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NOTE

Studies on pyranonigrins–isolation of pyranonigrin E and biosynthetic studies on pyranonigrin A

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Pyranonigrins A, B, C, D and S have been shown to be secondary metabolites produced by *Aspergillus niger LL*-LV3020 when grown on a solid culture.[1,2] The 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of pyranonigrin A was also reported in previous studies.[3,4] Pyranonigrins possess unique structures (Figure 1); however, biosynthetic studies on pyranonigrins have not been conducted.

We recently found that an acetone extract of *A. niger* NBRC5374 grown on potato dextrose agar shows a potent 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity and isolated a new pyranonigrin derivative (pyranonigrin E) in addition to pyranonigrins A and S as the antioxidative compounds. In this study, we report the isolation, structural determination and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of pyranonigrin E. We also investigated the biosynthesis of pyranonigrin A in feeding experiments using sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1,2-$^{13}C_2$] acetate and [1-^{13}C]glycine, and showed that it was composed of four units of acetate and one unit of glycine.

To produce and isolate pyranonigrin E, 1 ml of the *A. niger* NBRC5374 spore solution (1×10^6 spores per ml) was dropped on the potato dextrose agar agar (100 ml) in an Erlenmeyer flask and spread all over its surface. Twenty flasks were cultured statically for 48 h at 30 °C (spore formation started after 30 h and became stationary after 48 h).

Acetone (100 ml) was added to the flask cultured for 48 h and agar with mycelium and spores was cut into small pieces using a scoopula. The agar pieces and acetone from 20 flasks were combined in a 3-l beaker and extracted by stirring for 1 hr at room temperature. The solution was filtered and concentrated to a small volume (50 ml) to remove acetone and was then applied to a Diaion HP-20 column (i.d. 20 mm × 200 mm, Mitsubishi Chemical, Tokyo, Japan). The HP-20 column was washed with distilled water (200 ml) and eluted with MeOH (200 ml). The MeOH eluate was concentrated to dryness (250.8 mg) and subjected to Toyopearl HW-40F column chromatography (i.d. 20 mm × 800 mm, solvent: CH_2Cl_2-MeOH (1:1), Tosoh, Tokyo, Japan). Fractions containing the pyranonigrins were collected and concentrated to dryness (64.3 mg). After washing with *n*-hexane (5 ml), the precipitate (48.5 mg) was chromatographed on preparative octadecyl silane HPLC (CapselPak SG (i.d. 10 mm × 250 mm, Shiseido, Tokyo, Japan), solvent: 30% MeOH, flow rate: 3.0 ml min^{-1}, detect: 210–500 nm (potato dextrose agar)). Using this chromatography, pure pyranonigrin A, S and a new derivative (pyranonigrin E) were eluted at 10.9 min (12.2 mg), 15.7 min (0.7 mg) and 32.0 min (0.5 mg), respectively. Pyranonigrin A and S were identified by the comparison of ^{1}H, ^{13}C NMR and ESI-MS data with those of previously reported data.[2] This is the first report on the production of pyranonigrins by *A. niger* NBRC5374.

The molecular formula of pyranonigrin E was determined to be $C_{11}H_{11}NO_4$ by HR-APCIMS analysis, which implies one more methylene than pyranonigrin S. The ^{1}H NMR signals of pyranonigrin E were closely related to those of pyranonigrin S, whereas doublet methyl (δ_H 1.36) and quartet sp^3 methine (δ_H 4.56) signals, which coupled to each other, were observed only in pyranonigrin E, and the singlet sp^3 methylene (δ_H 4.31, H-7) observed in pyranonigrin S disappeared in pyranonigrin E. Consistent with the molecular formula of pyranonigrin E and the differences in the ^{1}H NMR signals between pyranonigrin E and S, pyranonigrin E was speculated to be 7-CH_3 pyranonigrin S. The structure was confirmed by the ^{1}H–^{13}C long-range couplings observed from δ_H 4.56 (H-7) to δ_C 165.1 (C-5) and δ_C 177.4 (C-7a) and from δ_H 8.27 (NH-6) to δ_C 49.6 (C-7) and δ_C 177.4 (C-7a) observed in the heteronuclear multiple bond correlation experiment on pyranonigrin E. Pyranonigrin E was a novel compound.

SPECTRAL DATA FOR PYRANONIGRIN E

UV (MeOH) λ_{max} nm (ε) 210 (18 000), 250 (8800), 315 (13 000). HR-APCIMS calculated for $C_{11}H_{12}NO_4$ [$(M+H)^+$], 222.07663; found 222.07681. ^{1}H NMR (DMSO-d_6) δ: 1.36 (d, J = 6.4 Hz, 3H, H-1″), 1.92 (d, J = 7.0 Hz, 3H, H-3′), 4.56 (q, J = 6.4 Hz, 1H, H-7), 6.42 (dq, J = 7.0, 16.5 Hz, 1H, H-2′), 6.58 (d, J = 16.5 Hz, 1H, H-1′), 8.27 (s, 1H, NH-6). ^{13}C NMR (DMSO-d_6) δ: 17.1 (C-1″), 18.2 (C-3′), 49.6

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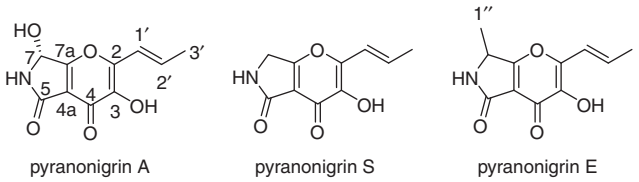

Figure 1 The structures of pyranonigrins A, S and E.

Table 1 Incorporation of ^{13}C-labeled precursors into pyranonigrin A

		Relative enrichments			
No.	*δ ^{13}C (p.p.m.)*	*[1−^{13}C] acetate*	*[2−^{13}C] acetate*	*[1,2−$^{13}C_2$] acetate*	*[1−^{13}C] glycine*
2	145.8	5.03	0.92	1.22 (67.4)[a]	0.92
3	142.1	1.25	1.54	1.25 (57.0)	1.00
4	168.9	7.82	1.00[b]	1.06 (57.0)	1.91
4a	111.6	1.00[b]	1.37	1.00[b] (65.2)	1.00[b]
5	164.9	6.67	0.80	1.17 (65.2)	0.92
7	75.0	1.27	2.70[c]	1.33 (53.5)	1.91
7a	175.1	8.25[c]	1.73[c]	1.54 (53.5)	39.2
1′	118.9	1.36	1.40	1.03 (67.4)	0.91
2′	131.5	7.29	1.25	1.05 (42.4)	1.29
3′	18.6	2.38	1.31	1.35 (42.4)	1.00

The ^{13}C enriched signals were underlined.
[a]J_{cc}-values are in parentheses (Hz).
[b]Relative enrichments were normalized to peak intensities for these signals.
[c]Indirect enrichment was observed.

(C-7), 111.9 (C-4a), 119.2 (C-1′), 131.0 (C-2′), 142.1 (C-3), 145.8 (C-2), 165.1 (C-5), 168.9 (C-4), 177. (C-7a). $[\alpha]_D^{22}$ (*c* 0.05, MeOH) −43.6°.

The 2,2-diphenyl-1-picrylhydrazyl radical scavenging activities[3] of pyranonigrins A, S and E were examined, and their IC_{50} values were 110 μM (pyranonigrin A), 52 μM (pyranonigrin S) and 156 μM (pyranonigrin E), respectively. (α-tocopherol: IC_{50} 27 μM) All the compounds showed moderate scavenging activities. Further biological tests on pyranonigrins were prevented by the lack of materials.

To study the biosynthetic origins of the carbons in pyranonigrin A, we performed feeding experiments of ^{13}C precursors (sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1,2-$^{13}C_2$] acetate and [1-^{13}C]glycine). The production and isolation of pyranonigrin A was carried out in the same manner as those of pyranonigrin E. In the feeding experiments, a ^{13}C compound solution (0.1 g in 1 ml sterilized water) was gradually dropped onto the mycelium using a sterilized Pasteur pipette for each flask at 24 h (10 flasks were used for each feeding experiment). After adding the ^{13}C compounds, the flasks were cultured for an additional 24 h ([1-^{13}C]glycine) or 48 h (sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate and sodium [1,2-$^{13}C_2$] acetate) until the spore formation became stationary. Throughout the feeding experiments, the yields of pyranonigrin A were 5.2–9.4 mg, whereas the yields of pyranonigrins S and E were <0.5 mg. Thus, we investigated the biosynthetic carbon origins of pyranonigrin A using ^{13}C NMR spectra.

The ^{13}C-enriched ratio in the feeding experiment of each precursor and ^{13}C–^{13}C coupling constants observed in the feeding experiment of [1,2-$^{13}C_2$] acetate are listed in Table 1. As shown in Table 1, the enrichment of carbons C-2, 4, 5, 2′ by [1-^{13}C]acetate and C-3, 4a, 7, 1′, 3′ by [2-^{13}C]acetate was observed. In addition, the analyses of ^{13}C–^{13}C coupling constants observed in [1,2-$^{13}C_2$] acetate feeding experiment proved which carbons were derived from intact acetate units.

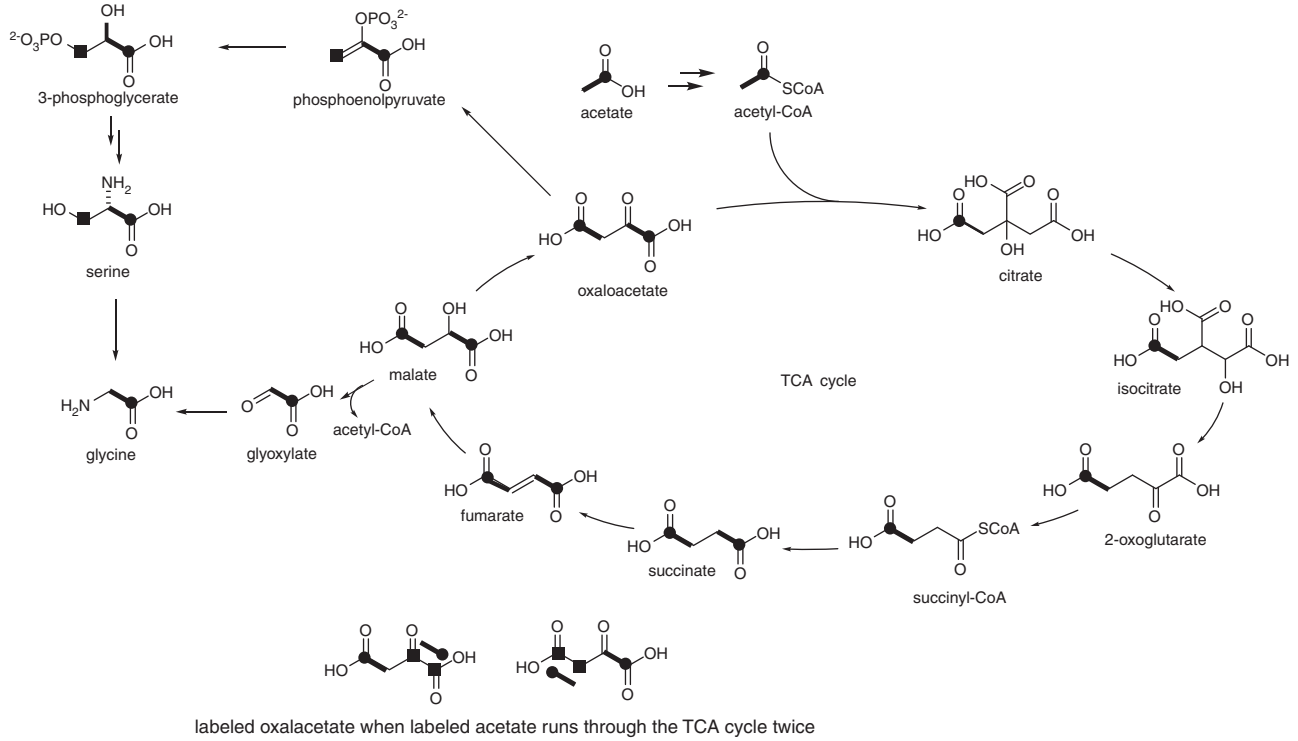

Figure 2 Proposed metabolic pathway from acetate to glycine.

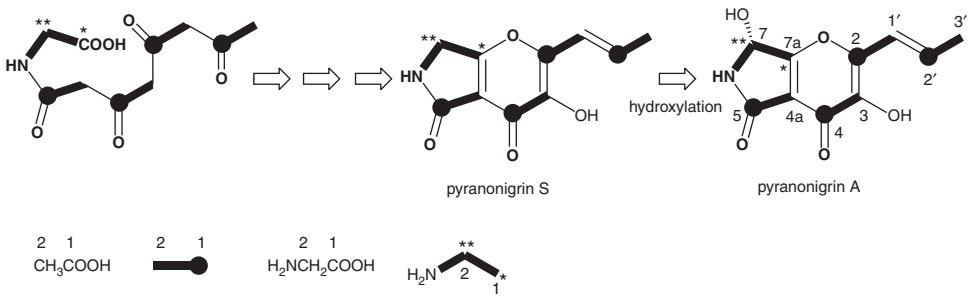

Figure 3 Proposed biosynthetic pathway of pyranonigrins A and S.

These results indicate the incorporation of acetate into a tetraketide chain (C-3′ to C-5).

A very high level enrichment of carbon C-7a (Table 1) was observed in the [1-^{13}C]glycine feeding experiment, which indicated the intact incorporation of glycine into the N-6, C-7 and C-7a positions. Further, the enrichment of carbons C-7a by [1-^{13}C]acetate and C-7 by [2-^{13}C]acetate was also observed (Table 1). Connection between acetate metabolism and amino acid metabolism can be found in the tricarboxylic acid cycle. Thus, these enrichments were reasonably explained by the metabolism of acetate to glycine through the route (acetate→citrate→ →oxaloacetate→phosphoenolpyruvate→serine→glycine) as shown in Figure 2. In another possibility, malate synthase is capable of converting (*S*)-malate from the tricarboxylic acid cycle into glyoxylate and acetyl-CoA.[5] Glyoxylate is well-known precursor of glycine (Figure 2). In both cases, the intact acetate could be incorporated into glycine. The enrichment of carbon C-7a by [2-^{13}C acetate] could be through oxaloacetate, which were constructed when labeled acetate was run through the tricarboxylic acid cycle twice (Figure 2).

The results obtained from the above feeding experiments with ^{13}C-labeled precursors demonstrated that one mole of pyranonigrin A was biosynthesized from four moles of acetate and one mole of glycine. Hence, the origin of all the carbon atoms of pyranonigrin A was established and has been summarized as shown in Figure 3.

In the feeding experiment with [1-^{13}C]glycine, we successfuly isolated pyranonigrin S (0.3 mg). In the ^{13}C NMR of this pyranonigrin S, labeled glycine was highly incorporated into pyranonigrin S similar to pyranonigrin A (data not shown). Considering the strucrures of pyranonigrins A and S, the biosynthetic pathway to pyranonigrins A and S can be considered as shown in Figure 3. Pyranonigrin S may be hydroxylated enzymatically to pyranonigrin A.

We could not measure the ^{13}C NMR spectrum of pyranonigrin E in each feeding experiment due to its low productivity. Therefore, the discussion on the biosynthesis of pyranonigrin E is difficult. We suppose the carbon origins, except for N-6, C-7, C-7a and C-1″, are likely to be common to those of pyranonigrin A, and the exception part (N-6, C-7, C-7a, and C-1″) may be derived from alanine or serine.[6] This part may also come from a glycine (N-6, C-7, C-7a) and methionine (C-1″).[7]

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