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ORGANOMETALLIC FLAVONOID DERIVATIVES AS SPECTROSCOPIC PROBES

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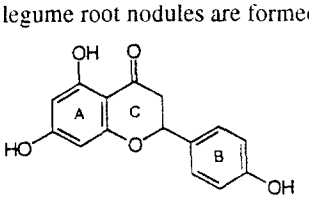
Abstract: Derivatives of naringenin have been synthesized with organometalcarbonyl reporting groups for IR spectroscopy attached at C-2, C-3', or C-6, and the products have been tested for the induction of *nod* gene expression using a *Rhizobium leguminosarum* strain which contains the *Escherichia coli lacZ* (β-galactosidase) gene fused to *nodABC*. Derivatives with an OMe substituent within the reporting group moiety showed residual gene induction activity.

Organometalcarbonyl derivatives[1] of peptides,[2] oligonucleotides,[3] and steroids[4] have been prepared in order to exploit the exceptional spectroscopic properties of the metal carbonyl group. The pioneering work of Jaouen with the estradiol receptor,[1,4] and the subsequent development of the CMIA method,[5,6] have established the utility and practicality of the incorporation of metal carbonyl groups in biological systems, and the value and exceptional sensitivity of FTIR spectroscopy when applied in this field. By including an unnatural organometallic moiety as a reporting group (RG), it is possible to measure its spectroscopic properties in such systems with minimal interference from other substances. Furthermore, signals from several organometallic compounds can be measured separately yet simultaneously in a single spectroscopic experiment, distinguishing two probes (multi-CMIA)[6] or protein-bound and free probes.[2e] In this paper, we report the first examples of bioflavonoids tagged with an organometalcarbonyl reporting group. Residual biological activity was detected by screening the products as inducers[7,8] of *nodABC* gene expression in *Rhizobium*, indicating that compounds of this type are candidates for the spectroscopic study of flavonoid/protein interactions in molecular signaling events that occur during *nodABC* gene induction when legume root nodules are formed.



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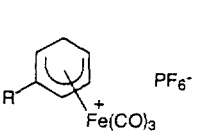
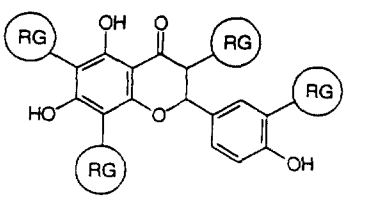


2 a : R = H
b : R = OMe



3 : C-6; **4** : C-8; **5** : C-6 and C-8; **6** : C-2; **10** : C-3'

Figure 1. Flavanones with covalently attached organometalcarbonyl reporting groups: nodulation-inducing flavanone naringenin **1** and derivatives **3**, **4**, **5**, **6** and **10** with reporting groups at C-6, C-8, C-2, or C-3' [reporting group: RG = $(\eta^4\text{-}C_6H_6R)Fe(CO)_3$ derived from **2a,b**]. For root nodulation activity, see Table 1.

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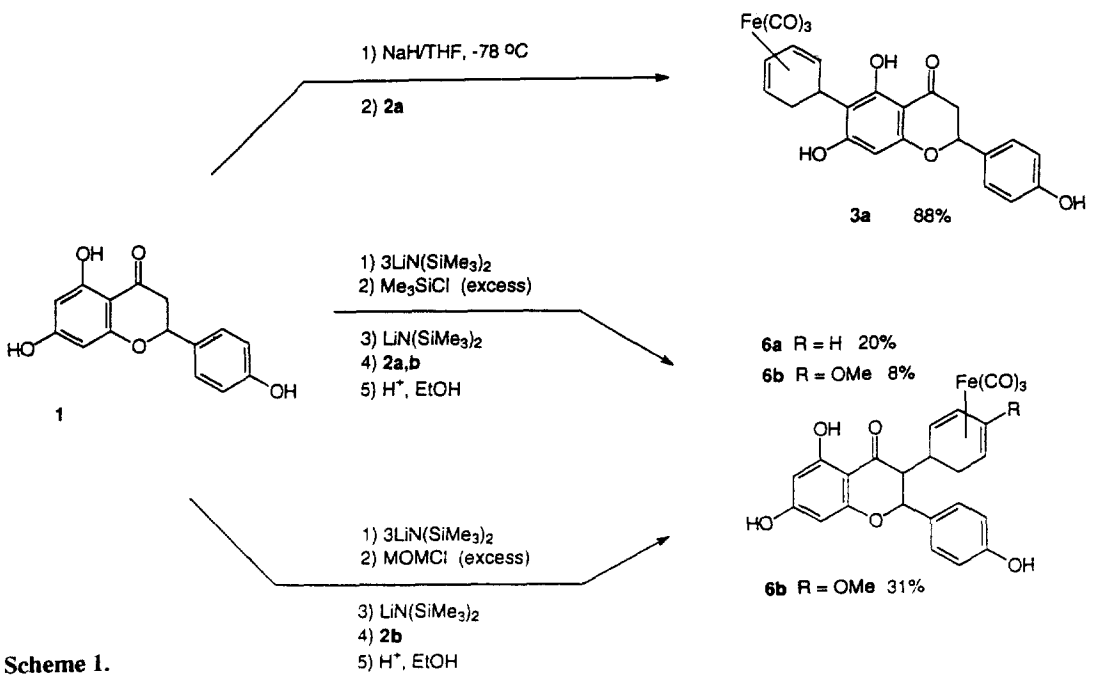
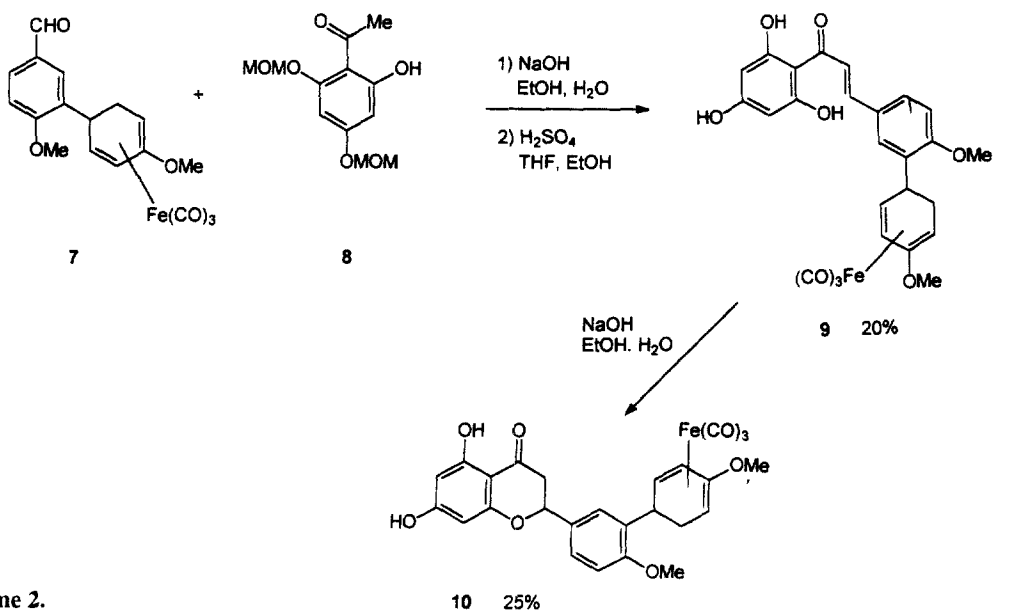
Flavonoid *nod* gene inducers require oxygenation on the A and B rings.[8] We have chosen the commercially available *nod* gene-inducing trihydroxyflavanone naringenin (**1**) for our investigations, and have developed procedures for the introduction of organometalcarbonyl-bearing structures at each of the A, B and C rings of the natural product (Figure 1). Addition of electrophilic η^5-cyclohexadienyl tricarbonyliron complexes **2a,b** by direct reaction with the flavanone in the presence of triethylamine was examined first. The reaction lacked control, giving adducts at C-6 and C-8 on the A ring, and double adducts in which both positions were substituted, (**3**, **4** and **5**, respectively). However, since these compounds could be separated by chromatography, this simple one-step procedure afforded samples for biological evaluation. A more efficient preparation has been demonstrated in the case of the C-6 mono-adducts. Complete deprotonation of the OH groups with sodium hydride in THF at -78 °C, followed by addition of the cyclohexadienyl complex, afforded the C-6 mono-adduct **3a** in 88% yield (Scheme 1).

For the central C ring, a similar approach was adopted. Our objective was to use the high reactivity of the η^5-cyclohexadienyl electrophiles to trap C ring enolates before ring-opening destroyed the flavanone through the formation of chalcones. Naringenin was treated with three equivalents of $LiN(SiMe_3)_2$, followed by trimethylsilyl chloride to effect an *in situ* protection of the OH groups. A fourth equivalent of base was then added, followed by the organometallic electrophile. In this way, the adduct **6a** could be obtained in 20% yield as the free hydroxy compound by deprotection during the reaction work-up. The 2-methoxy-substituted cyclohexadienyl complex **2b** is less reactive than **2a**,[9] and worked less well in the trapping procedure. By the use of an *in situ* protection by a MOM group, however, the yield of the C-ring adduct **6b** was improved to 31%.



Scheme 1.

The generation of C-ring enolates offered a strategy for protection at this position to shift reactivity to ring B. If the central carbonyl group could be protected as an enol ether, metallation at ring B should produce an intermediate in which the metallated ring would react more rapidly than the enol ether with the organometallic electrophile. Deprotonation of naringenin with four equivalents of the disilazide base was followed by addition of an excess of trimethylsilyl chloride to form the tetrasilyl derivative. The metallation step was attempted by reaction with *n*-BuLi, and the cyclohexadienyl complex was added. In this case, however, no organometallic derivative could be isolated from the reaction. We turned instead to a step-wise synthetic route (Scheme 2), in which the organometallic portion was introduced before the completion of the flavanone ring system. The starting material, 4-hydroxybenzaldehyde, was brominated, *O*-methylated, and protected as the acetal. Metallation by lithium/halogen exchange was followed by addition of $CuBr.SMe_2$ and the cyclohexadienyl complex **2b**. In this way, the intermediate **7** was obtained in 67% yield. This product was condensed with the partially protected trihydroxyacetophenone **8**, and removal of the MOM groups afforded **9** in 20% yield. Cyclisation to the flavanone is a difficult reaction, but the product **10** was obtained in sufficient quantities to permit biological evaluation. By these procedures, the first examples of organoiron flavanone derivatives have been prepared, and compounds with the metal-bearing unit at either the A, B, or C rings have been made available for testing for *nod* gene inducer activity.



Scheme 2.

For biological evaluation, the flavanones were purified by HPLC using a Dynamax preparative C18 column with gradient elution (methanol/water). The purity of each product was checked by re-injection to an analytical column, since even slight traces of metal-free flavonoids could invalidate the results of the *nod* gene induction assay, if levels of gene induction activity were significantly lowered by the presence of the bulky organometallic group. The pure organometallic derivatives were tested by an established procedure[7] using *Rhizobium leguminosarum* strain 8401 carrying plasmids pIJ1518 and pIJ1477, which contain the *nodD* gene

Table 1. Nodulation gene induction activity of organometallic flavonoid adducts.

Compound	R	n[a]	Units[b] (at concentration of flavonoid) 20 μM	2 μM	Activity
3a	H	1	80 ± 20	0	+/-
5a	H	2	0	0	-
3b	OMe	1	510 ± 160	0	+
5b	OMe	2	0	0	-
6a	H	1	45 ± 15	10 ± 10	+/-
6b	OMe	1	480 ± 200	70 ± 45	+
10	OMe	1	25 ± 5	15 ± 5	+/-
Naringenin (**1**)	-	0	4100 ± 50	4100 ± 50	+[c]
Eriodictiol[d]	-	0	4500 ± 100	4500 ± 100	+[c]

[a] Number of tricarbonyl(cyclohexadienyl)iron moieties attached. [b] Units of activity are the results of at least 3 assays and are corrected for control values (parallel experiments in the absence of the inducer) which range from 60-250 units in different tests. [c] Known inducers (ref. 8) for which units of activity were remeasured and corrected to be comparable to the data presented for the organometallic derivatives. [d] Eriodictiol is one of the most active of the *nod* gene inducing flavonoids.

(pIJ1518) and the *E. coli lacZ* (β-galactosidase) gene fused to the *nodABC* genes from *R. leguminosarum* (pIJ1477). In this way, *nod* gene induction can be assessed by spectrophotometric measurement of β-galactosidase activity arising from induction of the *nodABC-lacZ* gene fusion. Strong inducers can give positive results in this test even at 0.2 μM levels. When the organometallic derivatives were added at 2 μM to the growth medium of this strain, a low level of gene induction could be seen in some cases (complexes **6a**, **6b** and **10**). At 20 μM, several of the organometallic compounds showed levels of activity substantially higher than controls tested at the same time. The values observed with compound **3b** were somewhat variable but were consistently higher than the controls. The reason for this variability is not clear although we did observe some growth inhibition by this compound and this could have significantly influenced the values obtained. The results (Table 1) show that only organometallic derivatives with OMe substituents on the metal-bound cyclohexadiene moiety were active. This raises the possibility that this substituent may be mimicking the crucial role of natural A/B ring oxygenation in the interactions with the receptor structure.

A preliminary FTIR experiment[10] (Figure 2), has been performed with protein-containing fractions from lysed cell extracts of *R. leguminosarum* [a wild-type strain WT(8400pRL1) carrying *nodD* on pIJ1518] in which enhanced concentrations of the regulator protein NodD are present. This was compared with the corresponding fractions from an isogenic strain lacking *nodD*. These experiments have demonstrated that it is possible to record unobscured IR vibrational bands in relatively crude extracts derived from *Rhizobium* cells, even in the aqueous-buffered conditions needed for our application and at concentrations as low as 50 μM, although the level of activity of this first series of organometallic derivatives was too low for differential detection of the receptor-bound and free inducer molecules. Current work addresses the synthesis of organometallic flavonoid derivatives which can bind to the receptor more strongly to improve the prospects to distinguish the IR spectra of bound and un-bound molecules.

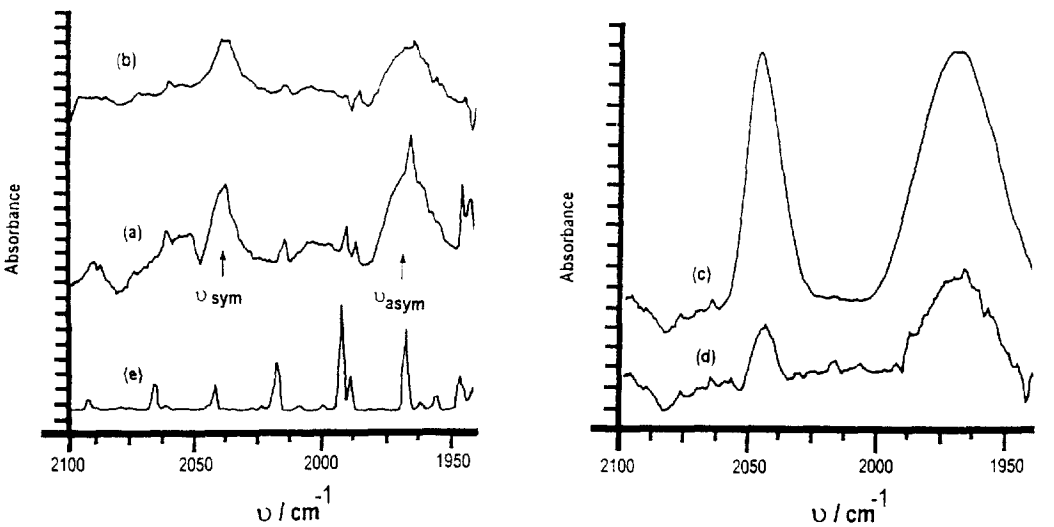


Figure 2. FTIR spectra of compound **6b** at 50 μM measured using protein extracts (25 mg ml^{-1}) of (a) *R. leguminosarum* (8400pRL1) carrying *nodD* on pIJ1518, and (b) an isogenic strain lacking *nodD* (on an absorbance scale of 0.002 units per ten divisions). For comparision, spectra of the flavonoid derivative at 2 mM and 50 μM concentrations in 0.2 M phosphate buffer are presented (traces (c) and (d), respectively; absorbance scale of 0.01 and 0.001 units per ten divisions), together with a water vapour background spectrum (trace e).

In these investigations, we have demonstrated a series of procedures for the attachment of organoiron reporting groups to flavanones, the feasibility of the spectroscopic examination of the products, and that *nod* gene induction activity is not completely blocked by the organometallic unit. On the basis that the metal-bound methoxycyclohexadiene may be capable of taking on the recognition role of an oxygenated aromatic ring in the natural inducer, better results may be obtained with analogues in which the methoxycyclohexadiene unit replaces one of the aromatic rings in the flavonoid, so providing more effective organometallic *nod* gene inducers for spectroscopic investigation.

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10. The spectra were recorded in a 0.1 mm pathlength solution sample cell fitted with ZnSe windows using a Nicolet Magna-IR 750 infrared spectrometer equipped with an MCT/A detector and KBr beam splitter; 10000 scans were recorded at 2 cm^{-1} resolution (aperture = 12).