# On the Biosynthesis of $C_{30}$ Carotenoic Acid Glucosyl Esters in *Pseudomonas rhodos*. Analysis of *car*-Mutants

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*car*-Mutants of *Pseudomonas rhodos* were isolated and classified into four types according to their blockage in the biosynthetic pathway. A biosynthetic scheme could be established. Three new structures could be identified (4,4′-diapocarotene-4,4′-dial, 4,4′-diapocarotene-4-al-4′-oic acid, glucosyl-4,4′-diapocarotene-4-oate).

## Introduction

We have recently described the identification of the carotenoid pigments from the soil bacterium *Pseudomonas rhodos* as derivatives of a symmetrical 4,4′-diapocarotene structure [1]. The pigments were 4,4′-diapocarotene-4-oic acid, di($\beta$,D-glucosyl) 4,4′-diapocarotene-4,4′-dioate, and $\beta$,D-glucosyl-4,4′-diapocarotene-4-oate-4′-oic acid. These structures are shown in Fig. 1 as (**5**), (**6**), and (**7**), respectively. We report now the analysis of several *car*-mutants of *Pseudomonas rhodos* which provides evidence for the biosynthetic pathway of these pigments and led to the structural elucidation of three new $C_{30}$ carotenoids.

## Results and Discussion

The selection of *car*-mutants based on differences on colony color. This method is sufficiently exact, at least in the present case, since each biosynthetic step in the formation of the carotenoid pigments in *P. rhodos* is supposed to be accompanied by an alteration within the chromophor, *i.e.* by a different absorption.

Three main classes of *car*-mutants were observed: cells showing the normal pigment pattern with, however, an increased or decreased pigment content and cells with an altered pigment pattern. Within the latter class four types could be distinguished (Fig. 1).

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Type I produced white colonies. In none of these mutants 4,4′-diapophytoene could be detected as the first $C_{30}$ intermediate. This is unexpected, since normally in white mutants either the phytoene synthase or the phytoene dehydrogenase could be affected. This is known from numerous $C_{40}$ carotenoids producing organisms and also from *Staphylococcus aureus* forming $C_{30}$ pigments [2].

Type II mutants were orange in color. They contained 4,4′-diapolycopene (**1**) as the only carotenoid. In these mutations the introduction of oxygen into the carotenoid molecule, probably by a mixed-function oxidase (see type III mutants), is blocked. Interestingly, less dehydrogenated intermediates such as 4,4′-diapophytofluene, 4,4′-diapo-$\zeta$-carotene, or 4,4′-diaponeurosporene were not present. Such intermediates have been described from *Streptococcus faecium* [3] and from *car*-mutants of *Staphylococcus aureus* [2, 4].

Type III mutants were red to violet in color. They contained 4,4′-diapocarotene-4-al (**3**) and 4,4′-diapocarotene-4,4′-dial (**2**) as the main pigments. A structure identified as 4,4′-diapocarotene-4-al-4′-oic acid occurred in one of these mutants in trace amounts. The latter two structures have not been described before (for identification see the Experimental Part). The introduction of oxygen on the terminal methyl groups to give the aldehydes is probably brought about by a mixed-function oxidase. Mutants containing intermediates with corresponding hydroxy functions could not be found. This is in accordance with observations made for *Staphylococcus aureus* [2]. Apparently, in the type III

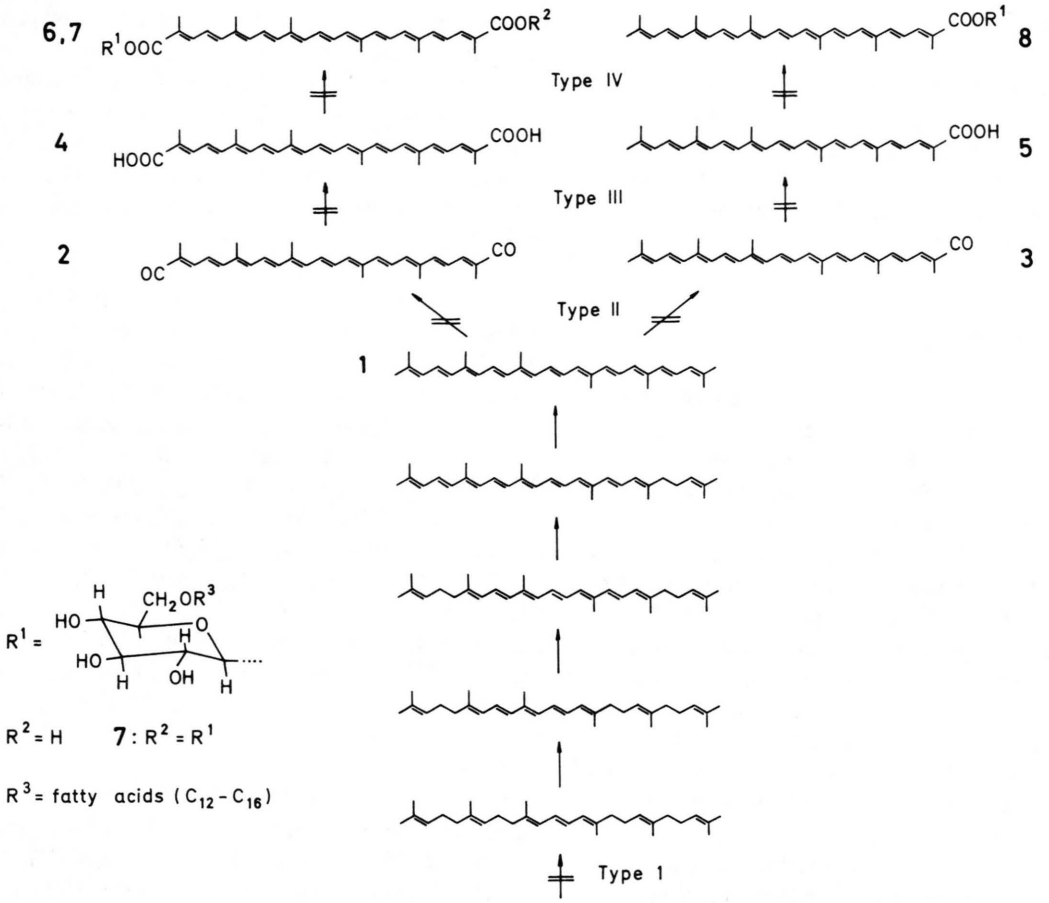


Fig. 1. Proposed biosynthetic pathway of the $C_{30}$ carotenoids in *Pseudomonas rhodos*. The identified pigments are numbered from **1** to **8**. The *car*-mutations type I to type IV are indicated.

mutants an aldehyde dehydrogenase is affected which converts the aldehydes to the corresponding acids.

Type IV mutants were bright red in color and, thus, well distinguishable from the deep red parent strain. The free acids **4** and **5** were found in these mutants as the main pigments which means that the esterifying enzyme connecting the carboxyl groups with monoacylated glucose to the end products **6, 7,** and **8** of the pathway was inactive. Structure **8**, a hithertoo unknown structure, was isolated from a mutant having a fourfold increased pigment content. In the parent strain it occurred only in very trace amounts. For the structural elucidation of this pigment see the Experimental Part.

The results suggest the biosynthetic pathway shown in Fig. 1. The symmetrical structure of the pigments [1] indicates that the $C_{30}$ pathway is initiated by the condensation of two molecules of farnesyl pyrophosphate. As pointed out above, 4,4′-diapophytoene (structure not numbered in Fig. 1) was not detected in any of the *car*-mutants of *P. rhodos*. An explanation of this finding would be that squalene instead of diapophytoene is the first $C_{30}$ intermediate, as has been recently discussed for *Streptococcus faecium* and *Staphylococcus aureus* [5]. The squalene synthase would then be affected in type I mutants of *P. rhodos*. The question whether a phytoene synthase is present or not in *P. rhodos* will be subject of further studies. The proposed pathway in Fig. 1 is similar to that suggested for the monosubstituted 4,4′-diaponeurosporene derivatives in *Staphylococcus aureus* [2].

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## Experimental Part

*Pseudomonas rhodos* strain B9 [6] was grown in a medium containing 0.8% nutrient broth (Merck) and 0.3% yeast extract (Difco) in 100 ml shaken cultures at 30 °C. The cultures were illuminated with three fluorescent tubes (Osram, 40 W/25-1).

Mutagenesis of exponentially growing *P. rhodos* was carried out in nutrient broth containing 300 µg N-methyl-N′-nitro-N-nitrosoguanidine for 5 h at 30 °C. Cells were washed, resuspended in fresh nutrient broth, grown overnight, and plated on minimal glucose medium (50 mM $K_2HPO_4$, 15 mM $KH_2PO_4$, 0.4 mM $MgSO_4$, 7.6 mM $(NH_4)_2SO_4$, 2% D-glucose, 1.6% Difco Agar). Pigment mutants were identified by colony color and isolated after 7 days of incubation at 30 °C.

Isolation of pigments, chemical methods, and instrumentation have been described recently [1].

*4,4′-Diapolycopene (4,4′-diapocarotene)* (**1**). VIS (ethanol): 440, 467, 498 nm. – MS: *m/z* 400(20, M); 341(1); 308(2); 294(2); 209(5); 157(11); 145(13); 119(14); 109(20); 105(18); 91(18); 81(41); 69(100); 55(26); 41(42).

*4,4′-Diapocarotene-4-al* (**3**). VIS (ethanol): 491 nm; reduced form: 442, 468, 500 nm. – Formation of a monoacetate of the reduced form upon acetylation. – MS: *m/z* 414 (M).

*4,4′-Diapocarotene-4,4′-dial* (**2**). VIS (ethanol): 501 nm; reduced form: 442, 468, 501 nm. – Formation of a diacetate of the reduced form upon acetylation. – MS: *m/z* 428 (M).

*4,4′-Diapocarotene-4-al-4′-oic acid.* VIS (ethanol): 494 nm; reduced form: 445, 471, 503 nm. – Formation of a monomethyl ether upon methylation using diazomethane. – Formation of a monoacetate of the reduced form upon acetylation. – A molecular ion with MS could not be obtained.

*β,* D-*Glucosyl-4,4′-diapocarotene-4-oate,* occurring as fatty acid ester (**8**). VIS (ethanol): 460, 484, 505 nm; as methyl-4,4′-diapocarotene-4-oate: 458, 480, 509 nm. – Formation of a monomethyl ether upon saponification using methanolic KOH with the spectral and chromatographic properties of methylated (5). – MS of peracetylated **8**: *m/z* 956(2, $M_1$: $R^3=C_{16}H_{31}O$); 954(5, $M_2$: $R^3=C_{16}H_{29}O$); 942(1, $M_3$: $R^3=C_{15}H_{29}O$); 928(6, $M_4$: $R^3=C_{14}H_{27}O$); 914(15, $M_5$: $R^3=C_{13}H_{25}O$); 900(17, $M_6$: $C_{12}H_{23}O$); 886(2, $M_7$: $R^3=C_{11}H_{19}O$); 848(2, $M_2-C_8H_{10}$); 836(2, $M_3-C_8H_{10}$); 822(7, $M_4-C_8H_{10}$); 808(10, $M_5-C_8H_{10}$); 794(7, $M_6-C_8H_{10}$); 780(1, $M_7-C_8H_{10}$); 525(5); 499(5); 485(10); 471(9); 429(5); 307(4); 239(4, $C_{16}H_{31}O$); 237(10, $C_{16}H_{29}O$); 211(18, $C_{14}H_{27}O$); 197(35, $C_{13}H_{25}O$); 183(37, $C_{12}H_{23}O$); 169(90); 109(65); 43(100).

$^1$H-NMR of peracetylated **8** (270 MHz, 0.2 mg in 0.2 ml $CDCl_3$): 0.87 ppm (tr, ca 3H, $CH_3(CH_2)$); ca 1.22 (m, ca 25H, $(CH_2)_n$); 1.52 (m, 2H, $CH_2$); 1.83 (s, 6H, $CH_3$–C(5)); ca 1.98 (2, ca 18H, $CH_3$–C=); 2.03, 2.04, and 2.09 (3 × s, 3H each, OAc); 2.24 (tr, 2H, $COCH_2$); 3.89 (m, 1H, H–C(5″)); 4.12 (d × d, 12.5 and 1.5 Hz, H–C(6″)); 4.33 (d × d, 12.5 and 4.3 Hz, 1H, H–C(6″); 5.17 (m, 1H, =CH of fatty acid chain); ca 5.30 (m, 3H, H–C(2″), H–C(3″) and H–C(4″)); 5.27 (~ d, 8 Hz, 1H, H–C(1″)); 5.95 (d, $J \sim 11$ Hz, 1H, H–C(6)); 6.2–6.7 (m, ca 15H, olefinic H′s); 7.43 (d, 11.3 Hz, 1H, H–C(6′)).

For the *β,* D-glucosyl structure see [1].

The identification of the other pigments has been described recently [1].

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