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ESTERIFICATION OF 3-NITROPROPANOIC ACID TO GLUCOSE BY SUSPENSION CULTURES OF *CORONILLA VARIA**

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Key Word Index—*Coronilla varia*; Leguminosae; crownvetch; suspension culture; aliphatic nitro compounds.

Abstract—*Coronilla varia* suspension cultures incorporated exogenous 3-nitropropanoic acid into 3-nitropropanoyl-D-glucopyranoses, increasing their levels 600-fold over control values. The resulting levels of 3-nitropropanoyl-D-glucopyranoses were still lower than those in *C. varia* plants but were high enough for isolation of hiptagin (1,2,4,6-tetra(3-nitropropanoyl)-β-D-glucopyranose), coronarian and cibarian. Structural assignments were partially made on the basis of 360 MHz ^{1}H NMR spectra.

INTRODUCTION

During a study of the regeneration of *Coronilla varia* L. plants from callus tissue cultures [1], we detected 3-nitropropanoyl-D-glucopyranoses in callus at much lower levels than those reported in aerial parts of *C. varia* plants [2]. Concentrations of 3-nitropropanoyl-D-glucopyranoses in *C. varia* suspension cultures were increased by adding 3-nitropropanoic acid (NPA) to the culture media. This paper describes the isolation and identification by ^{1}H NMR spectroscopy of three of these compounds, cibarian, coronarian and hiptagin. Cibarian and coronarian, as well as other 3-nitropropanoyl-D-glucopyranoses, have been isolated from *C. varia* cv. Penngift [2, 3]. Hiptagin has not been previously reported in *C. varia* but has been isolated from other plants [4]. No 3-nitropropanoyl-D-glucopyranoses have been previously isolated from suspension cultures.

RESULTS AND DISCUSSION

To increase the 3-nitropropanoyl-D-glucopyranose levels in *C. varia* suspension cultures, we supplemented the medium with 0.01 M NPA. LH-20 chromatography of acetone extracts from cells incubated for 72 hr yielded three fractions. Fraction I contained **1**, **2** and **3**, compounds not previously isolated from *C. varia*. Fraction II contained the triesters coronillin (**4**), karakin (**5**) and corollin (**6**); fraction III contained NPA and the diesters coronarian (**7**) and cibarian (**8**). Insufficient material precluded isolation of **1** and **2** or identification of **4**, **5** and **6** by means other than TLC. The ^{1}H NMR spectra of **7** and **8** were consistent with published data for coronarian [2] and cibarian [5], respectively.

Table 1. ^{1}H NMR data for **3** and its acetylated derivative, **9**

Proton	3	9
H-1	5.82 *d* ($J_{1,2}$ = 8.3)	6.02 *d* ($J_{1,2}$ = 8.3)
H-2	5.03 *dd* ($J_{2,3}$ = 9.5)	5.16 *dd* ($J_{2,3}$ = 9.8)
H-3	4.08 *t* ($J_{3,4}$ = 9.4)	5.52 *t* ($J_{3,4}$ = 9.6)
H-4	5.06 *t* ($J_{4,5}$ = 10)	5.24 *t* ($J_{4,5}$ = 9.6)
H-5	4.05 *ddd* ($J_{5,6a}$ = 2.2)	4.28 *m*
H-6a	4.19 *dd* ($J_{6a,6b}$ = 12.4)	4.28 *m*
H-6b	4.29 *dd* ($J_{5,6b}$ = 5.2)	4.28 *m*

Spectra were determined at 360 MHz (**3**) and 200 MHz (**9**) in Me_2CO-d_6. Chemical shifts are in δ; J values are in Hz.

Analysis of the ^{1}H NMR data (Table 1) indicated that **3** was hiptagin, 1,2,3,4-tetra-(3-nitropropanoyl)-β-D-glucopyranose. A low-field doublet at δ5.82 (1 H) denoted esterification at the anomeric carbon, while the coupling constant ($J_{1,2}$ = 8.3 Hz) indicated that **3** was a β-anomer. The C-6 methylene proton signals were observed at δ4.29 and 4.19 as two doublets of doublets ($J_{6a,6b}$ = 12.4 Hz, $J_{5,6b}$ = 5.2 Hz, $J_{5,6a}$ = 2.2 Hz, 2 H), the low-field position indicating esterification. As with 1,4,6-tri-(3-nitropropanoyl)-β-D-glucopyranose, the H-6a proton signal is upfield relative to the H-6b proton signal, indicating shielding by an ester carbonyl group at C-4 [6]. This is in contrast to 3-nitropropanoyl-β-D-glucopyranoses unesterified at C-4, where H-6a is downfield from H-6b due to deshielding by the C-4 hydroxyl group. The low-field position of the multiplet at δ5.04 (2H) indicated esterification. Irradiation of the doublet resonance at δ5.82 collapsed the multiplet to a doublet at δ5.03 and triplet at δ5.06, which were assigned to H-2 and H-4, respectively. A triplet (1H) at δ4.08 and a quartet of doublets (1H) at δ4.05 were assigned to the protons at unesterified positions because of the high-field position. Decoupling indicated that these were H-3 and H-5, respectively; irradiation of the H-6a resonance collapsed the quartet of doublets to a quartet. Multiplets at δ4.79 (8H) and 3.07 (8H) were assigned to CH_2-NO_2 and CH_2 CO-methylene groups of 3-nitropropanoyl residues. Decoupling experiments at major resonances gave results consistent with these assignments, which are in agreement with partial explications of the 60 MHz spectra of hiptagin [7, 8].

Acetylation of **3** yielded **9**. The signal for H-3 of **9** was found at δ5.52, compared to δ4.08 for **3**. The large downfield shift of only one proton signal indicated

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acetylation of a free hydroxyl group at that position and supported the assigned structure.

Colorimetric determination of NO_2 indicated 3.08, 0.96 and 5.7 mmol of NO_2 in fractions I, II and III, respectively accounting for 49% of the 20 mmol NPA added. Fraction III contained 1.11 mmol of free NPA. Thus, 43% of the added NPA was incorporated into aliphatic nitro compounds.

The aerial parts of *C. varia* contain NPA in the form of glucose esters at levels as high as 420 μmol NO_2/g dry wt, as measured by aliphatic NO_2 [2]. We found levels between 63 and 164 μmol NO_2/g fr. wt in the aerial parts of *C. varia*. The levels in cultured tissue were far lower. Prior to the addition of NPA, the suspension culture used in this study contained 0.026 μmol NO_2/g fr. wt of cells. Suspension cultures incorporated exogenous NPA into 3-nitropropanoyl-D-glucopyranoses to the extent of 15.6 μmol NO_2/g fr. wt, a 600-fold increase. Thus, the levels of 3-nitropropanoyl-D-glucopyranoses in *C. varia* appear to be limited by the availability and biosynthesis of NPA. Hiptagin has not been isolated from *C. varia*, although it has been reported in other plants [4]. Other than hiptagin, 3-nitropropanoyl-D-glucopyranoses produced in culture upon addition of exogeneous NPA are qualitatively the same as those found in plants, but relative amounts differ. In culture, the major esters are cibarian and hiptagin, whereas in plants, cibarian and the triesters karakin and coronillin predominate. This may be due to a difference in cultivars. Suspension cultures used in this study were derived from *C. varia* cv. Chemung. Previous isolations of 3-nitropropanoyl-D-glucopyranoses were done with cv. Penngift [2, 3].

EXPERIMENTAL

^{1}H NMR spectral data are reported as δ values (ppm from internal TMS in Me_2CO-d_6) and were run at 200 MHz (**9**) and 360 MHz (**3**, **7**, and **8**). Greiss Ilosvay colorimetric determinations of aliphatic NO_2 compounds were done in accordance with ref. [9]. TLC identification of **4** **8** was in accordance with refs. [2, 6].

Suspension culture. *C. varia* callus tissue initiated from hypocotyl and maintained on D20K2 solid medium [1] was transferred to liquid D20K2 medium (205 g tissue/20 × 125 ml flasks, 25 ml medium/flask). The medium vol. was doubled 7 and 14 days after initiation of suspension cultures, resulting in 40 flasks, each with *ca* 50 ml suspension culture. At 21 days, 20 mmol NPA was neutralized with 1.0 M NaOH, filter-sterilized, added to cultures at 0.5 mmol per flask, and incubated for 72 hr. Cells were collected by vacuum filtration.

Isolation. Pooled Me_2CO extracts of collected cells (550 g) were reduced to an aq. phase, which was extracted with hexane, followed by EtOAc. EtOAc extracts of the aq. phase and the culture filtrate were combined and chromatographed in Me_2CO on Sephadex LH-20, yielding fractions I, II and III. Fraction I yielded 183 mg **3** upon crystallization from Me_2CO-CCl_2H_2. Fraction III yielded 292 mg **8** (crystallization from Me_2CO–CCl_2H_2) and 40 mg **7** by silica gel CC (CCl_2H_2–EtOAc-HCO_2H, 35:64:1).

Identification. Acid hydrolysis of **3** yielded compounds co-chromatographing with NPA (silica gel TLC, $CHCl_3$–EtOAc–HCO_2H, 10:89:1) and glucose (cellulose TLC, pyridine–EtOAc–H_2O–HOAc, 36:36:21:7). (^{1}H NMR data of **3** are inconsistent with the presence of any hexopyranose other than glucose; coupling constants indicate that all ring protons are axial.) Transesterification with MeOH, EtOH and *iso*-PrOH produced compounds co-chromatographing on silica gel TLC (hexane-EtOAc–HCO_2H, 60:40:1) with authentic Me-, Et- and 2-propanyl 3-nitropropanoate, respectively. Colorimetric determination of NO_2 indicated 4 mol NO_2/mol of **3**. Acetylation of 35 mg **3** in 0.5 ml HOAc and 0.5 ml Ac_2O yielded 28 mg **9** upon addition of 4 ml H_2O and recrystallization from Me_2CO-H_2O.

Analysis. Mp uncorr. Hiptagin (**3**): mp 112.5 113.5° (lit. values 110° [10] and 102 104° [7, 8]). (Found: C, 36.72; H, 3.71; N, 9.4. $C_{18}H_{24}N_4O_{18}$ requires: C, 36.98; H, 4.11; N, 9.59%.)

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