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The ^{13}C-NMR Assignment of Nor-rubrofusarin having Strong Radical Scavenging Effect on 1,1-Diphenyl-2-picrylhydrazyl Radical

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Abstract - The ^{13}C-NMR spectrum of nor-rubrofusarin showing strong radical scavenging effect has been assigned by using HMBC and HMQC experiments.

Key words - *Cassia tora*, Leguminosae, NMR assignment, radical scavenging effect.

Introduction

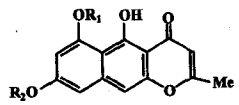
In the course of investigating biologically active natural compounds from the Korean medicinal plants, we reported that the methanolic extract of the seeds of *Cassia tora* (Leguminosae) exerts a radical scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Choi *et al.*, 1993, 1994). From this methanolic extract, cassiaside (**2**, nor-rubrofusarin glucoside) and rubrofusarin gentiobioside (**4**) were isolated as active principles, respectively (Choi *et al.*, 1994).

Because these two compounds demonstrated their effect as glycosides, we compared the radical scavenging effect with their respective genin, nor-rubrofusarin (**1**) and rubrofusarin (**3**) obtained by acid or enzymatic hydrolysis, to find a compound more effective than glycosides. We further compared the effect with isolated compounds having similar structure from raw or roasted *C. tora* (Lee *et al.*, 1997) to evaluate the radical scavenging effect and structure-effect relationship of each compound.

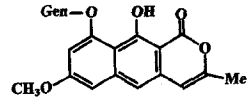
Though the structure of nor-rubrofusarin has already been elucidated (Ashley *et al.*, 1937), the assignments of the ^{13}C resonances to their respective carbon atoms have not been reported so far. The ^{13}C-NMR assignment of nor-rubrofusarin was obtained by utilizing HMBC and HMQC experiments.

Experimental

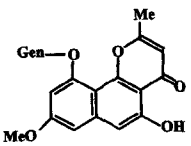
The ^{1}H- and ^{13}C NMR spectra were recorded at 600 MHz and 125 MHz, respectively on a Bruker AM 300 spectrometer with tetramethylsilane as the internal



1(nor-rubrofusarin) : R_1=R_2=H
2(cassiaside) : R_1=Glu R_2=H
3(rubrofusarin) : R_1=H R_2=CH_3
4(rubrofusarin gentiobioside) : R_1=Gen R_2=CH_3



5(toralactone gentiobioside)



6(iso-rubrofusarin gentiobioside)

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standard. The chemical shifts were referenced to residual solvent ppm {(2.49 ppm in ^{1}H-NMR (600 MHz) and 39.5 ppm in ^{13}C-NMR (125 MHz)} and were recorded δvalues. Multiplicities of ^{1}H- and ^{13}C-NMR signals are indicated as s(singlet), d(doublet) and t (triplet).

Isolation of cassiaside(2), toralactone gentiobioside(5), rubrofusarin gentiobioside(4) and isorubrofusarin gentiobioside(6) - Cassiaside, toralactone gentiobioside, rubrofusarin gentiobioside and isorubrofusarin gentiobioside were isolated according to the procedure in previous papers (Choi *et al.*, 1994, Lee *et al.*, 1997) and identified by direct comparison with authentic samples (^{1}H- and ^{13}C-NMR).

Acid hydrolysis of rubrofusarin gentiobioside acetate - The solution of rubrofusarin gentiobioside acetate (300 mg) in methanolic 7% H_2SO_4 (70 ml) was heated on a boiling water bath for 5 hr. The reaction mixture was diluted with water and extracted with dichloromethane, and the dichloromethane solution was washed with water, dried, and evaporated, and chromatographed on silica gel using dichloromethane. The yellow fluorescent band was eluted and recrystallized from methanol to give orange red needles of rubrofusarin (**3**, 70 mg). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 15.86 (1H, s, -OH), 6.95 (1H, s, H-10), 6.54 (1H, s, H-9), 6.44 (1H, s, H-7), 5.99 (1H, s, H-3), 3.87 (1H, s, 8-OMe), 2.36 (1H, s, 2-Me).

Enzymatic hydrolysis of cassiaside - The solution of 100 mg of the cassiaside in 5% HCl (30 ml) was heated on a boiling water bath for 10 min. The reaction mixture was diluted with water and extracted with EtOAc. The EtOAc solution was washed with water, dried, evaporated and enzymatic hydrolyzed. To a solution of EtOAc solution in acetate buffer (pH 5.4) β-glucosidase (80 mg)(Fluka, 1 mg of this enzyme liberates approx. 3.4 μmoles glucose per min) was added and the mixture was incubated at 37°C for 15 hr. The reaction mixture was diluted with water and extracted with EtOAc, the EtOAc layer was evaporated to dryness and rechromatographed on silica gel using EtOAc to form yellow needles of nor-rubrofusarin (**1**, 18 mg). ^{1}H- and ^{13}C-NMR : see Table 1.

Table 1. ^{1}H- and ^{13}C-NMR data for **1** in DMSO-d_6

position	**1**	
	δH	δC
2		169.76
3	6.17 s	106.06
4		183.48
5		162.34
6		158.45
7	6.32 s	100.66
8		161.90
9	6.57 s	100.74
10	6.97 s	99.75
11		151.00
12		101.68
13		105.38
14		140.00
2-CH_3	2.37 s	20.30
5-OH	15.75 s	
6-OH	9.84 s	
8-OH	10.24 s	

[a] Assignment are based on the analysis of HMQC and HMBC data

DPPH radical scavenging effect - Evaluation on the DPPH radical scavenging effect was carried out according to the method first employed by M. S. Blois (Blois, 1958). Four milliliters of MeOH solution of varying sample concentrations was added to 1.0 ml DPPH methanol solution (1.5×10^{-4} M). After standing at room temperature for 30 min., the absorbance of this solution was determined at 520 nm using a spectrophotometer and the remaining DPPH was calculated. The results were calculated by taking the mean of all triplicate values.

Results and Discussion

NMR assignments of nor-rubrofusarin (1) - Enzymatic hydrolysis of cassiaside (**2**) gave nor-rubrofusarin (**1**) as yellow needles. The ^{1}H-NMR spectrum of **1** in $CDCl_3$

(Table 1) exhibited the presence of two hydroxyl (δ 9.84, 10.24) as well as one hydrogen-bonded hydroxyl (δ 15.75), a methyl (δ 2.37) group and four aromatic protons ascribable to two isolated (δ 6.17, 6.97) and a pair of *meta*-coupled ones (δ 6.32, 6.57). These spectral data were consistent with the structure of nor-rubrofusarin. Detailed analysis of the ^{1}H- and ^{13}C-NMR spectra (Table 1), aided by HMQC (Summers *et al.*, 1986) and HMBC (Bax and Summers, 1986) experi- ments, enabled establishment of full assign- ments of **1**. ^{13}C-Signals of the protonated carbons in **1** were readily assigned by careful analysis of HMQC spectrum (Fig. 1) and by comparisons with the ^{13}C-NMR data of related naphthopyrones (Koyama and Natori, 1989; Gorst-Allman *et al.*, 1980; Lesper and Staunton, 1984). In the HMQC spectrum, the signals at δ 106.06, 100.66, 100.74 and 99.75 were assigned to C-3, C-7, C-9 and C-10 respectively. The signals of C-2 (δ 169.76) and C-4 (δ 183.48) showed coupling with CH_3 protons and H-3 respectively, in HMBC (Fig. 1). Of the carbons bearing hydroxyl function (C-5, C-6, and C-8), C-6 (δ 158.45) and C-8 (δ 161.90) showed couplings with H-7 and H-9 respectively, in HMBC. Accordingly, the remaining carbon signal at δ 162.34 was assigned to C-10 bearing intramolecular hydrogen bonded hydroxyl group. Among the four quaternary carbons at the ring junctions (C-11, C-12, C-13, and C-14), C11 (δ 151.00) and C12 (δ 101.68) showed strong couplings with H-10 and H-3, respectively in HMBC. And C-13 (δ 105.38) showed weak coupling with H-10. Thus, the ^{13}C assignments of nor-rubrofusarin (**1**) were all straightforward as shown in Table 1.

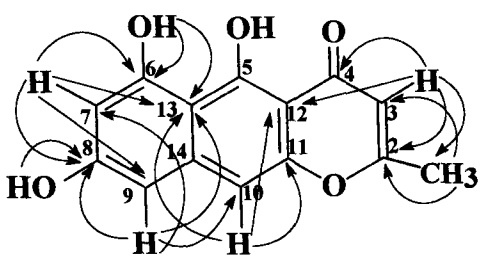

Fig. 1. HMBC corelations of **1**.

The radical scavenging effect of isolated components on DPPH radical - The radical scavenging effect of compounds (**1-6**) isolated from raw or roasted *C. tora* on DPPH radical were tested. The DPPH stable radical loses its characteristic purple color when supplied with electrons or hydro- gen ions. The capacity of the tested samples to donate electrons can be estimated from the degree of their loss of color (Blois, 1958). As shown in Table 2, the IC_{50} of the three compounds (**1, 3** and **4**) showed scavenging activity on DPPH radical at concentrations of 5.45, 8.53 and 7.57 μM respectively. These radical scavenging activities were more potent than that of L-ascorbic acid or BHT, which are well known radical scavengers. Among them, nor-rubrofusarin (**1**) exhibited higher scavenging activity on DPPH radical. However, naphtho-α-pyrone (**5**) and angular naphtho-γ-pyrone (**6**) were found to exhibit weak activities even at the higher concentrations.

A comparison of the radical scavenging activity of nor-rubrofusarin and its glucoside, cassiaside, shows that glycosylation by glucose decreased the activity. But this effect was not demonstrated in the case of compounds **3** and **4**, which lack free hydroxyl groups at C-8. This means that hydroxyl

Table 2. The radical scavenging effect of compounds **1-6** on DPPH radical

Sample	50% reduc.(μM)
1	5.45
2	17.36
3	8.53
4	7.57
5	96.23
6	194.62
L-ascorbic acid	11.50
BHT	10.78

[a] Amount required for reduction of DPPH after 30 min.

groups at C-6 and C-8 both seemed to be important for maximal scavenging activity. As nor-rubrofusarin has a resorcinol moiety with an adjacent additional hydroxyl group at C-5, the antioxidative potency of this compound may be attributable to this moiety. Adjacent hydroxyl group at C-5 hydrogen bonds with the keto group and induces maximal radical scavenging potency. Radical scavenging effect of phenolic compounds isolated from natural sources has been widely studied (Yoshida *et al.*, 1989). The radical scavenging potency of phenolic acids are interrelated. These compounds react with the free radicals formed during autoxidation, and generate a new radical which is stabilized by the resonance effect of the aromatic nucleus (Cuvelier *et al.*, 1992). The higher radical scavenging property of resorcinol phenolic acids is probably due to a superior stability of radical derived from resorcinol with the adjacent additional presence of hydroxyl group which hydrogen bonds with the keto group, compared to that of phenoxyl radical (Ruiz-Larrea *et al.*, 1994). The present work indicates that nor-rubrofusarin, as well as its glucoside, cassiaside, and rubrofusarin, as well as its gentiobioside, may be useful for the treatment of oxidative damage.

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