Phytochemistry, Vol 22, No 1, pp 207–210, 1983
Printed in Great Britain

0031–9422/83/010207–04$03 00/0

THE PIGMENTS OF *BREVIBACTERIUM LINENS*: AROMATIC CAROTENOIDS*

WERNER KOHL†§, HANS ACHENBACH‡§ and HANS REICHENBACH†

†Gesellschaft fur Biotechnologische Forschung, Mascheroder Weg 1, D-3300 Braunschweig, West Germany, §Chemisches Laboratorium der Universitat, Albertstr 21, D-7800 Freiburg, West Germany

(*Revised received* 30 *April* 1982)

Key Word Index—*Brevibacterium linens*, coryneform bacteria, aromatic carotenoids, isorenieratene, leprotene, phenolic carotenoids, chemotaxonomy

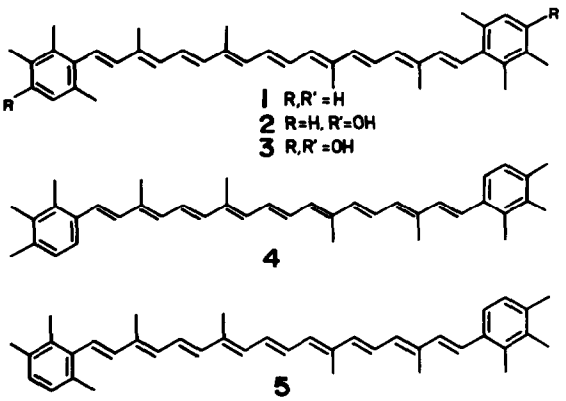
Abstract—The main pigment of *Brevibacterium linens* is the aromatic carotenoid 3,3′-dihydroxy-isorenieratene (ϕ, ϕ-carotene-3,3′-diol) Minor components are the corresponding monohydroxy compound and the hydrocarbon, isorenieratene The dihydroxyphenyl carotenoid is responsible for the colour shift from yellow-orange to pink-purple observed when colonies of *B linens* are covered with an alkaline solution The mass spectra of the three pigments and of the acetates of the hydroxy compounds are presented for the first time

INTRODUCTION

The flexirubin-type pigments, discovered in certain gliding bacteria [1, 2], seem to have a rather narrow distribution among prokaryotes They occur, with slight structural modifications, in terrestrial *Cytophaga*-like bacteria, including *Flexibacter* and *Sporocytophaga*, and in several of those flavobacteria possessing DNA with a low guanosine + cytosine content [3–5] Thus the flexirubin-type pigments are valuable chemosystematic markers, particularly as their presence can be demonstrated by a simple test Thus when the yellow or orange colonies are covered with a 20% potassium hydroxide solution, they immediately change their colour into purple or brown This colour shift can be fully reversed by replacing the potassium hydroxide with hydrochloric acid It is a phenolate reaction and thus not necessarily specific for the flexirubin-type pigments The reliability of the colour reaction as an indicator of the taxonomic groups mentioned above obviously depends on the absence of pigments with a similar behavior in unrelated bacteria With Gram-negative bacteria, we have not found any other yellow strains which change their colour upon addition of alkali But there are several Gram-positive bacteria with this property Our attention was kindly drawn by Dr H J Parish, Leeds, to *Brevibacterium linens*, for which it was known for some time that its yellow colonies quickly turn into pinkish purple if covered with an alkaline solution As apparently nothing was known about the pigments of this organism, we decided to investigate the matter It turned out that *B linens* contains unusual aromatic carotenoids

RESULTS

B linens contained three pigments, in order of increasing polarity (Si gel) **1**, **2**, and **3** Dilute solutions of all three pigments were yellow-orange and had virtually identical electron spectra (λ_{max}^{MeOH} 454 nm) Addition of alkali shifted the absorption maximum of **3** by 12 nm to longer wavelengths (MeOH) This bathochromic shift could be fully reversed by the addition of acid With **1** and **2**, alkali did not give a bathochromic effect



In the mass spectrum of the main component, **3**, the $[M]^+$ was at m/z 560 ($C_{40}H_{48}O_2$) The $[M]^+$'s of **2** and **1** were 16 and 32 amu lower, corresponding to $C_{40}H_{48}O$ and $C_{40}H_{48}$, respectively In all three spectra, the ions $[M-92]^+$, $[M-106]^+$ and $[M-158]^+$ were significant, as is characteristic for carotenoids [6–8] Strong fragments were observed at m/z 133 (**1**), 133 and 149 (**2**), and 149 (**3**) These fragments are characteristic for carotenoids with aromatic end groups [6, 7, 9], analogous fragments are found also in other ω-phenyl polyenoic compounds like the flexirubin-type pigments [10] From these data it was concluded, that the pigments of *B linens* were bicyclic aromatic C_{40} carotenoids, and that **3** contained two

*Part 24 in the series "Investigations on Metabolites of Microorganisms" For Part 25 see Achenbach, H, Bottger-Vetter, A, Fautz, E and Reichenbach, H *Arch Microbiol* (in press)

‡Present address Lehrstuhl fur Pharmazeutische Chemie, Universitat Erlangen-Nurnberg, D-8520 Erlangen, West Germany

monohydroxylated trimethylbenzene rings, **2** one such ring besides a non-hydroxylated ring, and **1** two non-hydroxylated rings To confirm these conclusions, **2** and **3** were reacted with acetic anhydride in pyridine As expected, **2** gave a monoacetate ($[M]^+$ *m/z* 586) and **3** a diacetate ($[M]^+$ *m/z* 644) The mass spectra of both compounds showed the characteristic fragments $[M-42]^+$, $[M-92]^+$, $[M-106]^+$, *m/z* 149 and, in the case of **2** acetate, *m/z* 133

Among the aromatic carotenoids described in the literature (for a review, see ref [11]), the three pigments renierapurpurin (**4**), renieratene (**5**), and isorenieratene (**1**), which were originally isolated from the sea-sponge *Reniera purpurea*, or *Halichondria panicea* [12], had the same elemental composition as pigment **1**

Comparison of the electron spectra of these compounds showed that pigment **1** [$\lambda_{max}^{C_6H_6}$ nm 430 (sh), 462, 492] was neither renierapurpurin [$\lambda_{max}^{C_6H_6}$ nm 464 (sh), 487, 519 [13]] nor renieratene [$\lambda_{max}^{C_6H_6}$ nm 457 (sh), 476, 507 [13]], but that it did have the same absorption characteristics as isorenieratene [$\lambda_{max}^{C_6H_6}$ nm 430 (sh), 463, 492 [13]]

The pigments isorenieratene (**1**), 3-hydroxy-isorenieratene (**2**) and 3, 3′-dihydroxy-isorenieratene (**3**) have been described from the bacterium, *Streptomyces mediolani* [14, 15] To prove the identity of these pigments with **1**, **2** and **3**, respectively, from *B linens*, the *Streptomyces* pigments were isolated from an extract kindly supplied by Dr Arcamone, and compared with the corresponding pigments from *Brevibacterium* by high performance TLC and HPLC There was full agreement in all three cases We then prepared the peracetates of the hydroxylated *Streptomyces* pigments and compared them with the acetates of **2** and **3** from *B linens* Again, there was complete agreement In addition, the IR-spectra of **3** and dihydroxy-isorenieratene were in agreement We therefore conclude that **1** is isorenieratene, **2** its 3-hydroxy-, and **3** its 3, 3′-dihydroxy derivative The exact position of the hydroxyl group(s) was deduced from the following observations (a) The hydroxyls must be located on the aromatic nucleus This was shown by the strong $[M-149]^+$ fragments in the mass spectra, and by the acidic nature of the hydroxyls, which easily form methyl ethers and phenolates (b) The electron spectra allowed only a 1, 2, 5-methylation pattern of the aromatic nucleus [13], therefore, the hydroxyls must be located at either positions 3 or 4 (c) The *para* (3) position was indicated by the blue oxidation product of **3**, which has been demonstrated to have a *para*-quinoid structure [9], and by the pronounced bathochromic shift in the electron spectrum caused by the phenolate reaction, which can be expected only if the hydroxyl was in the *para* position (d) Finally, **2** and **3** and their acetates appeared to be identical with the above-mentioned *Streptomyces* pigments, for which the 3-position of the hydroxyls had been unequivocally proved by oxidative degradation, yielding 2, 3, 6-trimethyl-4-hydroxy benzaldehyde, and by total synthesis [15]

DISCUSSION

The pigments of *B linens* are aromatic carotenoids They are thus chemically completely different from the flexirubin-type pigments [3], although they give a similar colour reaction with alkali The occurrence of the latter is therefore still limited to certain *Cytophaga*-like bacteria and to flavobacteria with DNA with a low guanosine + cytosine content [4]

There are several simple methods which can be used to distinguish between phenolic carotenoids and flexirubin-type pigments While the main absorption maxima are in approximately the same range (*ca* 450 nm in ethanol, at least as far as the more common octaene flexirubin-type pigments are involved), the bathochromic shift upon addition of alkali is much smaller in the case of 3,3′-dihydroxy-isorenieratene (12 nm) than with flexirubin-type pigments (38 nm) Therefore colonies of *B linens* change colour from yellow-orange to pink-purple when covered with potassium hydroxide solution, and not from yellow-orange to red or brown-violet as is the case with *Cytophaga*-like bacteria The mono-hydroxylated isorenieratene (**2**) does not give a colour reaction with alkali, as reported in the literature [9] Further, the phenolic carotenoids, particularly the diol, are relatively sensitive to oxidation [9] On thin-layer chromatograms they quickly turn light blue when exposed to iodine vapours, and solutions of crude or purified pigment in acetone may soon become deep green or blue, even when stored in the deep-freeze The flexirubin-type pigments are more stable in solution, although they too become brown or deep violet on thin-layer chromatograms when exposed to air As the biosynthetic pathways are different for the two classes of pigments, it is also possible to distinguish between the two by allowing the bacteria to incorporate radioactive specific precursors into their pigments [16]

Isorenieratene and its hydroxy derivatives seem to be relatively unusual natural compounds Isorenieratene itself was originally described in 1937 as leprotene from a strain of *Mycobacterium leprae*, later identified as *M phlei* (for a survey of the older literature in this field, see ref [17]) The structure of leprotene was not known at that time, but its identity with isorenieratene was immediately suspected and soon proved by comparison with authentic samples [18–20], after isorenieratene had been isolated from the sea-sponge *Reniera purpurea* and its structure had been elucidated as the first natural aryl-carotene [12, 18] In addition to the examples mentioned above, isorenieratene has been found so far only in several other strains of *Mycobacterium* [17], in the phototrophic bacterium '*Phaeobium*', a brown *Chlorobium* species [21], in *Corynebacterium fascians* [22] and in *Streptomyces mediolani* [14, 15] The two hydroxy derivatives of isorenieratene were discovered in the latter organism, but seem not to have been found in any other organism since With the discovery of 3-hydroxy-, and 3,3′-dihydroxy-isorenieratene in *B linens*, these compounds are now readily accessible

The very restricted and patchy distribution of isorenieratene and its hydroxy derivatives has two interesting aspects Firstly, the hydroxy compounds could be useful for the diagnosis of *B linens* because of the characteristic colour reaction of the diol with alkali Preliminary results indicate that all tested strains of *B linens* produce these pigments, but it remains to be seen whether the pigments are really limited to the species To answer this question, it is helpful that the taxonomy of *Brevibacterium* is presently under study [23] Secondly, in spite of its occurrence in obviously unrelated organisms, isorenieratene could be a valuable chemosystematic marker, because there are sufficiently large gaps between the different groups containing this unusual carotene Most organisms from which isorenieratene has been isolated so far belong into

the large group of the actinomycetes and related bacteria The taxonomic position of *B linens* is somewhat uncertain at present It has coryneform morphology and is regarded by some as closely related to, if not identical with *Arthrobacter*, in particular *A globiformis* The latter hypothesis is not supported by the pigments, however At least those two strains of *A globiformis* which we could check (DSM 20124 = ATCC 8010 and DSM 20125 = ATCC 11822) did not change their colour upon addition of potassium hydroxide solution, while all of our eight named *B linens* strains (kindly supplied by Dr Fiedler, Munich, and Dr Teuber, Kiel) did However, it seems likely that more producers of isorenieratene will be discovered upon careful examination of the coryneforms and related bacteria

EXPERIMENTAL

Organism B linens strain NCIB 8546 (= ATCC 9175) was kindly supplied by Dr H J Parish, Leeds Mass cultivation was done in large glass jars aerated via three glass-sinter outlets with sterile air from a compressed air supply Each jar contained 9 l Fx A 1 m–peptone from casein, tryptically digested (Merck, Darmstadt) 1%, yeast extract (Difco) 0 2%, $MgSO_4$ $7H_2O$ 0 1%, pH 7 2, autoclaved, with 1 ml silicone antifoam emulsion M-30 (Dow-Corning) added Each 9 l jar was inoculated with 900 ml log-phase shake culture and incubated at 30° After 20 hr the pH of the culture, which was not regulated, was in the range 8 0–8 6, and the cells were harvested by centrifugation and frozen at −25° The yield from 10 cultures (99 l) was 590 g wet wt (110 g dry wt, after extraction)

Instruments MS AEI MS 9, resolution of M/ΔM = 1000, direct inlet system, 70 eV The elementary composition of the $[M]^+$s was determined by the peak-matching method, at a resolution of M/ΔM = 15000

Extraction of the pigments The pigments were extracted from 310 g bacterial wet mass with MeOH The concd soln was extracted with toluene (× 3) Removal of insoluble material and the solvent gave 800 mg crude pigment

Separation and purification of the pigments The crude pigments were freed of contaminating material by CC on Fractogel PVA-2000 (Merck, Darmstadt) with CH_2Cl_2–MeOH (1 1) The pigment-containing fractions were combined, and chromatographed on a Si gel column (prepacked Lobar column size B, Merck, Darmstadt) with toluene–EtOAc (9 1) Three coloured bands appeared and were collected separately Fraction 1 contained 4 mg, fraction 2, 4 mg, fraction 3, 10 mg The separated pigments were then purified on a Séphadex LH-20 column with CH_2Cl_2–MeOH (1 1) Fraction 1 gave 3 mg pure pigment **1**, fraction 2, 4 mg **2**, fraction 3, 9 mg **3**

Characterization of the pigments Comparative HPLC was performed on LiChrosorb RP-18 (25 cm × 4 mm, 7 μm, Merck, Darmstadt) with MeOH–Me_2CO–H_2O (15 5 1) as the eluant Detection was at 436 nm TLC was performed on Si gel (Kieselgel 60, precoated aluminum sheets) with toluene–EtOAc (9 1) as the solvent

Complete mass spectra of isorenieratene, its hydroxy derivatives, and the acetates of the latter seem never to have been published We therefore present here the complete set of our mass data

Pigment **1** (= *isorenieratene* = *leprotene* = *ϕ,ϕ-carotene*) R_f = 0 9 $[M]^+$ at 528 3750, corresponding to $C_{40}H_{48}$ (calc 528 3756) MS *m/z* (rel int) 528 $[M]^+$, (34), 527 (13), 436 (11), 422 (6), 370 (13), 265 (7), 264 (10), 263 (9), 239 (9), 237 (9), 223 (7), 221 (11), 211 (10), 209 (14), 207 (13), 199 (13), 197 (11), 195 (11), 193 (9), 185 (9), 183 (14), 181 (9), 179 (9), 174 (9), 173 (46), 172 (9), 171 (29), 170 (11), 169 (16), 167 (9), 165 (9), 159 (13), 158 (17), 157 (30), 156 (20), 155 (14), 147 (9), 145 (20), 144 (16), 143 (26), 142 (14) 141 (16), 134 (14), 133 (100), 132 (11), 131 (11), 130 (9), 129 (20), 128 (10), 119 (21), 105 (20) Ratio $[M-92]^+/[M-106]^+$ = 1 83, corresponding to nine double bonds in the chain [24]

Pigment **2** (= *3-hydroxy-isorenieratene* = *ϕ,ϕ-carotene-3-ol*) R_f 0 6, mp 178° (lit 180–185° [14]) $[M]^+$ at 544 3701, corresponding to $C_{40}H_{48}O$ (calc 544 3705) MS *m/z* (rel int) 544 $[M]^+$ (30), 452 (17), 438 (7), 386 (14), 272 (9), 215 (21), 211 (12), 209 (17), 207 (14), 199 (14), 197 (14), 195 (14), 189 (38), 187 (24), 185 (12), 183 (14), 174 (17), 173 (36), 172 (14), 171 (24), 170 (12), 169 (14), 159 (19), 158 (17), 157 (26), 156 (21), 155 (14), 150 (14), 149 (100), 145 (26), 144 (17), 143 (24), 142 (14), 141 (17), 135 (14), 133 (62), 131 (14), 129 (17), 128 (14), 119 (21), 115 (12), 105 (26) Ratio $[M-92]^+/[M-106]^+$ = 2 42, corresponding to nine double bonds in the chain [9, 24]

Pigment **3** (= *3,3'-dihydroxy-isorenieratene* = *ϕ,ϕ-carotene-3,3'-diol*) R_f 0 4, mp 229° (lit 200° decomp [14], 230° authentic reference material from *Streptomyces mediolani*) $[M]^+$ at 560 3650, corresponding to $C_{40}H_{48}O_2$ (calc 560 3654) MS *m/z* (rel int) 560 $[M]^+$ (28), 559 (5), 469 (5), 468 (11), 454 (5), 402 (8), 281 (6), 280 (8), 279 (8), 267 (8), 255 (6), 253 (9), 251 (6), 239 (5), 237 (6), 227 (6), 215 (20), 213 (6), 211 (6), 209 (10), 207 (9), 199 (8), 197 (8), 195 (6), 190 (8), 189 (43), 187 (23), 186 (6), 185 (9), 183 (8), 181 (5), 179 (6), 175 (9), 174 (13), 173 (14), 172 (11), 171 (8), 169 (8), 165 (5), 161 (9), 160 (11), 159 (14), 158 (8), 157 (13), 156 (8), 155 (6), 150 (15), 149 (100), 148 (9), 147 (6), 146 (6), 145 (16), 144 (6), 143 (10), 142 (8), 141 (8), 136 (10), 135 (14), 133 (8), 131 (9), 129 (10), 128 (9), 121 (10), 119 (15), 117 (6), 115 (9), 107 (6), 106 (8), 105 (20) Ratio $[M-92]^+/[M-106]^+$ = 2 20, corresponding to nine double bonds in the chain [9, 24]

Acetylation of **2** *and* **3** **2** (1 mg) and **3** (2 mg), respectively, were treated with Ac_2O in C_5H_5N (1 1, 0 5 ml) for 12 hr at room temp The crude product was purified on a Si gel column (prepacked Lobar column size B) with toluene–EtOAc (9 1), to give 0 5 mg **2** acetate, and 1 mg **3** acetate

2-*acetate* (= *3-acetoxy-isorenieratene*) R_f 0 95 with toluene-EtOAc (9 1) and 0 5 with toluene–hexane (7 3) Electron spectrum not changed by acetylation, no bathochromic shift with alkali MS *m/z* (rel int) 586 $[M]^+$ (44), 544 (22), 494 (11), 480 (7), 452 (10), 438 (5), 386 (8), 272 (9), 253 (8), 251 (9), 239 (8), 237 (8), 215 (22), 211 (10), 209 (16), 199 (12), 197 (12), 195 (9), 189 (50), 187 (22), 185 (12), 183 (12), 174 (13), 173 (33), 171 (22), 169 (17), 161 (11), 159 (18), 157 (28), 150 (17), 149 (100), 145 (28), 143 (25), 141 (22), 133 (61), 129 (17), 119 (22), 105 (28)

3-*diacetate* (= *3 3'-diacetoxy-isorenieratene*) R_f 0 90 with toluene–EtOAc (9 1) and 0 3 with toluene–hexane (7 3) Mp 184° (186° lit [14]) Electron spectrum not changed by acetylation, no bathochromic shift with alkali MS *m/z* (rel int) 664 $[M]^+$ (40), 602 (18), 552 (18), 538 (12), 486 (8), 453 (10), 387 (7), 297 (8), 280 (13), 279 (13), 255 (11), 253 (11), 234 (14), 227 (14), 215 (36), 209 (26), 190 (13), 189 (75), 187 (25), 185 (8), 183 (8), 174 (15), 173 (15), 159 (12), 157 (12), 150 (20), 149 (100), 145 (17), 143 (11), 135 (14), 119 (10), 105 (10)

Acknowledgements—We wish to thank Miss Susanne Petersen for growing and extracting the bacterium, Miss Barbara Witte for doing most of the chromatography, the Fermentation Department of the GBF for harvesting the mass cultures, and the Spectroscopy Department of the GBF for recording most of the spectra Our special thanks are due to Dr F Arcamone from Farmitalia, Milano, for providing an extract of *Streptomyces mediolani* Furthermore, we thank the Fonds der Chemischen Industrie for financial support

REFERENCES

1 Reichenbach, H , Kleinig, H and Achenbach, H (1974) *Arch Microbiol* **101**, 131
2 Achenbach, H , Kohl, W and Reichenbach, H (1976) *Chem Ber* **109**, 2490
3 Achenbach, H , Kohl, W and Reichenbach, H (1978) *Rev Latinoam Quim* **9**, 111
4 Reichenbach, H , Kohl, W and Achenbach, H (1981) in *The Flavobacterium–Cytophaga Group* (Reichenbach, H and Weeks, O B , eds) pp 101–108 Verlag Chemie, Weinheim
5 Weeks, O B (1981) in *The Flavobacterium–Cytophaga Group* (Reichenbach, H and Weeks, O B , eds) pp 109–114, Verlag Chemie, Weinheim
6 Kjøsen, H , Liaaen-Jensen, S and Enzell, C R (1971) *Acta Chem Scand* **25**, 85
7 Vetter, W , Englert, G , Rigassi, N and Schwieter, U (1971) in *Carotenoids* (Isler, O , ed) pp 189–266 Birkhauser, Basel
8 Johannes, B , Brzezinka, H and Budzikiewicz, H (1974) *Org Mass Spectrom* **9**, 1095
9 Nybraaten, G and Liaaen-Jensen, S (1971) *Acta Chem Scand* **25**, 370
10 Achenbach, H and Kohl, W (1979) *Chem Ber* **112**, 209
11 Straub, O (1971) in *Carotenoids* (Isler, O , ed) pp 771–850 Birkhauser, Basel
12 Yamaguchi, M (1957) *Bull Chem Soc Jpn* **30**, 111
13 Cooper, R D G , Davis, J B and Weedon, B C L (1963) *J Chem Soc* 5637
14 Arcamone, F , Camerino, B , Cotta, E , Franceschi, G , Grein, A , Penco, S and Spalla, C (1968) *Experientia* **24**, 241
15 Arcamone, F , Camerino, B , Franceschi, G and Penco, S (1970) *Gazz Chim Ital* **100**, 581
16 Fautz, E and Reichenbach, H (1980) *FEMS Microbiol Letters* **8**, 87
17 Goodwin, T W and Jamikorn, M (1956) *Biochem J* **62**, 269
18 Yamaguchi, M (1958) *Bull Chem Soc Jpn* **31**, 51
19 Liaaen-Jensen, S (1964) *Acta Chem Scand* **18**, 1562
20 Liaaen-Jensen, S and Weedon, B C L (1964) *Naturwisserschaften* **51**, 482
21 Liaaen-Jensen, S (1965) *Acta Chem Scand* **19**, 1025
22 Prebble, J (1968) *J Gen Microbiol* **52**, 15
23 Fiedler, F , Schaffler, M J and Stackebrandt, E (1981) *Arch Microbiol* **129**, 85
24 Enzell, C R , Francis, G W and Liaaen-Jensen, S (1968) *Acta Chem Scand* **22**, 1054