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 preceeding 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 (IV, UV, ^{1}H RMN, ^{13}C RMN, HMBC, EIMS).

Physical and spectroscopic measurements

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

ratus. The ^{1}H 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). $[\alpha]_D^t$ −9.8° ($CHCl_3$; *c* 0.107). IR υ_{max}^{KBR} cm^{-1} 3392, 2921, 2850, 1645, 1628, 1491, 1463, 1374, 1265, 1185, 1075. UV λ_{max}^{MeOH} nm (ε cm^{-1} 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. ^{1}H NMR: Table I. ^{13}C NMR: Table II. EIMS (70 eV) *m/z* (rel. int.): $[M]^+$ 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 υ_{max}^{KBR} cm^{-1} 3040, 2980, 2930, 1655, 1620, 1580, 1495, 1450, 1360, 1130, 1060. UV λ_{max}^{MeOH} nm (ε cm-^{1} 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. ^{1}H NMR: Table I. ^{13}C NMR: Table II. EIMS (70 eV) *m/z* (rel. int.): $[M]^+$ 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 F_{254} 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*, $C_{25}H_{24}O_6$ (*m/z* 420 $[M]^+$ in EIMS), was obtained as white crystals. The ^{1}H RMN and ^{13}C RMN spectra (Tables I and II, respectively) showed characteristic signals of an isoflavone structure (proton signals at $\delta_{C\text{-}5\text{-}OH}$ 13.25 *s*, $\delta_{H\text{-}2}$ 7.82 *s* and carbon signals at $\delta_{C\text{-}2}$ 152.6 *d*, $\delta_{C\text{-}3}$ 123.3 *s*, $\delta_{C\text{-}4}$ 180.9 *d*). The IR spectrum showed the cor-

Table . ^{1}H 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 ($\upsilon_{C=O}$ 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 ($\delta_{H\text{-}2'}$ 7.16 *sl*, $\delta_{H\text{-}5'}$ 6.83 *d*, $J_{5',6'}$ = 8.2 Hz, $\delta_{H\text{-}6'}$ 7.23 *dl*, $J_{6',5'}$ = 8.2 Hz, $\delta_{H\text{-}3''}$ 5.63 *d*, $J_{3'',4''}$= 9.8 Hz, $\delta_{H\text{-}4''}$ 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 isoderrone (**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 ($\delta_{H\text{-}1'''A}$ 3.18 *dl*, $J_{1'''A,1'''B}$ = 14.6 Hz, $\delta_{H\text{-}1'''B}$ 2.92 *dd*, $J_{1'''B,1'''A}$ = 15.0 Hz, $J_{1'''B,2'''}$ = 8.0 Hz, $\delta_{H\text{-}2'''}$ 4.42 *d*, $J_{2''',1'''A/1'''B}$ = 7.5 Hz, $\delta_{H\text{-}4'''A}$ 4.99 *s*, $\delta_{H\text{-}4'''B}$ 4.87 *s* and $\delta_{H\text{-}5'''\text{-}Me}$ 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 isoderrone (**3**) (δ 6.32 *s*) and compound (**1**) (δ 6.46 *s*) (see Table I). The $\delta_{C\text{-}5\text{-}OH}$ 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* (C**H**$_3$-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, $C_{25}H_{22}O_5$ (*m/z* 402 $[M]^+$ 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 ressonances 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″ ressonances.

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 pterocarpans, (-)-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/activity 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 then 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.

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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