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A New Flavanone, Reflexin, From *Cuscuta reflexa* and Its Selective Sensing of Nitric Oxide

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Abstract

A new compound, reflexin, 5-hydroxy-7-methoxy-6-(2,3-epoxy-3-methylbutyl)-flavanone, is isolated from the stems of *Cuscuta reflexa* along with three other known compounds. This new compound has good potential for application especially in the photoactivity of reflexin. It was found to be sensitive to glutathione, forming a fluorescent product that is utilized for sensing nitric oxide (NO). The lowest detection limit of NO analysis was found to be 0.05 μ*M*.

Index Entries: Reflexin; nitric oxide; *Cuscuta reflexa*; glutathione; nitrosothiol.

Introduction

In a continuation of our chemical investigation *(1,2)* of *Cuscuta reflexa*, Roxb. (family: Convolvulaceae), a time-honored drug in the Indian system of medicine *(3,4)*, in this article we report on the isolation, characterization, and study of the biological properties of a new flavonoid, reflexin, designated as Fig. 1 along with other known compounds, namely *(5–7)* apigenin-7-*o*-glucoside, kaempferol-3-*o*-α-rhamnoside, and myricetin-3-*o*-α-rhamnoside. Several of such compounds are highly photochromic in nature, which led us to investigate whether such compounds could be of use in technological applications based on the measurement of photoactivity.

The detection of nitric oxide (NO) is important for human health because NO is involved in various biological processes such as brain ischemia, neurotransmission, immune regulation, and penile erection *(8–11)*

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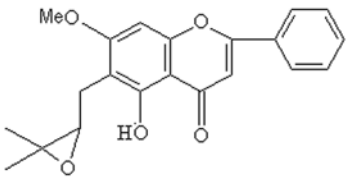

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Fig. 1. 5-Hydroxy-7-methoxy-6-(2,3-epoxy-3-methylbutyl)-flavanone.

and, accordingly, methods for detecting NO are important. Although some methods are available, there still exists a need for a new method for detecting NO mainly owing to the low sensitivity and selectivity of the available methods. Consequently, one of the protocols for detecting NO is based on fluorescence measurements. It has been shown that compounds containing the coumarin group form highly fluorescent products with compounds containing the thiol (–SH) group. Furthermore, it is known that NO forms nitrosothiol with similar thiol derivatives that are not fluorescent. Accordingly, sensitive NO detection could be conducted based on competitive binding of NO to the thiol group and subsequently monitoring the decrease in fluorescence of the resulting product. In the present investigation, while purifying and isolating reflexin, excellent sensitivity of reflexin to glutathione was observed. The reflexin–glutathione complex is fluorescent in nature, having an excitation wavelength of 380 nm and an emission wavelength of 450 nm. Therefore, our goal was to apply reflexin in NO detection. We report herein the sensing of NO utilizing reflexin.

Materials and Methods

Equipment

Melting point was measured on a Perfit melting point apparatus and was uncorrected. Infrared spectra were recorded on a JASCOFT/IR5300 spectrophotometer in KBr and ultraviolet (UV) spectra on a Cary 23 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-300 instrument at 300 MHz. Mass spectra were recorded on a JEOL JMSD300 spectrophotometer. Silica gel was used for column chromatography.

Isolation of Reflexin From C. reflexa

Air-dried and powdered stems (2 kg) were extracted successively with petroleum ether (60–80°C) and ethanol (5 mL) in a Soxhlet apparatus. The extracts were concentrated under reduced pressure.

After rotary evaporation, the ethanol extract (229.0 g) was dissolved in 300 mL of distilled water and extracted successively with petroleum ether (60–80°C), benzene, chloroform, and ethyl acetate. The ethyl acetate fraction (98 g) was concentrated under reduced pressure and chromato-

graphed over a silica gel column using a chloroform-methanol mixture as eluent. Chromatographic separation and extensive preparative thin-layer chromatography (chloroform/methanol 98/2) afforded pure apigenin-7-*o*-glucoside (60 mg), reflexin (10 mg), kaempferol-3-*o*-rhamnoside 3 (76 mg), and myricetin-3-*o*-rhamnoside 4 (80 mg). The new flavonoid, reflexin, was characterized as follows: yellow solid: λ_{max} (MeOH): 250, 262, 300 nm; (MeOH + MeONa): 245, 272 nm; (MeOH + $AlCl_3$): 250, 275, 330, 375 nm; δ_H: 1.16 (6H, br, s, 2Me), 2.62–2.74 (3H, m, $-CH_2-CH$), 3.91 (3H, s, OMe), 6.56 (1H, br, s, H-8), 6.70 (1H, s, H-3), 7.54 (3H, m, H-4', 2H-3',5'), 7.90 (2H, m, 2H-2',6'), 12.95 (1H, s, D_2O exchange, 5-OH); δc: 139.9 (C-4'), 131.3 (C-1'), 129.1 (2C-3',5'), 126.3 (2C-2'6'), 106.1 (C-3), 90 (C-8), 58.2 (C-3''), 67.2 (C-2''), 56.2 (OMe), 42.4 (C-1''), 28.5 (C-5''), 27.8 (C-4''); m/z (rel. in.): 352 [M^+] (60%), 351 (10%), 337 (15%), 324 (2%), 309 (6%), 191 (100%), 105 (20%), 102 (18%); found: [M^+] 352.378 $C_{21}H_{20}O_5$ requires 352.384.

Measurement of NO Concentration

A 0.01 m*M* sodium nitrite solution was made in 0.1 *N* HCl. A varying concentration (15–40 μ*M*) of this solution was added to glutathione solution (50 μ*M*) in 0.1 *N* HCl. The reaction mixture was incubated for 15 min followed by dilution of each nitrosothiol sample with 0.05 *M* phosphate buffer, pH 7.4, containing reflexin. The final concentrations were 0.15–0.40 μ*M* NO, 0.05 μ*M* glutathione, and 0.75 μ*M* reflexin. The fluorescence of each sample was recorded using a spectrofluorometer (excitation wavelength of 380 nm, emission wavelength of 450 nm).

It has been shown that compounds containing the thiol group, such as glutathione, form highly fluorescent products with a compound containing the coumarin group. Furthermore, it is known that NO forms nitrosothiol and the product of nitrosothiol; however, the present investigation was directed only at purely isolated reflexin. Fortunately, we made a very interesting observation on the sensitivity of reflexin toward glutathione. The reflexin–glutathione complex is fluorescent in nature, having an excitation wavelength of 380 nm and an emission wavelength of 450 nm.

Results and Discussion

The ethanolic extract of *C. reflexa* yielded, on repeated chromatographic separation and purification on silica gel, known flavonoids *(12,13)*, in addition to a new flavonoid, reflexin. Reflexin (melting point: 210–217°C; $C_{21}H_{20}O_5$) underwent a color test with magnesium-concentrated HCl. The UV spectral data (λ_{max} = 250, 262, and 300 nm) suggested that it is a flavone bearing a hydroxyl group at C-3 or C-5. The UV spectrum particularly resembled that of tectochrysin *(14)*. Because the compound readily gave a strong ferric chloride chelate reaction and showed a deshielded hydroxyl proton at δ_H 12.90 as well as the characteristic flavone H-3 signal (δ_H 6.60) in the NMR spectrum, it is clear that it is a 5-hydroxyflavone. The fragments m/z 105 (15%) and 102 (18%) in the mass spectrum support the unsubstituted nature of the flavonoid ring B. UV (MeOH + $AlCl_3$) 250,

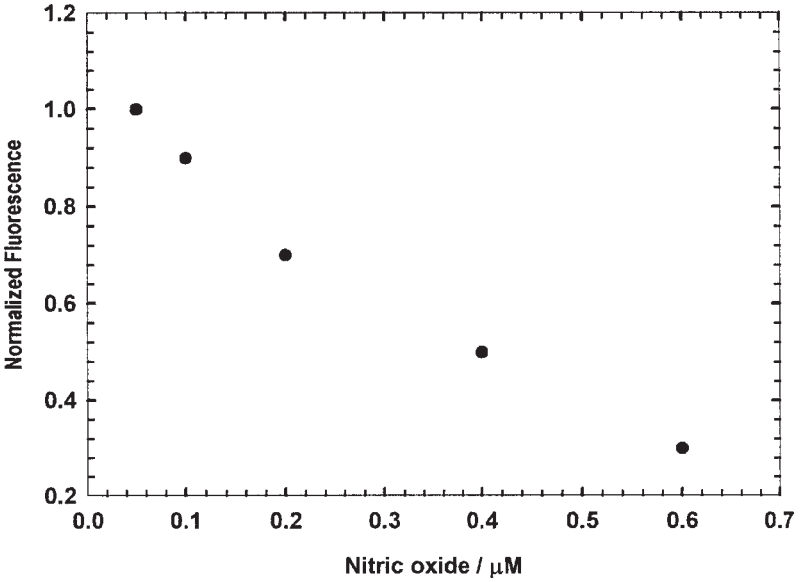

Fig. 2. Calibration curve for analysis of NO.

275, 330, 375 nm. The bathochromic shift of 75 nm of band I from λ_{max}^{MeOH} 300 nm to $\lambda_{max}^{MeOH/AlCl_3}$ 375 nm clearly indicates the presence of an –OH group at C-5. The ^{1}H-NMR spectrum showed signals for one methoxy group (δ_H 3.90), two isolated deshielded protons (δ_H 6.55 and 6.70), and a chelated hydroxyl proton (δ_H 12.90).

Two methyl groups on carbon carrying oxygen come at δ_H 1.16 and Ar-CH_2-CHO group at δ_H 2.62–2.74 (3H, m). These data suggest the presence of a side chain containing an epoxy function *(15,16)*. The epoxy function at C-6 is supported by the appearance of C-8 at δc 90, close to the value as in swertisin *(17)*.

The nitrosothiol of glutathione or cysteine can be made at pH 1.0 by the equimolar addition of NO and thiol compounds (glutathione or cysteine). The nitrosothiol and reflexin product is nonfluorescent at pH 7.0. If a constant concentration of thiol is treated with a varying concentration of NO, the concentration of the free thiol will decrease on increasing the concentration of NO. The addition of these samples containing a constant concentration of thiol with a varying concentration of NO to a constant concentration of reflexin will result in a fluorescent product as a function of the free thiol concentration. The fluorescent intensity of these samples provides quantitative information on the detection of NO, as shown in Fig. 2. The lowest detection limit is 0.05 μ*M*.

Conclusion

We have described a protocol for the isolation and purification of a novel flavonoid (5-hydroxy-7-methoxy-6-[2,3-epoxy-3-methylbutyl]-fla-

vanone) from *C. reflexa*, an Indian medicinal herb. The new flavanone was found to be highly sensitive, forming a fluorescent product with a thiol-containing compound. The analysis of NO was reported based on the activity of this new natural product.

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