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Robinlin: A Novel Bioactive Homo-monoterpene from *Robinia pseudoacacia* L. (Fabaceae)

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Abstract—A bioactivity-directed fractionation of the ethanolic extracts of *Robinia pseudoacacia* L. (Fabaceae) afforded robinlin (**1**), a novel homo-monoterpene. The structure of **1** was elucidated by spectral analyses of the parent compound as well as its derivatives; **1** showed strong bioactivity in the brine shrimp lethality test (BST). © 2001 Elsevier Science Ltd. All rights reserved.

Robinia pseudoacacia L. (Fabaceae), commonly known as the black locust tree, is a widely distributed species with interesting biological activities. It has been cited as having a number of interesting traditional medicinal uses[1,2] as well as being poisonous.[3–5] However, few chemical investigations have been conducted to identify the active principles in this plant. It is generally believed that a group of toxic glycoproteins, lectins, are responsible, at least partially, for the adverse effects on humans and animals.[3,4] However, the brine shrimp lethality test (BST)[6,7] and 7-day MTT cytotoxicity assays[7] on the partitioned ethanolic extracts showed significant toxicity that could not be associated with lectins.[8] As part of our continuing search for new and bioactive lead compounds from higher plants, we investigated the ethanolic extracts, using bioactivity-directed fractionation with the BST.[6] We herein report the structure of one of the unusual bioactive compounds, robinlin (**1**) (Fig. 1),[9] which is identified to be a novel C-11 homo-monoterpene.

Repetitive open column and HPLC chromatography, guided by the BST, resulted in a white amorphous solid, robinlin (**1**), mp 88.4–89.8 °C. Its molecular weight is suggested by the peak at *m/z* 199 $[M+H]^+$ in the CIMS. Its elemental formula, $C_{11}H_{18}O_3$, is determined by HRCIMS measurement of the $[M+H]^+$ ion at *m/z* 199.1334 (calcd 199.1334). Analysis of ^{13}C NMR and IR spectra revealed the presence of an α,β-unsaturated ketone moiety (δ_C 200.11, 129.52, and 161.33; IR ν_{max} 1672, 1667, and 1607 cm^{-1}). Since no proton signals are observed to correlate with the double bond carbons (δ_C 129.52 and 161.33) in the HMQC spectrum, the double bond must have been fully substituted. The β-carbon is further downfield than the α-carbon because of, at least partly, the electron-withdrawing effect of the carbonyl group. To account for the degree of unsaturation suggested by the molecular formula, **1** was, hence, suspected also to have a ring system.

Careful analyses of ^1H, ^{13}C, and HMQC NMR spectra (Table 1) disclosed that there are a total of three isolated methyl groups (δ_H/δ_C 1.207/29.52, 1.275/25.33, and 1.875/12.08) in the molecule. The methyls, δ_H/δ_C 1.207/29.52 and δ_H/δ_C 1.275/25.33, are established to be geminal dimethyls because HMBC correlations are observed between them. The HMBC correlations also disclosed their attachment to a quaternary carbon (δ_C 37.31). On the basis of HMBC correlations, the other two bonds of this quaternary carbon were proposed to connect to

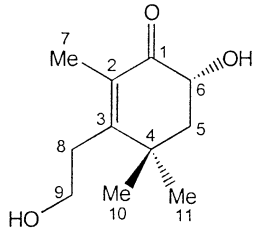

Figure 1. Structure of robinlin (**1**).

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carbons at δ_C 161.33 and 45.25, respectively (Table 1 and Fig. 2).

The combination (δ_H/δ_C 1.875/12.08) of a slightly downfield proton chemical shift and a relatively upfield carbon chemical shift is indicative that this methyl is attached to the double bond. In addition, the HMBC correlations between the methyl signal of δ_H 1.875 (H-7) and carbon signals at δ_C 200.11, 129.52, and 161.33 suggested that it is attached to the α-carbon of the α,β-unsaturated ketone moiety.

2-D NMR spectral data also suggested that **1** contains a hydroxyethyl group (δ_C 60.97 and 34.21; δ_H 3.717/3.732 and 2.525/2.649), which is established to connect to the β-carbon of the α,β-unsaturated ketone fragment based on their HMBC correlations with the β-carbon at δ_C 161.33 (Table 1 and Fig. 2).

An ABX spin system is observed (δ_H 1.751/2.143 and 4.250) in the COSY spectrum. Based on its coupling constants and splitting pattern, it was rationalized that the proton at δ_H 4.250 has an axial–axial relationship with δ_H 1.751, but an axial–equatorial relationship with δ_H 2.143 (Table 1). The HMBC experiment suggested that the hydroxylated methine (δ_H/δ_C 4.250/69.21) is connected to the ketone moiety and the methylene (δ_H/δ_C 1.751, 2.143/34.21) is bonded to the quaternary carbon at δ_C 37.31. Therefore, the planar structure of **1** was proposed to be 6-hydroxy-3-(2-hydroxy-ethyl)-2,4,4-trimethyl-cyclohex-2-enone. Double-relay COSY provided further evidence for the proposed structure of **1** (Fig. 2). It is named robinlin.

A NOESY experiment (with mixing time of 600 ms) was performed to determine the conformation of **1** (Fig. 3). Correlations are observed between the proton at δ_H 2.143 and each of the two methyl signals (δ_H 1.207 and 1.275); whereas the proton at δ_H 1.751 is observed to correlate with only one of the methyls (δ_H 1.207). Since it has been known that δ_H 1.751 occupied the axial position and δ_H 2.143 the equatorial position, it is concluded that the methyl at δ_H 1.207 was *cis* towards the proton δ_H 1.751 and *trans* towards δ_H 2.143. Subsequently, the

Table 1. ^{13}C (125 MHz) and ^{1}H (500 MHz) and NMR data of robinlin (**1**) and its Mosher esters (**1a** and **1b**)[a]

	$\delta^{13}C$	$\delta^{1}H$[b]	HMBC correlations[c]	$\delta^{1}H$ of **1a**	$\delta^{1}H$ of **1b**	$\Delta\delta^{1}H = \delta^{1}H_{1a} - \delta^{1}H_{1b}$
1	200.11					
2	129.52					
3	161.33					
4	37.31					
5	45.25	Ha: 1.751 (t, 14.0)	C-1, C-4, C-6, C-10, C-11	1.991(dd, 12.5/14.0)	2.150 (dd, 12.5/14.0)	−0.159
		Hb: 2.143 (dd, 6.0/14.0)	C-1, C-3, C-4, C-6, C-10, C-11	1.932 (dd, 6.0/12.5)	2.067 (dd, 6.0/12.5)	−0.135
6	69.21	4.250 (dd, 6.0/14.0)	C-1, C-5	5.650 (dd, 6.0/14.0)	5.620 (dd, 6.0/14.0)	
7	12.08	1.875 (s)	C-1, C-2, C-3	1.871 (s)	1.859 (s)	+0.012
8	34.21	Ha: 2.525 (ddd, 6.5/9.5/13.0)	C-2, C-3, C-4, C-9	2.59–2.72 (m)	2.592 (ddd, 6.0/9.0/13.0)[e]	
		Hb: 2.649 (ddd, 6.5/9.5/13.0)	C-2, C-3, C-4, C-9	2.59–2.72 (m)	2.715 (ddd, 6.0/9.0/13.0)[e]	
9	60.97	3.717 (ddd, 6.5/9.5/10.5)	C-3, C-8	4.390 (ddd, 6.5/10.0/11.0)[d]	4.29–4.38 (m)	
		3.732 (ddd, 6.5/9.5/10.5)	C-3, C-8	4.284 (ddd, 6.5/10.0/11.0) [d]	4.29–4.38 (m)	
10	25.33	1.275 (s)	C-3, C-4, C-5, C-11	1.291 (s)	1.302 (s)	−0.011
11	29.52	1.207 (s)	C-3, C-4, C-5, C-10	1.202 (s)	1.240 (s)	−0.038

[a]All measurements were taken in $CDCl_3$, using TMS as proton reference and $CDCl_3$ as carbon reference.
[b]Chemical shifts are followed by multiplicities and coupling constants in Hz in parentheses.
[c]Optimized for $J = 9.1$ Hz.
[d,e]Assignments could be reversed.

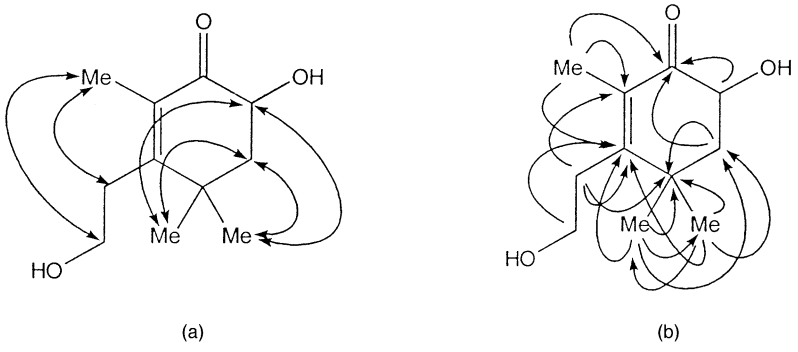

Figure 2. (a) Double-relay COSY and (b) selected HMBC correlations of **1**.

methyl at δ_H 1.275 is *cis* towards δ_H 2.143 and *trans* towards δ_H 1.751. Additionally, the fact that a cross-peak was observed between the methyl δ_H 1.275 and the proton at δ_H 4.250 substantiated the inference that they are in an axial–axial relationship. Difference NOE experiments helped to gain some insight on the average atomic distances within **1**. The proton at δ_H 1.751 attained a larger NOE enhancement than the proton at δ_H 2.143 (3.7% vs. 3.1%) when the methyl at δ_H 1.207 was saturated, and this observation indicated that this methyl is tilted towards the proton δ_H 1.751. Following similar reasoning, it was expected that the proton at δ_H 2.525 is in close proximity to both methyls, and the proton at δ_H 2.649 is a little further away. The lowest energy conformation of **1**, which was generated by the SPARTAN program based on semiempirical AM1 potential, confirmed this conclusion (Fig. 3). It was also observed that conformations with the hydroxyethyl side chain either α or β to the six-membered ring only differed in energy about 0.2%. The fact that when the methyl at δ_H 1.275 was saturated, the percent NOE enhancement of δ_H 4.250 (5.9%) was much larger than that of δ_H 2.143 (3.1%), is probably because δ_H 2.143 has more relaxation pathways than δ_H 4.250.

Thanks to the presence of the nucleophilic hydroxyl group at the chiral center C-6, Mosher ester methodology could be employed to solve the absolute stereochemistry.[10,11] Compound **1** was derivatized with the (*S*)-(+)- and (*R*)-(−)-forms α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPACl) to form Mosher esters, **1a** and **1b**, respectively. Both hydroxyl groups in **1** were esterified. Calculation of differences in chemical shifts of protons in **1a** and **1b** ($\Delta\delta^1H = \delta^1H$ for **1a**-δ^1H for **1b**), summarized in Table 1 suggested the absolute stereochemistry at C-6 to be *R*.

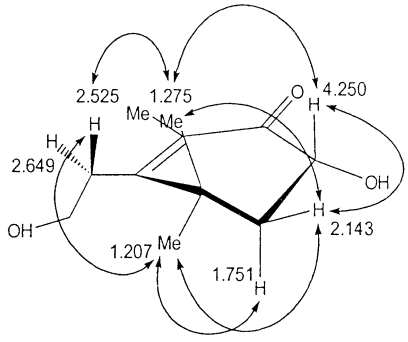

Figure 3. Selective NOESY correlations used to determine the relative stereochemistry of **1**.

Table 2. Bioactivities[7] of **1**

	(**1**)	Adriamycin
BST LC_{50} (μg/mL)	4.9 (1.2/8.3)	—
Human A-549	4.7×10	2.1×10^{-3}
Tumor MCF-7	4.6×10	5.8×10^{-2}
Cell HT-29	5.2×10	1.3×10^{-2}
Lines[5] A-498	>100	4.6×10^{-3}
ED_{50} PC-3	>100	2.4×10^{-3}
(μg/mL) PACA-2	>100	1.8×10^{-3}

The carbon skeleton of robinlin (**1**) suggests that it is derived from isoprenyl building blocks. It could either be a breakdown product of higher terpenes, such as the tetraterpene, β-carotene, via photo-oxygenation reactions,[12] or it could be a derivative from the monoterpene pathway via C-methylation. The same absolute stereochemistries of **1** and robinspirols A–C[13] at the position two carbons away from the geminal dimethyl moiety suggest that they are likely biogenetically related and may involve losses of units of two carbons from a sesquiterpene precursor. Nevertheless, **1** is classified into the homo-monoterpenes based on its C-11 backbone.

The biological activities of robinlin (**1**) are summarized in Table 2. The BST is a convenient, yet effective, bench top assay that is capable of detecting a broad spectrum of bioactivity arising from a variety of structurally diverse compounds.[6] **1** is active in the BST but is not significantly cytotoxic in one panel of six human solid tumor cell lines.[7] The potent BST results suggest that this unusual compound possesses pharmacological activities other than cytotoxicities.

Acknowledgements

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7. BST LC_{50} values <40 μg/mL for pure compounds are considered as active. The bioactivity in the BST is reported as LC_{50} values, followed by 95% confidence level in parenthesis. The 7-day MTT assays are in vitro cytotoxicity tests against six human solid tumor cell lines and were performed at the Cell Culture Laboratory, Purdue Cancer Center, following standard protocols. The six cell lines tested are A-548 (human lung carcinoma), MCF-7 (human breast carcinoma), HT-29 (human colon adenocarcinoma), A-498 (human kidney carcinoma), PC-3 (human prostate adenocarcinoma), and PACA-2 (human pancreatic carcinoma). Adriamycin is always used as a positive control in the same run; ED_{50} values less than 4 μg/mL are considered to be significantly active.

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9. 2.4 mg was isolated; mp 88.4–89.8 °C; $[\alpha]^{22} = +526°$ (*c* 0.1 $CHCl_3$); IR (film on NaCl plate) ν_{max} 3381, 2963, 2929, 2870, 1671, 1608, 1030 cm^{-1}; UV (MeOH) $\lambda_{max} = 249$ nm (log $\varepsilon = 4.60$).
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