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CAROTENOID GLUCOSIDE MYCOLIC ACID ESTERS FROM THE NOCARDIOFORM ACTINOMYCETES, *RHODOCOCCUS RHODOCHROUS*

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Key Word Index—*Rhodococcus rhodochrous*; Nocardioform actinomycetes; carotenoid; mycolic acid; β-D-glucoside; carotenoid glucoside mycolic acid ester.

Abstract—Six carotenoids, including carotenoid glucoside fatty acid esters, have previously been identified in *Rhodococcus rhodochrous* RNMS1. From its colour mutant, two carotenoids with apparently very low polarities on reversed phase HPLC were found to be carotenoid glucoside mycolic acid esters. From spectroscopic and chemical evidence, their structures were determined as 1′-[(6-*O*-mycoloyl-β-D-glucopyranosyl)oxy]-1′,2′-dihydro-β,ψ-caroten-4-one and 1′-[(6-*O*-mycoloyl-β-D-glucopyranosyl)oxy]-1′,2′-dihydro-β,ψ-carotene. Their mycolic acid residues were similar to those of the cellular mycolic acids. In all strains of *R. rhodochrous* tested, these carotenoid derivatives were found. Such carotenoid derivatives have never been reported in other organisms.

INTRODUCTION

Rhodococcus rhodochrous forms red to orange colonies [1]. The strain RNMS1 is due to carotenoid pigments. Six carotenoids have already been identified [2, 3]; a monocyclic carotenoid with a tertiary hydroxyl group at C-1′ (carotenoid **B**; 1′,2′-dihydro-β,ψ-caroten-1′-ol), a keto derivative of carotenoid **B** (carotenoid **K**; 1′-hydroxy-1′,2′-dihydro-β,ψ-caroten-4-one), their β-D-glucosides at C-1′ (carotenoid **B-G**; 1′-[(β-D-glucopyranosyl)oxy]-1′,2′-dihydro-β,ψ-carotene and carotenoid **K-G**; 1′-[(β-D-glucopyranosyl)oxy]-1′,2′-dihydro-β,ψ-caroten-4-one), and their fatty acid monoesters at C-6 of the D-glucoside moiety (carotenoid **B-G-FA**; 1′-[(6-*O*-acyl-β-D-glucopyranosyl)oxy]-1′,2′-dihydro-β,ψ-carotene and carotenoid **K-G-FA**; 1′-[(6-*O*-acyl-β-D-glucopyranosyl)oxy]-1′,2′-dihydro-β,ψ-caroten-4-one). The fatty acid composition of the carotenoid glucoside fatty acid esters is different from that of the total cellular lipids.

R. rhodochrous belongs to Nacardioform actinomycetes, which produce nocardio mycolic acids in the cell wall [1]. In RNMS 1, the total carbon atoms of the cellular mycolic acids range from C_{36} to C_{50}. The main carbon atoms of the β-chain are C_{26} and C_{28} with none or one double bond, and those of the α-chain are C_{14} and C_{16} [4].

In this study, new carotenoid derivatives in *R. rhodochrous* RNMS 1 were isolated and identified as carotenoid glucoside mycolic acid esters. Although the carotenoid glucoside fatty acid monoesters have been found in several species of bacteria [5, 6], this is the first report on carotenoid glucoside mycolic acid esters [7, 8]. These new carotenoids were also encountered in other strains of *R. rhodochrous*.

RESULTS AND DISCUSSION

Carotenoids from R. rhodochrous *RNMS1 and its mutant KA-2*

In previous reports [2, 3] two carotenoids (carotenoid **B** and **K**), two carotenoid glucosides (carotenoid **B-G** and **K-G**) and two carotenoid glucoside fatty acid esters (carotenoid **B-G-FA** and **K-G-FA**), have been found in *R. rhodochrous* RNMS1 on reversed phase HPLC using methanol as a mobile phase. Upon elution with methanol containing chloroform, some new carotenoids (**1** and **2** in the order of elution) were detected in the crude extract [Fig. 1(A)].

The carotenoid mutant KA-2 was found to produce a larger quantity of **1** and **2** [Fig. 1(B)] than the wild type strain, and the new carotenoids were purified

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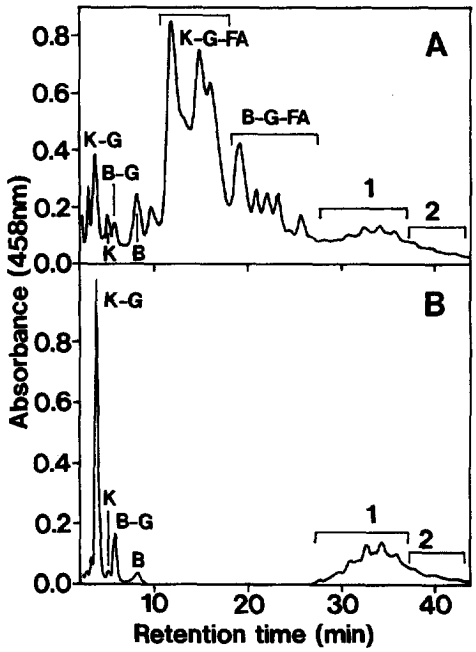


Fig. 1. Elution profiles of the crude carotenoid extracts from *R. rhodochrous* RNMS1 by reversed phase HPLC: (A) the wild type strain and (B) its mutant KA-2.

from this mutant. The total content of carotenoids in KA-2 was 0.18 nmol mg^{-1} (dry wt), which was similar to that of the wild type strain [3].

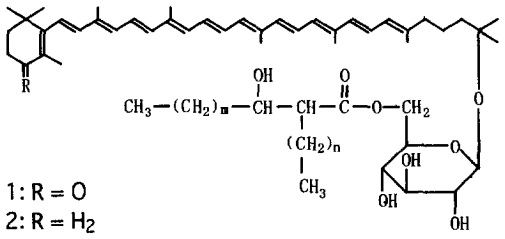
The absorption spectrum of **1** showed one broad peak at *ca* 470 nm and a shoulder at *ca.* 490 nm and was similar to that of carotenoid **K**. By reduction of **1** with $NaBH_4$, the broad absorption peak was changed into two peaks at 460 and 490 nm and a shoulder at *ca* 440 nm. The absorption spectrum was similar to that of carotenoid **B**. Therefore, the reduced pigment had properties compatible with a monocyclic carotenoid containing 11 conjugated double bonds and would be a derivative of β,ψ-carotene [9].

The carotenoid aglycone obtained by acid methanolysis of **1** was identified as carotenoid **K** based on the absorption spectrum, its R_f on HPLC and its M_r of 568 [2]. By saponification, **1** was transformed to two products, which were extracted with *n*-hexane. The carotenoid product was identified as carotenoid **K-G** based on the absorption spectrum, its R_f on HPLC and its M_r of 730 [2]. The colourless product was converted into its methyl ester and analyzed by

Table 1. ^1H and ^{13}C NMR spectral data of compound **1** (chemical shifts)*

H	δ_H	C	δ_C
Carotenoid moiety			
H_3-16,17	1.20 *s*	C-16,17	27.7
H_3-18	1.87 *s*	C-18	13.9
H_3-19	2.00 *s*	C-19	12.6
H_3-20	1.97 *s*	C-20	12.8†
		C-1	35.8
H_2-2	1.85 *t* (6.9)	C-2	37.4
H_2-3	2.51 *t* (6.9)	C-3	34.3
		C-4	199.4
		C-5	129.8
		C-6	161.3
H_3-16′	1.24 ‡*s*	C-16′	26.1§
H_3-17′	1.25 ‡*s*	C-17′	26.9§
H_3-18′	1.80 *s*	C-18′	16.8
H_3-19′	1.97 *s*	C-19′	12.9
H_3-20′	1.97 *s*	C-20′	12.8†
		C-1′	78.4
H_2-2′	~1.51	C-2′	41.6
H_2-3′	~1.51	C-3′	22.4
H_2-4′	2.09	C-4′	40.5
		C-5′	139.2
H-6′	5.94 *d* (11.0)	C-6′	126.0
Glucoside moiety			
H-1	4.45 *d* (7.8)	C-1	97.0
H-2	3.32 *t* (8.5)	C-2	73.8
H-3	3.56 *t* (7.8)	C-3	76.2
H-4	3.47	C-4	70.6¶
H-5	3.47	C-5	73.4¶
H-6	4.42 *d* (11.0)	C-6	63.6
H-6′	4.35 *d* (10.1)		
Mycolic acid moiety			
		C=O	175.6
H-α	2.44 *m*	C-α	51.8
H-β	3.67 *m*	C-β	72.5
H-γ	1.39 *m*	C-γ	35.4
H-δ	1.25	C-δ	25.7
H-β′	1.25	C-β′	29.0
H-γ′	1.25	C-γ′	27.5
–(CH_2)–	1.25	–(CH_2)–	29.7
–CH=	5.35 *m*	–CH=	129.9
–CH_2–CH=	2.02	–CH_2–CH=	27.2
H-χ,χ′	1.25	C-χ,χ′	31.9
H-ψ,ψ′	1.31	C-ψ,ψ′	22.7
H-ω,ω′	0.88 *t* (7.3)	C-ω,ω′	14.1

* Values in parentheses are the coupling constants in Hz.
†,‡,§,¶ Corresponding assignments may be reversed.



1: R = O
2: R = H_2
(Saturated mycolic acid moiety is shown.)

silica gel HP-TLC and KC-18 TLC. Its R_f values were compatible with those of the cellular mycolic acid methyl esters [4]. Therefore, **1** was a mycolic acid ester of carotenoid **K-G**.

Assignments of the ^1H NMR and ^{13}C NMR spectra of **1** (Table 1) were made by comparison with those of standards and by ^1H-^1H COSY and ^{13}C-^1H COSY data. In the ^1H NMR spectra, the signals of the ca-

rotenoid moiety were compatible with those of carotenoid **K-G-FA** [2]. In addition, the signals of the carotenoid moiety of the ^{13}C NMR spectra indicated that one end group (primed number) was identical with the 4-keto-β end group and the other (unprimed number) was identical with the 1-hydroxy-1,2-dihydro-ψ end group, except for the signals for C-16′, C-17′ and C-1′ [10]. These shift differences might be due to the glycosylation at C-1′.

Based on the ^1H-^1H COSY and ^{13}C-^1H COSY data, the glycoside moiety was identified as a β-D-glucoside esterified at the C-6 hydroxyl group (Table 1). This structure is the same as for carotenoid **K-G-FA** [2]. Furthermore, **1** also showed the typical signals of the mycolic acids, such as α, β, γ and β' protons and carbons (Table 1) [11, 12]. The signals of unsaturated double bonds in the mycolic acid moiety were also observed.

The FD-MS spectrum of **1** revealed several molecular ion peaks between m/z 1250 and 1450. This indicated that the mycolic acid moiety was composed of several molecular species. The main M_rs were 1292, 1320, 1346, 1348, 1374, 1376 and 1402. Accordingly, the esterified moiety was compatible with saturated C_{38}, C_{40}, C_{42} and C_{44}, and mono-unsaturated C_{42}, C_{44} and C_{46} mycolic acids. These molecular species of the mycolic acid moiety were similar to those of the cellular mycolic acids in *R. rhodochrous* RNMS1 [4]. After trimethylsilylation, the M_rs of **1** increased by 288 mass units (tetrasilyl derivatives), indicating that the presence of four hydroxyl groups in the structure of **1**. This result corresponded with three free hydroxyl groups in the glucoside moiety and one hydroxyl group in the mycolic acid moiety.

With this spectroscopic and chemical evidence, **1** was identified as a carotenoid glucoside mycolic acid monoester, 1′-[(6-*O*-mycoloyl-β-D-glucopyranosyl)-oxy]-1′,2′-dihydro-β,ψ-caroten-4-one, and referred to as carotenoid **K-G-Myc**. The molecular species of the mycolic acid moiety were similar to those of the cellular mycolic acids. Each peak on HPLC (Fig. 1) may correspond to one or two species of mycolic acids as in the case of the carotenoid glucoside fatty acid esters [2].

The absorption spectrum of **2** showed three absorption peaks (λ_{max} nm: (442), 461, 490) indicating that **2** was a derivative of β,ψ-carotene [9]. It was similar to that of carotenoid **B**. Compound **2** was strongly adsorbed both by silica gel column chromatography and by reversed phase HPLC (Fig. 1B), similar to carotenoid **K-G-Myc**. The FD-mass spectrum showed main M_rs of 1278, 1306, 1332, 1334, 1360, 1362 and 1388, which were smaller than those of carotenoid **K-G-Myc** by 14 mass units. This reduction was compatible with the loss of a keto group at the β-end group. Therefore, **2** is also suggested to be a carotenoid glucoside mycolic acid ester, and may be 1′-[(6-*O*-mycoloyl-β-D-glucopyranosyl)oxy]-1′,2′-dihydro-β,ψ-carotene. This is referred to as carotenoid **B-G-Myc**. Molecular species of the mycolic acid moiety were similar to those in carotenoid **K-G-Myc**.

In addition to the carotenoid glucosides (carotenoid **K-G** and **B-G**) and the carotenoid glucoside esters (carotenoid **K-G-FA** and **B-G-FA**) [2, 3], the presence of the carotenoid glucoside mycolic acid monoesters (carotenoid **K-C-Myc** and **B-G-Myc**) were also demonstrated in this bacterium. This is a new feature of the esterifying moiety. These are a new type of carotenoid derivative and they have not yet been reported in any other organism [7, 8].

Carotenoid compositions of four strains of R. rhodochrous *and carotenoid mutant KA-2*

Carotenoid mutant KA-2 produced a large quantity of carotenoid **K-G-Myc** and **B-G-Myc**, but no carotenoid **K-G-FA** and **B-G-FA** (Table 2). This may be due to a mutation in a gene for a fatty acid esterase of the carotenoid glucosides. A presumed precursor of carotenoid **K-G** was also accumulated.

Carotenoid compositions of the authentic strains of *R. rhodochrous*, JCM 3202^T, JCM 2157 and IFO 3338, were also compared (Table 2). All strains had eight carotenoids in different proportions, and also produced carotenoid glucoside mycolic acid esters (carotenoid **K-G-Myc** and **B-G-Myc**). Carotenoid **K-G-Myc** was the major component especially in strain JCM 2157. Compared with the HPLC elution profiles of the crude carotenoid extracts from four strains, the carotenoid glucoside mycolic acid ester region (carotenoid **K-G-Myc** and **B-G-Myc**) was similar to each other (data not shown). This result indicated that the compositions of the mycolic acids in carotenoid glucoside mycolic acid esters might be similar in all strains tested. In contrast, the elution profiles of the carotenoid glucoside fatty acid ester region (carotenoid **K-G-FA** and **B-G-FA**) were somewhat different to each other (data not shown). The compositions of fatty acids in the carotenoid glucoside fatty acid esters might consequently be different in each strain.

EXPERIMENTAL

Biological materials. Rhodococcus rhodochrous RNMS1 (IAM 13988, IFO 14894, JCM 7553) were used [4]. The authentic strains were obtained from Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (JCM 3202^T (=ATCC 13808^T) and JCM 2157) and Institute of Fermentation, Osaka (IFO 3338). They were cultured at 30° [2]. A carotenoid mutant KA-2 was obtained by a treatment of RNMS1 with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

Isolation and purification. Carotenoid pigments were extracted from the wet cells with $CHCl_3$–MeOH (1:2). All processes were carried out under a dim-light without saponification. Carotenoids extracted from KA-2 were submitted to silica gel 60 CC. After elution with *n*-hexane-$CHCl_3$, **1** and **2** were simultaneously

Table 2. Carotenoid compositions (% of total carotenoid) of some *R. rhodochrous* strains and mutant KA-2

Carotenoid	Strain				
	RNMS1	KA-2	JCM 3202^T	JCM 2157	IFO 3338
B	2	2	1	0	0
K	1	1	3	1	2
B-G	1	4	4	1	3
K-G	7	26	31	2	14
B-G-FA	25	0	4	20	14
K-G-FA	48	0	21	25	48
B-G-Myc	3	11	5	6	2
K-G-Myc	13	55	32	45	17

eluted with $CHCl_3$–MeOH (98:2) as a red eluate. This eluate was submitted to DEAE-Sepharose CL-6B CC [5]. Compound **2** was eluted with *n*-hexane–Me_2CO (7:3) as a yellow eluate, which was followed by a red eluate of **1**. Purification by CC was repeated once more. Finally, each carotenoid was purified by prep. HPLC.

HPLC equipped with a μBondapak C18 column (100 × 8 mm) was used analytically [5]. MeOH was the eluent for 6 min, followed by a linear gradient of $CHCl_3$ to 25%, and at 28.5 min by an isocratic $CHCl_3$–MeOH (1:3) at a constant flow rate of 2.0 ml min^{-1}. A preparative column (100 × 25 mm) of μBondapak C18 was also used. The eluent was $CHCl_3$–MeOH (1:4), and the flow rate was 6.67 ml min^{-1}.

Saponification of the carotenoid glucoside mycolic acid esters. After saponification of the purified compounds, mycolic acid residues extracted with *n*-hexane were esterified with trimethylsilyldiazomethane [13]. Mycolic acid methyl esters were identified by silica gel HP-TLC developed with *n*-hexane-Et_2O–HOAc (80:30:1) and by reversed phase TLC (KC-18) developed with $CHCl_3$–MeOH (4:1) detection by fluorescence of plimulin.

Spectroscopic analysis. Absorption spectra were obtained by the photodiode array detector which was connected to the HPLC system as described previously [9]. The molar absorption coefficient of carotenoids in the eluent of HPLC was assumed to be 128 mM^{-1} cm^{-1} at 458 nm [3]. The M_rs of the carotenoids and their derivatives were measured by FD-MS [14]. ^1H and ^{13}C NMR spectra data (500 and 125 MHz, respectively, $CDCl_3$, 25°) are shown in Table 1.

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