Tetrahedron Vol 39, No 1, pp 175 to 185, 1983
Printed in Great Britain

0040-4020/83/010175-11$03.00/0
Pergamon Press Ltd

INVESTIGATIONS ON THE BIOSYNTHESIS OF FLEXIRUBIN—THE ORIGIN OF BENZENE RING B AND ITS SUBSTITUENTS[1]

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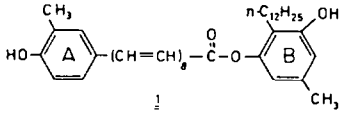
(*Received in Germany 5 February* 1982)

Abstract—Experiments on the biosynthesis of flexirubin (**1**) from the gliding bacterium *Flexibacter elegans* using ^{14}C- and ^{13}C-labelled acetate as well as investigations on the metabolites of a blocked mutant showed, that (a) all carbon atoms of ring B are derived from acetate; (b) 3-dodecylorsellinic and orsellinic acid are intermediates in the biosynthesis; and (c) the carboxyl group of orsellinic acid is lost before the ester linkage with the ω-phenyl-polyene carboxylic acid or its precursor is established to form **1**. ^{14}C-propionate is specifically incorporated into the $C_{13}H_{27}$ side chain of a higher homologue of **1**, which is produced besides **1** in very low concentration (<1% of **1**).

Flexirubin (**1**), whose structure was established recently,[2,3] is the main pigment of the gliding bacterium *Flexibacter elegans* and the first member of the new class of flexirubin-type pigments. These pigments are common in certain groups of gliding bacteria[3,4] and very recently



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were even found in a *Flavobacterium* species.[5,6] Their special structural feature is a ω-(4-hydroxyphenyl)-polyene carboxylic acid which is esterified with a 2,5-dialkylated resorcinol. In an earlier publication[7] we were able to show that of the two methyl groups present in **1** only the methyl group on benzene ring A is derived from the C_1-pool (CH_3 from methionine), whereas tyrosine is the precursor of ring A as well as of the three neighbouring carbon atoms of the polyene chain; furthermore, acetate was found to be the precursor for the major part of the polyene and ring B with its alkyl substituents. A calculation supported an equal distribution of labelled acetate units into all carbon atoms of this part of the molecule. However, since the polyene chain and the ring B system obviously should be derived via different biosynthetic pathways, it seemed necessary to prove this assumption and to do some additional experiments in order to gain evidence of possible intermediates, which might be involved in the biosynthesis of flexirubin.

The following experiments were conducted:

(1) degradation of ^{14}C-flexirubin dimethyl ether obtained by labelling with [1 – ^{14}C]acetate, [2 – ^{14}C] acetate and [1 – ^{14}C] butyrate,

(2) studies on the incorporation of ^{13}C-labelled acetates into flexirubin,

(3) analysis of accumulated metabolites in cultures of colourless mutants of *Fx. elegans*,

(4) feeding experiments with [1 – ^{14}C]acetate in the presence of an excess of supposed biosynthetic intermediates, and

(5) investigation of the distribution of the radioactivity found in flexirubin from cultures with [1 – ^{14}C]propionate.

RESULTS AND DISCUSSION

(1) *Degradation of ^{14}C-flexirubin biosynthesised from ^{14}C-acetates*

In a previous publication[7] we showed that 63% of the total radioactivity in ^{14}C-flexirubin from [1 – ^{14}C]acetate is found in ring B (and its alkyl substituents) and that the CH_3 from methionine can be excluded as a precursor for its CH_3-group. In order to determine the amount of radioactivity located in both of the alkyl substituents of ring B, we cleaved the ^{14}C-hexadecahydroflexirubin dimethyl ether (**3**) using lithium aluminum hydride and degraded the resulting 2-dodecyl-5-methyl-resorcinol monomethyl ether (**5**) by ozonolysis according to Scheme 1. The acids in the reaction mixture were converted into the p-bromo-phenacyl esters and separated by column chromatography to yield the pure esters **6** (from the dodecyl substituent) and **7** (from the methyl group).

The results of the radioactivity measurements of these degradation products from the feeding experiments with [1 – ^{14}C] acetate and [2 – ^{14}C] acetate (Table 5, experiments 1 and 2) are summarised in Table 1. The data clearly demonstrate that both the alkyl groups on ring B are derived from acetate and that the radioactive acetate units are apparently incorporated almost to the same extent into ring B and its substituents.

In addition, Table 1 shows the distribution of radioactivity, when [1 – ^{14}C]butyrate was administered as a possible precursor (e.g. for ring C-atoms C – 1′ to C – 3′ and CH_3; numbering according to Figure 2): the distribution of radioactivity from [1 – ^{14}C]butyrate differs only slightly from that obtained with the labelled acetates, and in particular **7** which contains C – 3′ and the methyl group of ring B is radioactive. This observation excludes butyrate as a specific precursor in the biosynthesis of ring B; presumably, this acid, whose rate of

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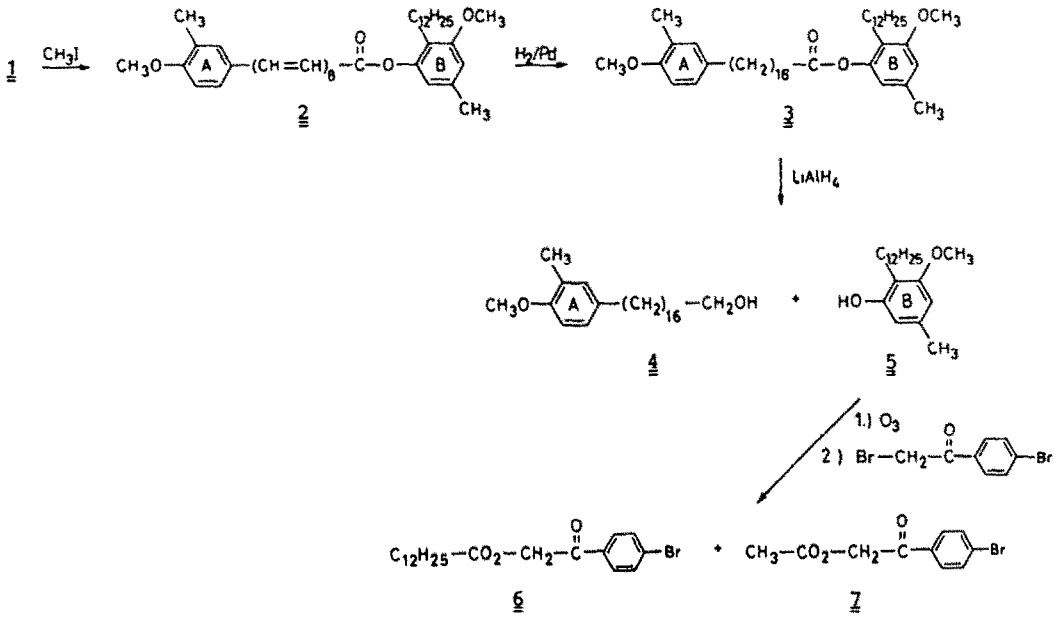


Scheme 1. Chemical degradation of labelled flexirubins.

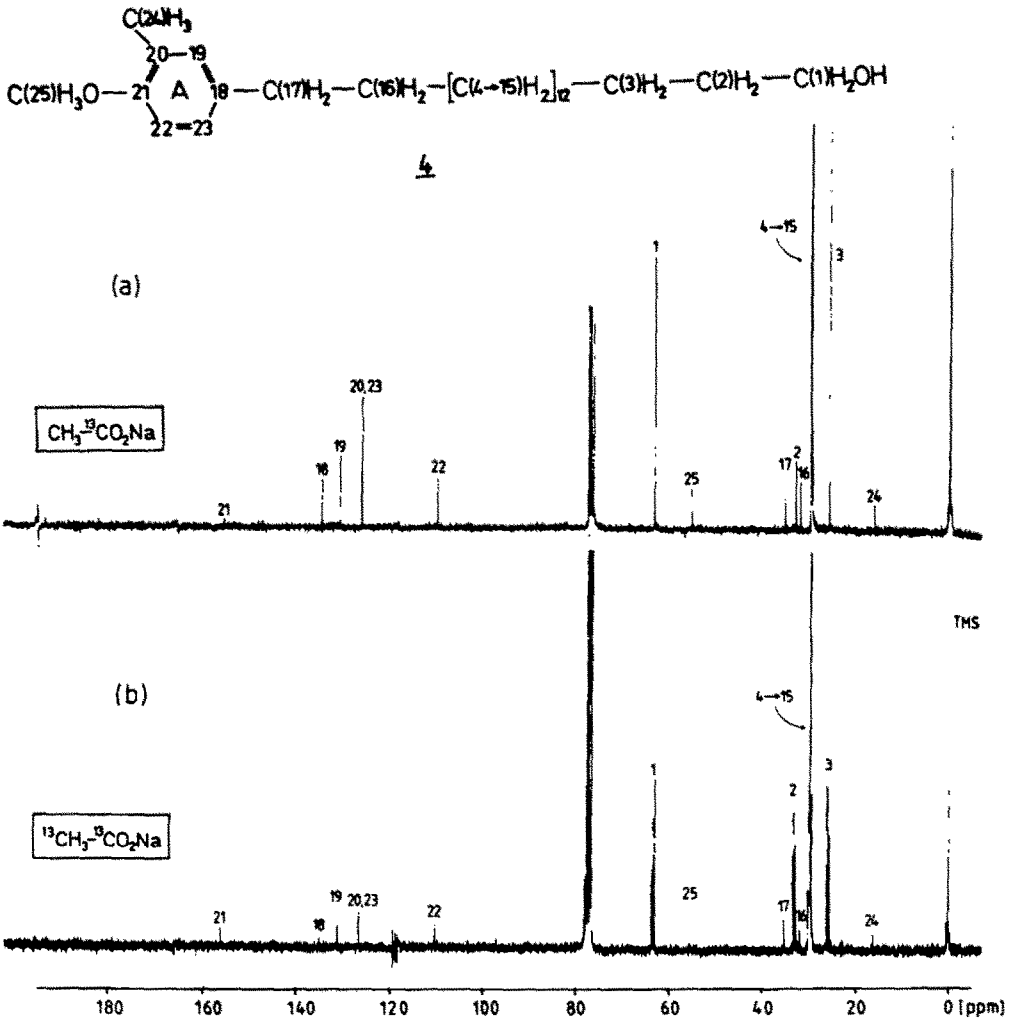


Fig. 1. ^{13}C-NMR spectra (noise decoupled) of degradation products 4 from flexirubins biosynthesised in the presence of (a) sodium [1 – ^{13}C] acetate and (b) sodium [1,2 – $^{13}C_2$]acetate.

Table 1. Distribution of radioactivity in degradation products of flexirubin from cultures with differently labelled precursors

Precursor	Rate of Incorporation into 1	Distribution of Radioactivity				
		Dimethylether 3	C_{25}-Alcohol (4)	C_{20}-Phenol (5)	6	7
[1-^{14}C]Acetate	0.07%	100%	41%	63%	39%	8%
[2-^{14}C]Acetate	0.03%	100%	40%	62%	38%	8%
[1-^{14}C]Butyrate	0.02%	100%	32%	67%	43%	7%

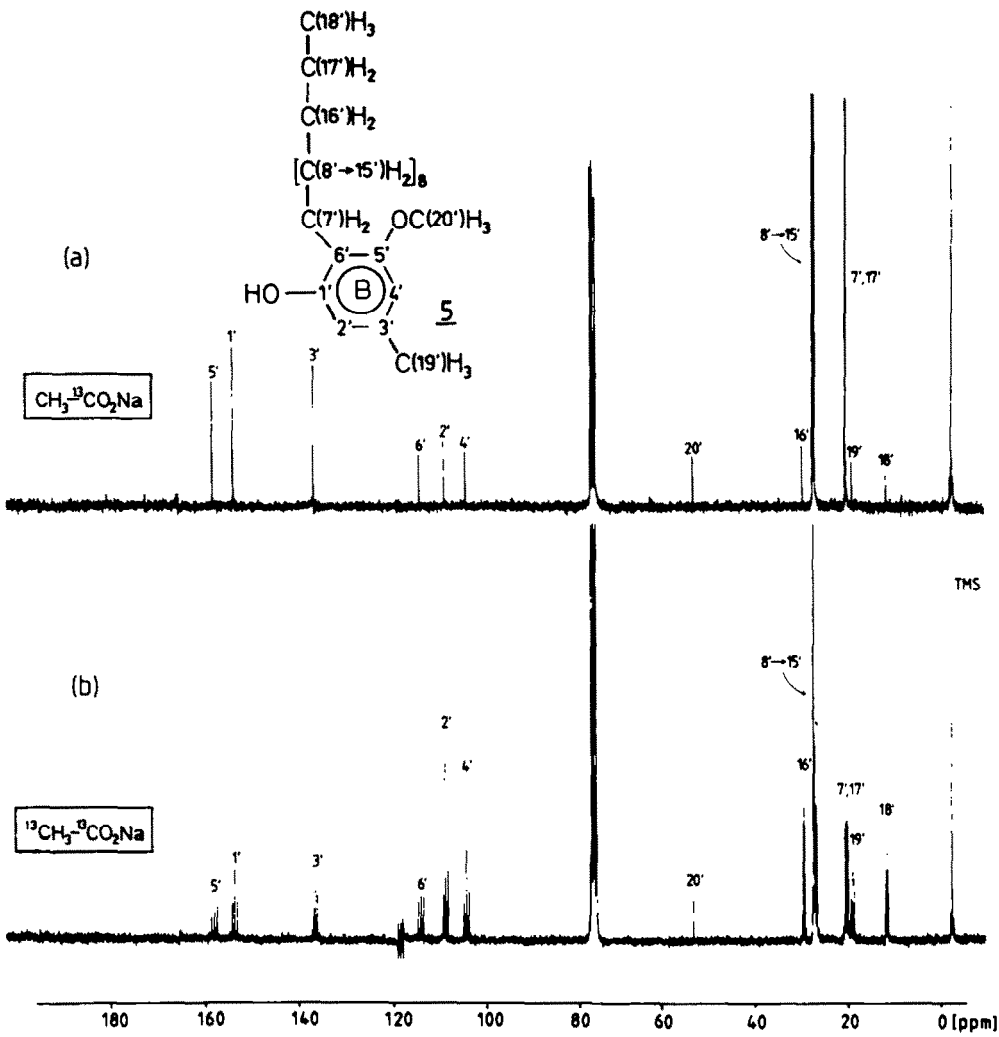


Fig. 2. ^{13}C-NMR spectra (noise decoupled) of degradation products 5 from flexirubins biosynthesised in the presence of (a) sodium [1 – ^{13}C]acetate and (b) sodium [1,2 – $^{13}C_2$]acetate.

incorporation is similar to that of the acetates, is incorporated via [1 – ^{14}C]acetate.

(2) *Studies with ^{13}C-labelled acetates*

^{13}C-NMR-spectroscopy in combination with ^{13}C-labelled acetates is a powerful technique for efficient study of the participation of acetate in biosynthetic pathways, particularly if doubly labelled acetate (= [1,2 – $^{13}C_2$]acetate) is used as the precursor.[8,9]

Because of comparatively poor incorporation of ^{14}C-acetates into flexirubin preliminary tests were carried out with highly diluted ^{14}C-acetates in order to optimise the fermentations and feeding conditions for maximum rates of enrichment (Table 5, experiments 9–12). In the two main experiments [1 – ^{13}C]acetate and [1,2 – $^{13}C_2$]-acetate were administered to cultures of *Fx. elegans*. The resulting flexirubins were isolated in the usual manner but without any addition of unlabelled flexirubin as a carrier.

The rates of total ^{13}C-enrichment in the isolated ^{13}C-

flexirubins were found to be 33% (with [1 – ^{13}C] acetate) and 51% (with 1,2 – $^{13}C_2$]acetate) by mass spectrometry.

The incorporation of ^{13}C can best be recognised and analysed in the cleavage products **4** and **5**, the ^{13}C-NMR spectra of which are shown in Fig. 1 and 2; these spectra will be discussed as follows:

Incorporation of ^{13}C-labelled acetate into the C_{25}-alcohol. Figure 1 presents the ^{13}C-NMR spectra of **4** from both the ^{13}C-acetate experiments.

The assignments and the observed alterations of signals caused by the ^{13}C-labelled precursors compared with unlabelled flexirubin are compiled in Table 2.

These data corroborate the facts already deduced from the ^{14}C-experiments:[7] the alcoholic carbon atom is derived from the carboxyl group of acetate. Acetate units obviously linked in the usual head-to-tail manner also build up the main part of the carbon chain. Ring A and the adjacent carbon atoms, however, do not originate from acetate. As mentioned above tyrosine is the precursor of this part of the flexirubin molecule.[7]

In the ^{13}C-NMR spectrum of the C_{20}-phenol (**5**) (Figure 2) the signals of 12 of the 20 carbon atoms can clearly be distinguished and unambiguously assigned as shown in Table 3 (column 1).

Table 3 also summarises the changes of the signals which are observed in Fig. 2 when ^{13}C-acetates were used as precursors. These data provide full proof that the dodecyl side chain originates from acetate units in a fatty acid mode and that the methyl group on ring B derives from C-2 of an acetate, the C-1 of which becomes the adjacent ring carbon atom.

It is of particular interest to consider the incorporation of ^{13}C from [1,2 – $^{13}C_2$]acetate into positions 2′ and 4′: the ^{13}C-signals of these two atoms show the same patterns which are significantly different from all other signals in the spectrum since each of them is composed of a doublet *and* an enhanced central singulet. Whereas the doublet originates from incorporation of an intact C_2-unit from acetate, the enhancement of the singulet in the [1,2 – $^{13}C_2$]-experiment must be caused by incorporation of one carbon atom of acetate combined with loss of the adjacent one. As can be seen by comparison with the corresponding signals in the spectrum of the C_{20}-phenol from the [1 – ^{13}C]acetate experiment, incorporation of the C-2 from acetate and loss of its carboxyl group is responsible for the enhanced singulets. After the decarboxylation step carbon atoms 2′ and 4′ obviously become equivalent; therefore, in the case of the [1,2 – $^{13}C_2$] acetate experiment, one of these carbon atoms collapses from a doublet to a singulet, whereas the other one remains a doublet since it originates from incorporation of an intact acetate unit.

This observation and the other data from Table 3 support the formation of the benzene ring B in flexirubin via orsellinic acid (**8**) or 3-dodecylorsellinic acid (**9**) according to Scheme 2. From the equivalent ^{13}C-resonance patterns of carbons 2′ and 4′ in the ^{13}C-labelled flexirubin (or its C_{20}-phenol) it can be concluded, that after the biosynthetic decarboxylation step of **8** or **9** has occured, this part of the flexirubin molecule must pass through a symmetrical structure (like **10** or **11**) before the ester bond with the polyene or its precursor is formed.

(3) *Investigations with colourless mutant of* Fx. elegans

Studies on accumulated metabolites in cultures of a colourless mutant of *Fx. elegans* supported the biosynthetic pathway outlined in Scheme 2: after cultivation in an [2 – ^{14}C]acetate-containing medium, the fermentation broth was investigated in order to establish the presence of radioactive **8**, **9**, **10** and/or **11** by the isotope dilution technique. The result was, that 3-dodecyl-orsellinic acid (**9**) and 2-dodecylorcine (**11**) could clearly be identified

Table 2. Assignments of ^{13}C-NMR resonances of **4** (Fig. 1) and alterations of signals when ^{13}C-labelled acetates were administered to cultures of *Fx. elegans*

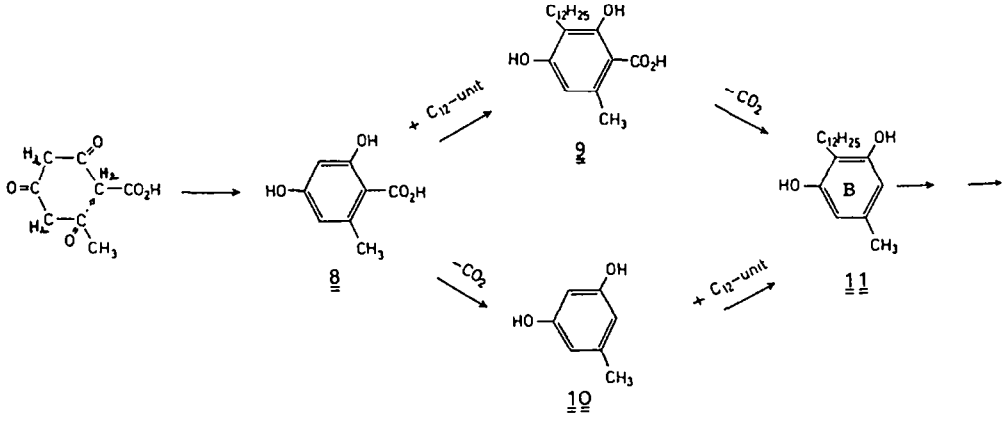
C-Atom	^{13}C-Resonance (δ-Values)	Observed alteration of signal after feeding with: [1-^{13}C]Acetate	[1,2-$^{13}C_2$]Acetate
1	63.2	Enhancement	Triplet
2	33.0	None	Triplet
3	25.9	Enhancement	Triplet
4 - 15	29.1 - 30.1	Partial Enhancement	Partial Triplets
16	31.9	None	None
17	35.2	None	None
18	134.9	None	None
19	131.0	None	None
20	126.5	None	None
21	156.1	None	None
22	110.1	None	None
23	126.5	None	None
24	16.2	None	None
25	55.5	None	None

Table 3. Assignments of ^{13}C-NMR resonances of **5** (Fig. 2) and alterations of signals when ^{13}C-labelled acetates were administered to cultures of *Fx. elegans*

C-Atom	^{13}C-Resonance (δ-Values)	Observed alteration of signal after feeding with: [1-^{13}C]Acetate	[1,2-$^{13}C_2$]Acetate
1'	154.3	Enhancement	Triplet
2'	109.1	None	Triplet[a]
3'	136.9	Enhancement	Triplet
4'	104.6	None	Triplet[a]
5'	158.7	Enhancement	Triplet
6'	114.4	None	Triplet
7'	23.0[b]	Enhancement	Triplet
8' - 15'	29.1 - 30.0	Partial Enhancement	Partial Triplets
16'	32.0	None	Triplet
17'	22.7	Enhancement	Triplet
18'	14.1	None	Triplet
19'	21.5	None	Triplet
20'	55.7	None	None

a) In addition the central signal significantly enhanced.

b) Assignment by single-frequency off-resonance ^{1}H-decoupling.



Scheme 2. Possible biosynthetic pathway to ring B of flexirubin (**1**).

among the radioactive metabolites, and orsellinic acid (**8**) was also radioactive, but to a considerably lower extent, whereas orcine (**10**) did not contain measurable radioactivity.

(4) *Competition experiments with ^{14}C-acetate and inactive intermediates*

In another experiment [1-^{14}C]acetate was administered to cultures of *Fx. elegans* together with (a) inactive orsellinic acid (**8**) and (b) inactive 3-dodecylorsellinic acid (**9**). The ^{14}C-flexirubins were isolated and degraded according to Scheme 1. Both additives did not significantly influence the rate of ^{14}C-incorporation into flexirubin. The observed distribution of radioactivity in flexirubin and its degradation products is given in Table 4.

The data in Table 4 again show that **9** is a biosynthetic intermediate of ring B in flexirubin: in the presence of this acid incorporation of acetate into **5** is suppressed and then it is mainly found in the C_{25}-alcohol **4**, which contains the polyene chain and the ring A. In agreement with this competing situation is the observation, that the distribution of the remaining radioactivity within **5** was found to be similar as in the experiment without addition of **9**.

Quite another effect was observed when the culture was grown in the presence of orsellinic acid: this acid does not influence the ratio of radioactivity between the cleavage products **4** and **5** so much, but it has a dramatic effect on the distribution of radio-activity within **5**: in the presence of orsellinic acid (**8**) the concentration of radioactivity is significantly enhanced in the C_{12}-substituent, which is apparently formed independently from acetate

Table 4. Distribution of radioactivity in degradation products of flexirubin from cultures with [1 – ^{14}C]acetate in the presence of orsellinic acid (**8**) and 3-dodecylorsellinic acid (**9**)

Precursor	Distribution of Radioactivity				
	Dimethylether **3**	C_{25}-Alcohol (**4**)	C_{20}-Phenol (**5**)	**6**	**7**
[1-^{14}C]Acetate	100%	41%	63% (=100%)	(62%)	(13%)
[1-^{14}C]Acetate + **8**	100%	48%	49% (=100%)	(86%)	(3%)
[1-^{14}C]Acetate + **9**	100%	75%	24% (=100%)	(56%)	(11%)

units and then linked to **8** to give **9**, which later on is decarboxylated and incorporated into flexirubin via **11** as outlined in Scheme 2. In summary our investigations on the biosynthesis of flexirubin allow to draw the following conclusions:

(a) all carbon atoms of ring B and its alkyl substituents are derived from acetate,

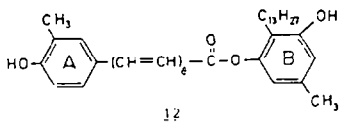
(b) this part of the flexirubin molecule is built up independently from the ω-phenylpolyene chain (or its precursor via orsellinic acid (**8**) → 3-dodecylorsellinic acid (**9**) → 2-dodecylorcine (**1**) (Scheme 2), and

(c) acetate is the building unit for carbon atoms C – 1 to C – 14 of the polyene chain, whereas tyrosine acts as precursor of ring A and carbon atoms C – 17 to C – 15; the methyl group on ring A is derived from methionine.[7]

(5) *The role of propionate in the biogenesis of flexirubin*

Preliminary screening tests of potential precursors for **1** revealed a good incorporation of radioactivity from [1 – ^{14}C]propionate (Table 5; experiment 13). The results from the experiments with ^{14}C- and ^{13}C-labelled acetates strongly suggested that an incorporation of C-1 from propionate into flexirubin eventually might occur by transcarboxylation reactions via acetate. But this assumption was in contradiction to degradation experiments, which showed, that more than 90% of the incorporated radioactivity from [1 – ^{14}C]propionate are located in the tridecanoic acid p-bromophenacyl ester (**6**), which is obtained from the alkyl substituent of ring B by ozonolysis according to Scheme 1.

As further investigations demonstrated, **6** contains a very small amount of tetradecanoic acid p-bromophenacyl ester. This higher homologue of **6** amounts to a concentration of approx. 0.8% of the C_{13}-acid ester and is therefore not detected in routine degradation experiments. However, separation of the C_{14}-acid ester by high performance liquid chromatography (HPLC) of the p-



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bromophenacyl esters showed that the highly purified C_{13}-acid ester is inactive and the C_{14}-acid derivative carries all the radioactivity from [1 – ^{14}C] propionate.

Thus, propionate acts as the starter molecule for the biogenesis of a C_{13}-side chain on the benzene ring B (Scheme 2), e.g. in a homoflexirubin **12**. Similar concentrations of the C_{14}-acid were also found in the C_{13}-acids derived from the chemical degradation of flexirubin from other fermentation experiments without the addition of propionate (e.g. with ^{14}C-acetate; Table 5; experiments 1 and 2). This hitherto unknown pigment, which accompanies flexirubin and which is synthesised under incorporation of propionate, therefore, appears to be a normal, but quantitatively very minor component of the standard pigment pattern of *Flexibacter elegans*.

EXPERIMENTAL

If not stated otherwise, instruments and experimental conditions were as described in earlier publications.[7] Radioactivity measurements were carried out as follows: 2–8 mg substance were dissolved in toluene: 2 ml of this solution together with 5 ml Lipoluma scintillation cocktail (Lumac) were put into a counting vessel and measured in a scintillation counter for 10 min simultaneously with a blank. UV/VIS-spectra were run in methanol (p.a.), except for flexirubin and flexirubin dimethyl ethers, for which hexane (Uvasol) was used. IR-spectra were recorded in chloroform with a Perkin-Elmer 457 instrument. If not stated otherwise, ^{13}C-NMR- and ^{1}H-NMR-spectra were recorded on a WM 250 instrument (Bruker-Physik) in deuterio-chloroform with tetramethylsilane as the internal standard; ^{13}C-NMR data are from noise decoupled spectra. If not stated otherwise, mass spectra were run on a SM 1B spectrometer (Varian-MAT) at 70 eV; resolution: M/ΔM = 1000. The gas chromatographic (GC) conditions were: 2m-Ni-columns (1/8 in.), filled with 2% SE 30 on Chromosorb W (AW-DMCS, 60/80 mesh); carrier gas: He (100 ml/min); detector: WLD (T = 300°); temperature program: starting at 100° with an increase of 6° min. For high performance liquid chromatography (HPLC) we used a Waters instrument equipped with a pump M 6000 A, an injection system U6K and a UV/VIS-photometer M 440. The column (4 mm × 30 cm) was filled with Nucleosil 10-C_{18} (Macherey-Nagel); solvent system: acetonitrile/water (85/15); flow rate: 1 ml/min; detection: at 254 nm; retention times: t_R = 19 min for tridecanoic acid (= C_{13} acid) p-bromphenacyl ester and t_r = 25 min for myristic acid (= C_{14} acid) p-bromophenacyl ester. If not otherwise stated, for column chromatography silica gel 60 (< 200 mesh; Macherey-Nagel) was used. Thin layer chromatography (TLC) was performed on Nano-plates SIL-20 UV_{254} (Macherey-Nagel) in benzene/ethyl acetate (9/1), if not otherwise stated. For preparative TLC we used the same solvent system and plates coated (0.5 mm) with silica gel P/UV_{254} (Macherey-Nagel) and prewashed with chloroform and methanol.

Microorganisms and culture conditions

Microorganisms and culture conditions were according to ref.[10] The mode of fermentation, the volume of cultures, the uptake of labelled compounds and their incorporation into flexirubin are summarised in Table 5.

Table 5. Precursor experiments: mode of fermentation, uptake of labelled compounds, incorporation into flexirubin

Experiment no.	Labelled precursor			Fermentation				Flexirubin			
	Compound (as sodium salt)	Specific radioactivity [Ci/mol] or enrichment [%]	Amount added to culture [mg]	Method	Volume of culture [l]	Time [h]	Cell material[a]	Specific radioactivity [DPM/µg]	[mCi/mol]	Rate - incorporation [%]	Absolute amount[b] [µg]
1	[1-^{14}C]Acetate	59.5	1.26	A	3	6	15	1530	440	0.07	970
2	[2-^{14}C]Acetate	60.2	0.345	B	0.75	16	11.5	418	120	0.03	370
3	[1-^{14}C]Butyrate	55	1.02	B	1.5	14	15.9	1409	370	0.02	170
4	[1-^{14}C]Acetate + 8[c]	58.7	0.188	C	0.75	16	24.1	1176	330	0.07	170
5	[1-^{14}C]Acetate + 9[d]	58.7	0.188	C	0.75	16	23.4	3375	9500	0.04	40
6	[1-^{13}C]Acetate	93%	5660	D	40	24	-	-	-	-	11200
7	[1,2-$^{13}C_2$]Acetate	C-1 ca. 93%; C-2 ca. 92%	5492	D	40	24	-	-	-	-	13500
8	[2-^{14}C]Acetate	60.2	0.42	A[e]	1.5	14	30.5	-	-	-	-
9a	[1-^{14}C]Acetate	59.1	0.014	A	0.05	14	16.5	239	69	0.07	61
9b		1.93	0.425	A	0.05	14	15.2	203	58	0.06	61
9c		0.2	4.115	A	0.05	14	7.2	213	61	0.06	60
10a		57.2	0.014	A	0.05	16	23.3	564	160	0.1	38
10b		0.4	2.06	A	0.05	16	21.3	536	150	0.095	39
10c		0.2	4.115	A	0.05	16	17.7	370	100	0.07	39
10d		0.1	8.2	A	0.05	16	12.1	305	88	0.06	42
10e		0.05	16.4	A	0.05	16	6.9	125	35	0.02	39
10f		0.033	24.6	A	0.05	16	5.2	122	34	0.02	39
11a		59.1	0.014	A	0.05	10	19.0	636	180	0.08	26
11b		0.1	8.2	A	0.05	10	9.0	236	66	0.02	19
11c		0.05	16.4	A	0.05	10	5.6	156	44	0.02	27
11d		0.033	24.6	A	0.05	10	3.5	111	32	0.01	26
11e		0.025	32.8	A	0.05	10	2.6	89	25	0.01	28
11f		0.02	41.0	A	0.05	10	2.1	71	20	0.008	25
12a		57.2	0.014	A	0.1	16	24.9	746	100	0.23	68
12b		0.04	20.5	A	0.1	16	9.4	276	75	0.09	73
12c		0.02	41.0	A	0.1	16	4.7	102	30	0.03	71
12d		0.01	82.0	A	0.1	16	1.6	47	13	0.02	81
13	[1-^{14}C]Propionate	59	0.813	B	1.5	14	3.7	322	92	0.02	690

a) percentage of added radioactivity found in the cells. - b) by photometry. - c) 7.5 mg orsellinic acid (8). - d) 7.5 mg 3-dodecylorsellinic acid (9). - e) colourless mutant.

Method A. The experiments were performed at 35° in Fx A liquid medium (Fx A lm) as shake cultures; optical density was 0.3 – 0.4 at time of addition of the precursors.

Method B. The cell material from a dense pre-culture was harvested by centrifugation, resuspended in a small volume of Fx A lm (adjusted to pH 4.5 with 1 N HCl) and then incubated with the labelled precursor. After 1 hour at 35° the culture was diluted by addition of fresh Fx A lm to an optical density of 0.3 – 0.4 and further fermented at 35°.

Method C. Conditions as method A, but simultaneously with the addition of radioactive precursors inactive compounds (10 μg/ml culture medium) were added.

Method D. The fermentations were performed in Fx A lm at 35° using a Biostat fermentor with continuous pH-regulation to pH 7.2 (with 2N H_2SO_4). For agitation a 3-turbine-stirrer was used and 1 ml silicone antifoam emulsion was added.

Colourless mutants of strain *Fx e*1 were obtained by treating cell suspensions in 0.05 M tris/HCl buffer at pH 7.1 with 100 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for 4 h. The cells were then harvested, washed, and transferred to Fx A lm. After 3 h of incubation the resulting cell population was plated on cy-agar. Pale colonies were isolated, cloned, and characterised with respect to their pigmentation. All colourless mutants still contained traces of flexirubin (0.05 μg/mg dry cells and less, compared with 0.70 μg/mg in the wild strain). Mutant B 27 was chosen for the experiments described in this article. Cultivation of the mutant was performed according to method A.

Flexirubin, which was needed for isotopic dilution, was isolated as described earlier.[3]

Derivation and degradation of labelled flexirubins

Methylation, hydrogenation and cleavage of the ester group were carried out as described earlier.[3] In the case of the ^{14}C-flexirubins the degradations were started with about 30 mg **2** (diluted with inactive **2**) and in case of the ^{13}C-labelled flexirubins with about 15 mg **2** (without dilution).

Preparation of p-bromophenacyl esters **6** *and* **7**: 9 to 13 mg of the C_{20}-phenols **5** were dissolved in 50 ml CH_2Cl_2; the temperature of this solution was held at 0° to −2°C and a stream of ozone/O_2 (0.4 g O_3/h) was passed through until O_3 could be detected at the exit of the reaction vessel (after 40 min). After warming up to room temperature 10 ml H_2O_2 (30%) and 20 μl formic acid (p.a.) were added, and the reaction mixture was stirred for 12 h at room temperature and then 1 h under reflux. After cooling to room temperature excess H_2O_2 was destroyed by adding *ca.* 1 mg platinum black and stirring for another 12 h. After filtration the reaction mixture was brought to pH 9 with methanolic KOH solution, evaporated to dryness *in vacuo* and the residue esterified with p-bromo-phenacyl bromide according to Ref.[11] Separation of the reaction products was achieved by column chromatography on 30 g silica gel using benzene/ethyl acetate (98/2) as solvent and yielded 10–13 mg tridecanoic acid p-bromophenacyl ester **6** and acetic p-bromophenacyl ester **7** as crystalline compounds. **6**: m.p. 74–75° (ref[12]. 75°), GC: T_D = 250°; **7**: m.p. 84–85° (ref.[12] 86°), GC: T_D = 160°.

^{14}C-Flexirubins, ^{14}C-labelled derivatives, and degradation products

The ^{14}C-flexirubins were usually separated from the cultures by extraction of the cells with acetone. If not stated otherwise, the purification was performed as described earlier.[10] In experiments No. 4 and 5 the ^{14}C-flexirubins were isolated from the crude acetone extracts (70 and 65 mg) of the bacteria by twofold preparative TLC using benzene/ethyl acetate (9/1) and chloroform/methanol (95/5). In the main experiments the isolation procedures yielded the quantities summarised in Table 6.

These ^{14}C-flexirubins were diluted to approximately 10 mg by addition of unlabelled **1** and converted into their dimethyl ethers. Degradation experiments were carried out with 1/3 of these ^{14}C-dimethyl ethers **2**, which for this purpose were again diluted by addition of unlabelled **2** as compiled in Table 7, then hydrogenated to the ^{14}C-hexadecahydroflexirubin dimethyl ethers **3**, whose specific radioactivity was determined precisely. Table 7 also summarises the specific radioactivities found in degradation products **4**, **5**, **6** and **7**.

Quantitative determination of the p-bromophenacyl ester of myristic acid (= C_{14}-acid) in the p-bromophenacyl ester **6** *obtained by ozonolysis of* **5** *from experiment No.* 13

(a) *By photometry.* About 1 mg of the p-bromophenacyl ester of the C_{13}-acid isolated from ozonolysis of the C_{20}-phenol was dissolved in CH_3OH (p.a.); 4 × 15 μl of this solution were injected into the HPLC system, and the fractions with t_R = 19 and t_R = 25 min were collected. According to planimetry of the HPLC peaks and photometry of the collected fractions the p-bromophenacyl ester of the C_{13}-acid contained 0.7 – 0.9% of the corresponding ester of C_{14}-acid.

(b) *By mass spectroscopy* (MAT 312/55200; Finnigan-MAT). For the determination we used the relative peak intensities of fragments $(M\text{-}Br)^+$ in the E.I. mass spectra (70 eV) of the p-bromophenacyl ester (*m/z* 331 in MS of pure C_{13}-acid ester and *m/z* 345 in MS of C_{14}-acid ester). The mass spectrum of the p-bromophenacyl ester of the C_{13}-acid isolated from the ozonolysis of the C_{20}-phenol showed relative peak intensities (*m/z* 331: *m/z* 345) = 133: 1; therefore, the isolated ester contained approx. 0.75% of a C_{14}-acid ester.

Determination of radioactivity in the p-bromophenacyl ester **6** *from experiment No.* 13

To a methanolic solution of 1.1 mg p-bromophenacyl ester of the long chain carboxylic acid derived from ozonolysis of **5** 1.3 mg of synthetic p-bromophenacyl ester of myristic acid (= C_{14}-acid) were added. 1.8 mg of this mixture were separated

Table 6. Amounts of ^{14}C-flexirubins isolated from cultures in experiments No. 1 to 5 and 13

Experiment no.	Precursor	^{14}C-Flexirubin isolated [mg][a]
1	[1-^{14}C]Acetate	0.97
2	[2-^{14}C]Acetate	0.37
3	[1-^{14}C]Butyrate	0.17
4	[1-^{14}C]Acetate + **8**	0.17
5	[1-^{14}C]Acetate + **9**	0.04
13	[1-^{14}C]Propionate	0.69

a) by photometry

Table 7. ^{14}C-Labelled flexirubin dimethyl ethers and their degradation products (amounts and specific radioactivities)

Experiment no.	2		3[a]		4		5		6		7	
	[mg]	Diluted to [mg]	[mg]	[cpm/μmol]	[mg]	[cpm/μmol]	[mg]	[cpm/μmol]	[mg]	[cpm/μmol]	[mg]	[cpm/μmol]
1	1.5	34.4	27.6	1118	10.2	414	9.4	697[b]	12.5	222	4.7	47
2	2.7	20.1	16.6	700	7.7	278	5.7	432[c]	11.3	131	6.5	26
3	3.1	37.6	28.5	1734	11.2	556	12.4	1152	12.4	739	4.2	127
4	3.0	15.8	16.0	2725	6.6	1308	5.6	1330[d]	10.9	489	6.4	17
5	2.3	17.7	14.6	620	6.3	465	5.2	147[e]	9.6	47	4.2	9
13	3.2	35.0	29.1	1244	11.4	68	12.8	1175	10.8	9[f]	3.8	0

a) Used for degradation. - b) Diluted to 355. - c) Diluted to 210. - d) Diluted to 575. - e) Diluted to 80. - f) After purification by HPLC.

by HPLC (30 injections à 60 µl). Fractions with $t_R = 19$ and $t_R = 25$ min were collected. Table 8 summarises the results of photometric and radioactivity measurements.

^{13}C-Flexirubins, ^{13}C-labelled derivatives, and degradation products

The dried acetonic extracts (experiment No. 6: 0.8 g; experiment No. 7: 1.2 g) of the wet bacteria were separated by column chromatography on 12 g Al_2O_3 (neutral, Woelm, activity II-III) using 50 ml benzene/ethyl acetate (9/1) and then chloroform/methanol (95/5). The coloured fractions were combined (experiment No. 6: 130 mg; experiment No. 7: 165 mg) and again separated on 30 g silica gel (column ø: 2.5 cm) with 200 ml benzene/ethyl acetate (9/1) into 40 fractions. According to TLC, fractions 12 to 25 contained the pure flexiburins. Yields were 11.2 mg ^{13}C-flexirubin (from experiment No. 6: [1-^{13}C]acetate) and 13.5 mg ^{13}C-flexirubin (from experiment No. 7: [1,2-$^{13}C_2$]acetate).

Methylations, catalytic hydrogenations, the reductive cleavages into **4** and **5**, and the separation by preparative TLC were carried out as described above.

From experiment No. 6 yields were: 4.3 mg [^{13}C]-**4**, MS: *M/Z* = 379(5%), 378(14), 377(33), 376(51, M^+ for $^{12}C_{25}$), 136(10) 135(100), 121(6). ^{1}H-NMR: $\delta = 6.96$(2 H, m), 6.74(2 H, d, J = 9 Hz), 3.81(3 H, s), 3.66(2 H, t, J = 6.5 Hz), 2.50(2 H, t, J = 8 Hz), 2.21(3 H, s), 1.64 - 1.43 and 1.43 - 1.17 (*ca.* 30 H), 0.88(1 H, m). ^{13}C-NMR: see Fig. 1 and Table 2.

[^{13}C]-**5**: 4.6 mg. MS: *m/z* = 308(5%), 307(11), 306(18, M^+ for $^{12}C_{20}$), 153(8), 152(35), 151(100), 121(10). ^{1}H-NMR: $\delta = 6.30$(1 H), 6.27(1 H), 4.59(1 H), 3.79(3 H, s), 2.56(2 H, t, J = 7.5 Hz), 2.27(3 H, s), 1.57(H_2O). 1.55 - 1.41(*ca.*3 H, m), 1.41 - 1.15 (*ca* 17 H), 0.88(3 H, t, J = 7 Hz). ^{13}C-NMR: see Fig. 2 and Table 3.

From experiment No. 7 yields were: 4.6 mg [^{13}C]-**4**. MS: *m/z* = 381(5%), 380(12), 379(14), 378(27), 377(24), 376(47, M^+ for $^{12}C_{25}$), 136(13), 135(100), 121(6). ^{1}H-NMR: cf [^{13}C]-**4** from experiment No. 6 ^{13}C-NMR: see Fig. 1 and Table 2.

[^{13}C]-**5**: 5.2 mg. MS: *m/z* = 310(5%), 309(6), 308(8), 307(10), 306(18, M^+ for $^{12}C_{20}$), 155(5), 154(13), 153 (27), 152(38). 151(100), 123(4), 122(4), 121(10). - ^{1}H-NMR: *cf.* [^{13}C]-**5** from experiment No. 6. ^{13}C-NMR: see Fig. 2 and Table 3.

Model compounds for ^{13}C-NMR spectroscopy (Positions of carbon atoms analogous to Figs. 1 and 2)

(a) *1,3-Dimethoxy-2-dodecyl-5-methylbenzene (2-dodecylorcinol dimethyl ether)*:[3] ^{13}C-NMR: $\delta = 158.4$(*C*-1, *C*-5), 136.4(*C*-3), 117.0(*C*-6), 1.4.9(*C*-2, *C*-4), 55.7(C^1-*O*CH_3, C^5-*O*CH_3), 32.0(C-16), 29.9 - 29.4(C-8 to C-15), 22.8(C-7 and C-17), 22.0(C-19), 14.1(C-18); C-7 was assigned by single-frequency off-resonance ^{1}H-decoupling.

(b) *1-Acetoxy-2-dodecyl-3-methoxy-5-methylbenzene (2-dodecyl-3-methoxy-5-methylphenyl acetate)*:[3] ^{13}C-NMR: $\delta = 169.7$(C^1-OCOCH$_3$), 158.6(C-5), 149.7(C-1), 136.8(C-3), 121.0(C-6), 115.3(C-2), 109.4(C-4), 55.7(C-20), 32.0(C-16), 29.9 - 29.3(C-8 to C-15), 23.9(C-7), 22.8(C-17), 21.5(C-19), 20.9(C^1-OCO*C*H$_3$), 14.1(C-18).

(c) *2-Dodecyl-1,3-dihydroxybenzene (2-dodecylorcinol)*:[3] ^{13}C-NMR: $\delta = 154.7$ (C-1, C-5), 137.2(C-3), 112.6(C-6), 108.9(C-2, C-4), 32.1(C-16), 29.9 - 29.4(C-8 to C-15), 23.1(C-7), 22.8(C-17), 21.1(C-19), 14.2(C-18).

(d) *5-(4-Methoxy-3-methyl-2-phenyl)-pentane-1-ol*: 30 mg 5-(4-methoxy-3-methyl-phenyl)-2,4-pentadiene-1-al[13] were dissolved in 5 ml ethyl acetate and hydrogenated in the presence of *ca* 10 mg Pd/C (10%) at room temperature for 1 h; purification by column chromatography on silica gel using benzene/ethyl acetate (9/1) yielded: 17 mg viscous oil, $R_F = 0.7$. - UV(CH_3OH): λ_{max} = 277, 283 nm. MS: *m/z* = 208 (19%, M^+), 191(14), 190(45), 189(16), 188(6), 175(6), 162(8), 161(15), 149(8), 148(8), 136(12), 135(100). ^{1}H-NMR: $\delta = 6.95$(2 H, m), 6.73(1 H, d, J = 9 Hz), 3.80(3 H, s), 3.71 - 3.31(2 H, m), 2.53(2 H, t, J = 7.5 Hz), 2.20(3 H, s), 1.70 - 1.48(4 H, m), 1.48 - 1.29(2 H, m). ^{13}C-NMR: $\delta = 156.0$(C-21), 134.4(C-18), 130.0(C-19), 126.4 and 126.3(C-20 and C-23), 110.0(C-20), 65.6(C-1), 55.4(C-25), 35.1(C-17), 31.7(C-16), 29.9(C-2), 26.1(C-3), 16.2(C-24).

Metabolites of the colourless mutant B 27 of Fx. elegans. 900 mg lyophilised cell material from 10 cultures each 150 ml were refluxed with 20 ml acetone for 5 h. The acetone extract was filtered and evaporated in vacuo giving 21 mg residue which was separated by preparative TLC with chloroform/methanol (9/1). The zone from $R_F = 0.52 - 0.72$ was extracted with chloroform/methanol (9/1), 25 mg of each 2-dodecylorcinol and orcinol were added and this solution separated on 30 g silica gel (column ø: 1.5 cm). With 60 ml benzene/ethyl acetate (9/1) 42 fractions, 2.5 ml each, were eluted; combined fractions No. 14-21 contained the 2-dodecyl-orcinol (**11**) and 31-36 the orcinol (**10**). Radioactivity measurement with **11**: 23 cpm/µmol; **10**: < 0.1 cpm/µmol (8.3 mg **10**: 265 counts/10 min; zero rate: 200 counts/10 min). The zone with $R_F = 0.0 - 0.15$ was suspended in acetone and methylated with methyl iodide using the standard procedure. After filtration and evaporation 14 mg of each methyl 2,4-dimethoxy-3-dodecyl-6-methyl-benzoate (permethylated 3-dodecylorsellinic acid) and methyl 2,4-dimethoxy-6-methylbenzoate (permethylated orsellinic acid) were added and this mixture separated like the above-mentioned orcinols. Fractions 11-15 contained permethylated 3-dodecylorsellinic acid and fractions 17-21 permethylated orsellinic acid. Further purification was achieved by chromatography on silica gel and on fractogel PVA 500 (Fa. Merck). Radioactivity measurement with permethylated **9**: 530 cpm/µmol; permethylated **8**: 5 cpm/µmol.

Orsellinic acid was synthesised from ethyl crotonate and ethyl acetoacetate according to ref.[14]

3-Dodecylorsellinic acid was synthesised by carboxylation of 2-dodecylorcinol (**11**) with methylmagnesium carbonate (2.5 M solution in dimethylformamide, Fluka).[15] The crude material was purified by chromatography over silica gel using chloroform/methanol (9/1) and then crystallised from methanol/water (6/4). 800 mg **11** yielded 520 mg colourless crystals with m.p.

Table 8. Results of photometric and radioactivity measurements of the HPLC-separated p-bromophenacyl esters after isotopic dilution

p-Bromophenacyl ester	Amount[a] [mg]	Specific radioactivity [cpm/µmol]
Prior to HPLC	2.4	1165
HPLC fraction: t_R=19 min (=C_{13}-acid ester)	0.57	9
HPLC fraction: t_R=25 min (=C_{14}-acid ester)	0.68	1218

a) by photometry

109/110°C. UV(CH_3OH): λ_{max} = 214, 257 nm. IR: 1655 cm^{-1} (CO). MS: m/z = 336(39%, M^+), 318(56), 301(19), 292(87), 164(59), 137(100). ^{1}H-NMR (360 MHz): δ = 11.72(1 H, s), 6.23(1 H, s), 5.17 (1 H, s, broad), 2.62(2 H, t, J = 7 Hz), 2.53(3 H, s), 1.69 – 1.43(2 H, m), 1.4 – 1.15(*ca.* 18 H, m), 0.89(3 H, t, J = 7.5 Hz).

Acknowledgements—This work was financially supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the Wissenschaftliche Gesellschaft in Freiburg.

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