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ALKYLCITRATE-DERIVED METABOLITES FROM *ASPERGILLUS NIGER*

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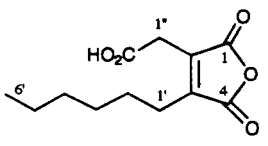
ABSTRACT.—The isolation and identification of a new metabolite, 2-methylene-3-(6-hydroxyhexyl)-butanedioic acid [**3**], and the known metabolites, 2-carboxymethyl-3-hexyl-maleic acid anhydride [**1**], and 2-methylene-3-hexyl-butanedioic acid [**2**], is described from a strain of *Aspergillus niger*. The biosynthetic implications of the co-occurrence of **1** and **2** are discussed.

As part of a study on the identification of plant-growth regulating substances produced by fungi, we have examined the secondary metabolites of an isolate of *Aspergillus niger* van Tieghem (Deuteromycotina). We now report on the isolation and identification of three major metabolites that accumulate in liquid cultures of this fungus: 2-carboxymethyl-3-hexyl-maleic acid anhydride [**1**], 2-methylene-3-hexyl-butanedioic acid [**2**], and 2-methylene-3-(6-hydroxyhexyl)-butanedioic acid [**3**]. Although metabolites **1** and **2** have previously been found individually in different isolates of *A. niger* (1,2), their formation in the same isolate is interesting from a biosynthetic point of view. Metabolite **3**, presumably a catabolite of **2**, has not been described previously.

The *A. niger* isolate was cultured in a liquid medium, either potato dextrose broth or Sabouraud broth. After removal of the mycelium, the liquid medium was extracted with EtOAc and the organic extract was separated by chromatography to yield three compounds. The metabolites could be detected on a tlc plate (Si gel) by coating the plate with a solution of bromocresol purple in EtOH, after which they appeared as yellow spots on a purple background.

Spectroscopic properties of the first metabolite indicated the presence of a 2,3-dialkyl maleic anhydride group (ν max 1770 cm^{-1}; δ_C 165.1, 2s; 148.0, s; 135.4, s), substituted by a carboxymethyl group (ν max 1720 cm^{-1}; δ_C 173.4, s; δ_H 3.59, s) and a hexyl group (δ_C, 13.8, q; and five t between δ_C 22 and 31). Structure **1** was inferred from this information and confirmed by comparison of spectroscopic data with that reported in the literature (1). Treatment with cyclohexylamine gave a crystalline triammonium salt (1). Although **1** is an oil at room temperature, it solidifies on storage at $-10°$.

The second metabolite isolated was assigned structure **2** (hexylitaconic acid) on the basis of its spectroscopic properties which clearly showed a 2-methylene-3-substituted butanedioic acid structure (δ_C, 179.6, s; 171.6, s; 137.3, s; 129.7, t; δ_H, 3.40, t; 5.83, s; and 6.52, s). This was confirmed by comparison with spectroscopic data reported for this compound (2). The optical rotation ([α]D +9°) did not increase to that reported for **2** ([α]D +15.3°) (2) despite repeated purification attempts.



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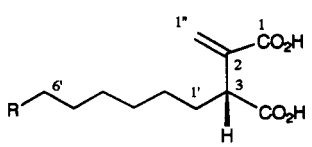


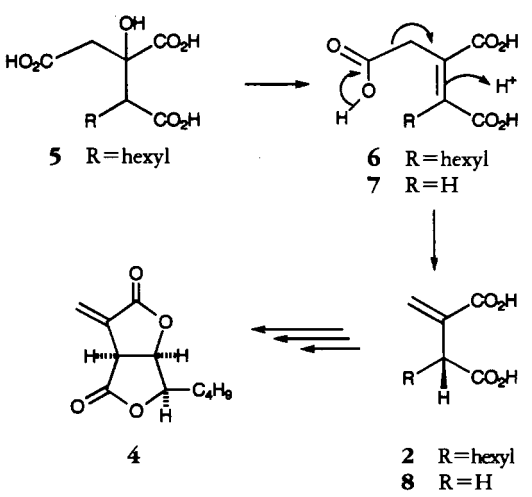
2 R=H
3 R=OH

The third metabolite showed similar spectroscopic properties to those of **2** with the exception that, in the nmr spectra, the signals for the terminal methyl group (δ_C 14.0, q; δ_H 0.85, t) in **2** were replaced by those attributable to a primary alcohol (δ_C 62.9, t; δ_H 3.54, t). The optical rotation of **3** ([α]D +10°) was similar to that of **2** ([α]D +9°) indicating that both have the same configuration. The absolute configuration of (+)-hexylitaconic acid has been assigned as *R*-on the basis of a very weak positive Cotton effect at 252 nm in the cd spectrum of the α-methylene-γ-lactone derived from **2** (3). However, the fact that 3*R*-hexylitaconic acid ([α]D +9-15.3°, MeOH) and the 3*R*-(15-oxohexadecanyl)-derivative ([α]D −5.9°, MeOH) (4), differing only at a site far removed from the single asymmetric carbon atom, show opposite signs of rotation merits further investigation.

The biosynthesis of hexylitaconic acid [**2**] has been studied indirectly in work on the biosynthesis of canadensolide [**4**] and related metabolites from *Penicillium canadense* (5-7). It was found that **2**, arising from hexylcitric acid [**5**], was efficiently incorporated into canadensolide and its congeners. The production of **1** and **2** in the same isolate points to a biogenetic relationship between the two metabolites and suggests the possibility that **1** (or its corresponding diacid [**6**]) is derived from hexylcitric acid [**5**] and is a likely precursor of hexylitaconic acid [**2**]. This transformation has a precedent in the conversion of *cis*-aconitic acid [**7**] into itaconic acid [**8**] in *A. terreus* (8). However, the latter reaction shows a point of sterochemical distinction in that addition of hydrogen occurs from the 2-*si*, 3-*re* face of *cis*-aconitic acid (numbering as in **1**). For a similar formation of (+)-hexylitaconic acid [**2**], the addition of H^+ must occur from the opposite face of the corresponding double bond (2-*re*, 3-*si* face). (+)-Hexylitaconic acid has the appropriate configuration (*R*) to act as a precursor of the canadensolides (e.g., **4**).

The production of **1–3** was investigated under two different growth conditions. Higher yields of **1** were obtained if the fungus was grown on a nutrient-poor medium (potato dextrose broth, 1/5 normal concentration) in still cultures. Better yields of **2** and **3** were obtained when the fungus was grown in shake cultures on a Sabouraud liquid medium at 25° over six days. It is worth noting that 74 mg of **2** were obtained from 5.2 liters of *A. niger* K-88 cultivated on Czapek-Dox medium over 10 days (2). In our case, the best yield obtained was 100 mg liter^{-1}, representing a seven-fold increase.

(+)-Hexylitaconic acid [**2**] has been shown to stimulate the root growth of



lettuce seedlings, at concentrations of 2 to 20 ppm, to a maximum of 250% relative to controls (2). Growth of rice shoots and roots were promoted at the same concentrations. Higher concentrations (100–500 ppm) inhibited the growth of shoots and roots of both plants. These effects mimic the action of indole acetic acid (9).

In the present work, compounds **1**–**3** did not significantly inhibit the growth of three fungal plant pathogens, *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia solani*, and *Phytophthora cinnamomi*, at concentrations up to 100 ppm in the antibiotic disk assay.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Experimental details have been recorded previously (10). Si gel 100, C-18 reversed phase (Fluka) was used for reversed-phase chromatography. Detection of metabolites on tlc plates (Si gel) was achieved by coating the plates with a solution of bromocresol purple (4 g in 50% aqueous MeOH, pH 10). The *A. niger* isolate (accession code: *A. niger* 5, no. 22) was obtained from the fungal culture collection at the Department of Botany, University of Western Australia.

GROWTH OF *A. NIGER*.—The organism was cultured in two media; (a) potato dextrose broth (PDB) at 1/5 (5 g liter^{-1}) of the normal concentration, and (b) Sabouraud broth (40 g dextrose, 10 g peptone in 1 liter of H_2O adjusted to pH 5.6). In the first case, the inoculated culture was allowed to stand at 25°. Mycelial growth was slow and, typically, production of metabolites reached its maximum after 4 weeks. When the fungus was grown on Sabouraud broth and the culture was shaken at 25°, equivalent amounts of metabolites were produced after an incubation period of 6 days.

ISOLATION AND SEPARATION OF METABOLITES.—The culture broth (1 liter) from a 9-week-old sporulating culture of *A. niger*, grown on 1/5PDB, was extracted with EtOAc, the organic layer dried over $MgSO_4$, and the solvent was removed under vacuum. The oily extract (422 mg) was chromatographed on Si gel 60 (vlc) with gradient elution from petroleum ether to CH_2Cl_2-EtOAc (3:1) to give 3 fractions: **1** (139 mg), **2** (67 mg), and **3** (25 mg). Similar extraction of a 6-day-old culture, grown on Sabouraud broth (2 liters), yielded 580 mg of crude extract. Separation of a portion (150 mg) of this extract was achieved by reversed-phase silica chromatography and gradient elution from H_2O-MeOH (1:1) to MeOH. In this way, three fractions were obtained which, in order of elution, corresponded to **3** (31 mg), **1** (4 mg), and **2** (40 mg). Mixed fractions of **1** and **2** were also obtained. The fortuitous observation that **2** is selectively dissolved in $CHCl_3$ in preference to **1** allowed a facile separation of the two metabolites. Acidification of the broth left after the first extraction and re-extraction with EtOAc gave a further quantity (201 mg) of crude extract containing **1** and **2**.

2-Carboxymethyl-3-hexyl maleic anhydride [**1**].—Oil that solidified when stored at −10°; R_f 0.68 (Si gel; diisopropyl ether-EtOAc, 3:7 with 2 drops of HOAc). Ir ν max 3600–3400, 1770, 1720 cm^{-1}; ^{1}H nmr (300 MHz, $CDCl_3$) δ 0.88 (3H, t, J=6.7 Hz, H_3-6′), 1.3–1.6 (8H, H-2′–H-5′), 2.51 (2H, dd, J=7.6 and 7.9 Hz, H_2-1′), 3.51 (2H, s, H-1″), 10.52 (1H, s, OH); ^{13}C nmr (75.5 MHz, $CDCl_3$) δ 13.8 (q, C-6′), 22.3 (t), 24.8 (t), 27.4 (t), 29.0 (t), 29.0 (t), 31.1 (t), 135.4 (s), 148.0 (s), 165.1 (2s, C-1, C-4), 173.4 (s, C-2″); eims *m/z* 222 (M^+−18) (2), 194 (8), 170 (13), 166 (13), 152 (66), 140 (10), 126 (76), 125 (15), 124 (23), 44 (50), 43 (100).

2-Methylene-3-hexylbutanedioic acid (hexylitaconic acid) [**2**].—Gum, R_f 0.5 (Si gel, diisopropyl ether-EtOAc, 4:6 and a drop of HOAc); [α]D +9° (c=0.8, MeOH). Ir ν max 3600–3400, 1770, 1720 cm^{-1}. The nmr and ms parameters corresponded closely to those in the literature (2).

2-Methylene-3-(6-hydroxyhexanyl)-butanedioic acid [**3**].—Gum, R_f 0.2 (Si gel, diisopropyl ether-EtOAc, 4:6 and a drop of HOAc); [α]D +10° (c=0.4, MeOH). Ir ν max 3600–3400, 1770, 1720 cm^{-1}; ^{1}H nmr (300 MHz, MeOH-d_4) δ 1.3 (6H, s), 1.45 (2H, m), 1.65 (2H, s), 1.85 (1H, m), 3.45 (1H, t, J=7.0 Hz, H-3), 3.54 (2H, t, J=7.0 Hz, H_2-6′), 5.70 (1H, s, H-1″a), 6.30 (1H, d, J=1 Hz, H-1″b); ^{13}C nmr (75.5 MHz, $CDCl_3$) δ 26.7 (t, C-4′), 28.6 (t, C-3′), 30.3 (t, C-2′), 32.2 (t, C-1′), 33.5 (t, C-5′), 48.3 (d, C-3), 62.9 (t, C-6′), 126.7 (t, C-1″), 141.2 (s, C-2), 169.9 (s, C-1), 177.33 (s, C-4); eims *m/z* 212 (M^+−18) (1), 199 (M^+−31) (5), 182 (3), 166 (16), 152 (11), 149 (12), 148 (13), 143 (15), 138 (13), 130 (46), 129 (58), 112 (95), 95 (30), 84 (41), 55 (100), 43 (70).

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