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Aspects of the Biosynthesis of Carolic and Carlosic Acids in *Penicillium charlesii*. A ^{13}C NMR Study

TORSTEN REFFSTRUP and PER M. BOLL

Department of Chemistry, Odense University, DK-5230 Odense M, Denmark

The origin of two metabolic products of *Penicillium charlesii* NRRL 1887, carolic (*5*) and carlosic acids (*2*) has been reinvestigated in a ^{13}C labelling study. The main biosynthetic pathway was established to be the combination of a C_6 polyketide unit with a C_4 compound closely related to succinic acid in agreement with earlier findings. Repeated experiments showed that a bilateral mixing of intact acetate units occurred between the acetate pool and the incorporated C_4 unit. Dehydrocarolic acid could be converted to carolic acid in a cell free extract of *P. charlesii*, whereas an earlier proposal of carlosic acid as a precursor of carolic acid could not be confirmed. A new biosynthetic scheme is proposed considering oxalacetate as the actual C_4-precursor. On reaction with a β-ketocaproyl moiety the formed α-(α-ketosuccinyl)-β-ketocaproate (*1*) is considered the key intermediate in the biosynthesis of the 3-acyltetronic acids of the 5-methyl series as well as of the 5-carboxymethyl series. The occurrence of viridicatic acid (*6*) as a metabolite of *P. charlesii* was established.

The mould *Penicillium charlesii* has been shown to produce a number of tetronic acid derivatives.[1] The

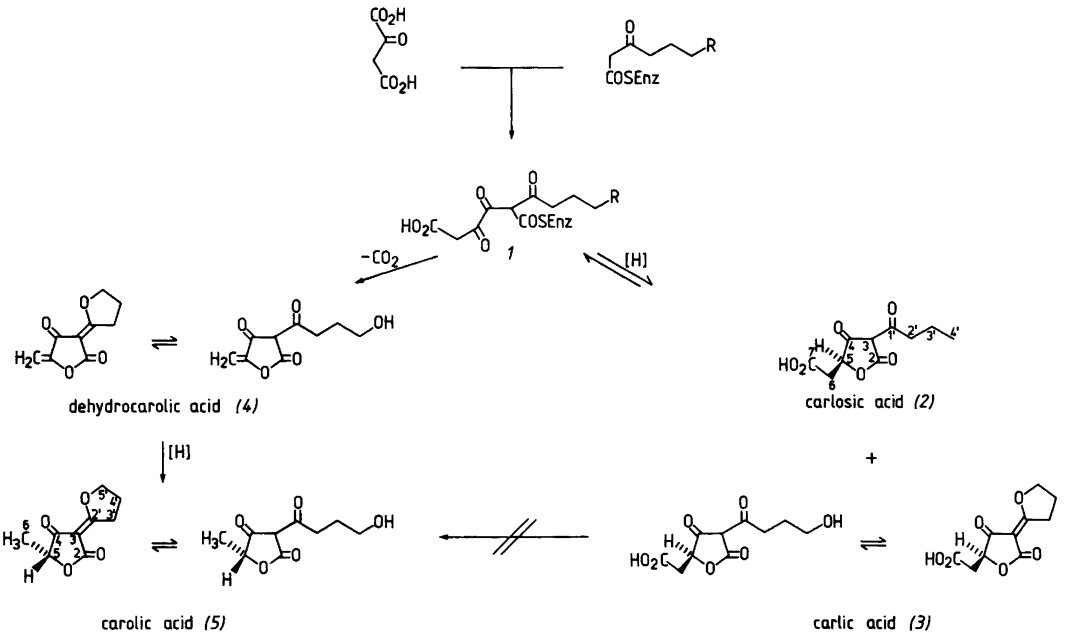

Fig. 1. Possible biosynthetic pathways for formation of tetronic acid derivatives from oxalacetate.

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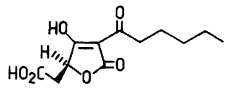
biosynthesis of two of the acids formed, carolic acid (*5*: (*E,R*)-5-methyl-3-(2′-tetrahydrofurylidene)tetrahydrofuran-2,4-dione) and carlosic acid (*2*: (*S*)-5-carboxymethyl-3-butanoyltetrahydrofuran-2,4-dione), has previously been studied using a degradation technique on ^{14}C-labelled *5* and *2* formed from ^{14}C-labelled precursors fed to the mould.[2,3] Another paper[4] to some extent contradicts these results. Consequently we undertook the task by means of ^{13}C-tracer experiments to investigate in greater detail the biosynthesis of tetronic acids in *P. charlesii*. The experiments were performed analogous to Ref. 3 using the following precursors: [1-^{13}C], [2-^{13}C] and [1,2-^{13}C]acetate, [1-^{13}C] and [2-^{13}C]pyruvate, [1-^{13}C]propionate, [1,4-^{13}C] and [2,3-^{13}C]succinic acid, [4-^{13}C]5-methyltetronic acid (3 % label), and [1′-^{14}C] (*R,S*)-carlosic acid. A total of 23 experiments were carried out.



viridicatic acid *(6)*

In one preliminary large scale experiment a mixture of viridicatic (*6*: (*S*)-5-carboxymethyl-3-hexanoyltetrahydrofuran-2,4-dione), carlosic, and carolic acids was isolated. It was not possible to isolate viridicatic acid in pure state, but the mass spectrum was in full agreement with that of synthetic viridicatic acid.[5] This is, to our knowledge, the first reported finding of viridicatic acid as a metabolite of *P. charlesii*. However, in the following experiments viridicatic acid was only isolated in trace amounts and no labelling data were obtained.

EXPERIMENTAL

Culturing of the fungus and isolation of metabolites. The culture medium[3] was distributed in 1 l flasks (300 ml/flask) which were sterilized, inoculated evenly with a spore suspension of *Penicillium charlesii* NRRL 1887 and kept at 24 °C during the culture period of 12 days. The different precursors (*cf.* Table 1) were added on the eighth or ninth day.

Although the culturing conditions were rigorously specified, cultures of the same batch were found to develop unevenly, and consequently the cultures chosen for monitoring by UV measurements[3] were not always found representative.

The isolation of the tetronic acids was performed according to Ref. 3. The carolic acid was found in chloroform extracts of the acidified culture medium, and itaconic and carlosic acids, together with some carolic acid, were found in the ether extracts. The co-presence of these latter acids were established by mass spectrometry.

Attempts to separate carlosic and itaconic acids by thin-layer chromatography revealed that the content of carlosic acid in the ether extracts was low. Ion exchange chromatography on an ion exchange resin in formate form[6] established the carlosic acid content to be lower than 7 %. During the course of the investigation the mould apparently lost its ability to produce itaconic acid and the ether extracts appeared as red oils which proved very difficult to work up.

The evaporated ether extracts from experiments 1, 10 and 15 afforded upon solution in water and extraction, first by chloroform to remove carolic acid together with the coloured impurities and then by ether, carlosic acid in trace amounts. The carlosic acid content of the samples was too low to obtain any information with respect to the labelling pattern through the ^{13}C NMR spectra. Only from experiment 8 was a pure sample of carlosic acid obtained. Details of the performed isolations are given in Table 1.

Interpretation of the results. The ^{13}C NMR spectra of the enriched samples were compared to spectra of unenriched samples as references. For samples enriched with solitarily labelled precursors the enrichments given in Table 2 are divided into two groups consisting of high intensity and low intensity peaks, respectively. In the spectra of the enriched samples, the peak of lowest enrichment within each intensity group was adjusted in peak height to the height of the corresponding peak in the unenriched reference spectrum, and the adjustment factors found were used to normalize the peak heights of the signals of the enriched spectra, thus eliminating scattered background. The enrichment of each carbon is calculated as the increment in adjusted peak height relative to the height of the corresponding peak in the unenriched reference sample. An enrichment is regarded as specific if the enrichment, calculated as above, exceeds 0.50.

For samples exhibiting ^{13}C–^{13}C couplings the enrichment was found as the fraction of the sum of height of peaks exhibiting couplings to the height of the peak at the centre of the "triplet" in every carbon signal (Tables 3 and 4). The observation of a coupling was considered as a specific incorporation of an intact unit.

The assignments of the lines were made in accordance with earlier publications[7,8] with the exception of the assignments of C-1′ and C-4 in carlosic acid (Table 4) which had to be interchanged in order to explain the observed couplings.

In experiments 17 and 18 no specific incorporations were observed of the 3 % enriched [4-^{13}C]5-

Table 1. Tabulation of precursors fed and yields of pure metabolites isolated from incorporation experiments on *P. charlesii* NRRL 1887. Exp. No. 8 yielded 5 mg spectroscopically pure carlosic acid/flask; all other experiments did not yield any carlosic acid.

Exp. No.	Number of flasks	Precursor fed	mg precursor/ flask	mg spectroscopically pure carolic acid/flask
1	2	[1-^{13}C]sodium acetate	100	150
2	3	[1-^{13}C]sodium acetate	100	60
3	3	[1-^{13}C]sodium acetate	100	110
4	3	[1-^{13}C]sodium acetate	100	74
5	2	[2-^{13}C]sodium acetate	100	225
6	2	[1,2-^{13}C]sodium acetate	100	235
7	3	[1,2-^{13}C]sodium acetate	100	40
8	3	[1,2-^{13}C]sodium acetate	100	36
9	3	[1,2-^{13}C]sodium acetate	100	215
10	3	[2-^{13}C]sodium pyruvate	170	310
11	10	[1,4-^{13}C]succinic acid	100	186
12	1	[2,3-^{13}C]succinic acid	83	30
13	1	[2,3-^{13}C]succinic acid	83	50
14	1	[2,3-^{13}C]succinic acid	83	120
15	3	[1,4-^{13}C]succinic acid	100	310
16	2	[1,4-^{13}C]succinic acid	100	155
17	2	[4-^{13}C]γ-methyltetronic acid (3 % label)	75	75
18	2	[4-^{13}C]γ-methyltetronic acid (3 % label)	75	135
19	2	[1-^{13}C]sodium propionate	100	40
20	2	[1-^{13}C]sodium propionate	100	60
21	2	[1-^{13}C]sodium pyruvate	100	65
22	1	[1′-^{14}C](*R*,*S*)carlosic acid	70	160
23	1	[1′-^{14}C](*R*,*S*)carlosic acid	70	90

Table 2. Incorporation into carolic acid of solitarily ^{13}C-labelled precursors.[a]

Precursor added	[1-^{13}C]sodium acetate				[2-^{13}C]sodium acetate	[2-^{13}C]sodium pyruvate	[1,4-^{13}C]succinic acid		
Experiment No.	1[b]	2[b]	3[b]	4[b]	5[b]	10[b]	11[b]	15[c]	16[c]
C-6	0.49	0.23	0.14	0	0.24	0.18	0.02	0.39	0.60
C-4′	2.54	1.31	1.02	1.18	0	0.83	0.25	0	0
C-3′	2.02	0.60	0.44	0.47	1.11	0	0.05	0.29	0.51
C-5′	0.44	0	0.19	0.15	1.06	0.05	0.09	0.29	0.52
C-5	0	0.14	0	0.04	0.03	0.38	0	0.10	0.33
C-3	0	0	0	0	0.52	0	0	0	0
C-2	1.85	1.93	1.64	1.20	0.09	1.13	1.00	0.87	0.52
C-2′	1.46	2.18	1.54	0.55	0.29	0.54	0.69	0.41	0.39
C-4	0.08	0.61	0.98	0.29	0	0.05	2.38	1.02	1.47

[a] The signals are listed in increasing value of the chemical shift relative to TMS with deuteriochloroform as solvent. [b] Recorded on a JEOL Fx 60 NMR spectrometer. [c] Recorded on a JEOL Fx 60 Q NMR spectrometer.

Table 3. Incorporation into carolic acid of precursors ^{13}C-labelled at neighbouring carbon atoms.[a,b]

Precursor added	[1,2-^{13}C]sodium acetate				[2,3-^{13}C]succinic acid		
Experiment No.	6	7	8	9	12	13	14
C-6	0	0	0	0	0.14	0.17	0.27
C-4′	1.07	0.61	0.37	0.63	0	0	0
C-3′	1.04	0.50	0.38	0.59	0	0	0
C-5′	0.97	0.51	0.55	0.30	0	0	0
C-5	0.13	0.09	0.08	0.13	0.11	0.18	0.38
C-3	0.75	0.33	0.33	0.37	0	0	0
C-2	1.11	0.30	0.28	0.45	0	0	0
C-2′	0.82	0.28	0.27	0.35	0	0	0
C-4	0.14	0.06	0.11	0.09	0	0	0

[a] The signals are listed in increasing value of the chemical shift relative to TMS with deuteriochloroform as the solvent. [b] Recorded on a JEOL Fx 60 Q NMR spectrometer.

methyltetronic acid fed to the mould. Likewise experiments 19, 20, and 21 with propionate and pyruvate did not give rise to marked incorporation, and no conclusive evidence can be drawn concerning the incorporation of these compounds into the tetronic acids investigated.

All obtained samples of carolic acid and carlosic acid were recrystallized to satisfactory m.p.'s and were found spectroscopically pure.

Viridicatic acid. Upon isolation of carlosic acid from the ether extract obtained from 3 l of culture medium the remaining mother liquor contained a mixture of carlosic, carolic, and viridicatic acids judged from mass spectrometry. Some purification was obtained upon recrystallization from ether. The mass spectrum of the impure viridicatic acid was compared with that of synthetic viridicatic acid.[5] (Found for M^+: 256.0909. Calc. for $C_{12}H_{16}O_6$: 256.0947).

Table 4. Incorporation of [1,2-^{13}C]-sodium acetate into carlosic acid.[a,b]

Carbon No.	Experiment No. 8
4′	2.05
3′	1.62
6	0
2′	1.91
5	0
3	1.65
7	0
2	2.18
1′	1.98
4	0

[a] The signals are listed in increasing value of the chemical shift relative to deuteriomethanol as the solvent. [b] Recorded on a Bruker HX 270 NMR spectrometer.

[4-^{13}C]5-Methyltetronic acid (3 % label). [4-^{13}C]Carolic acid (1.0 g from experiment 11) was degraded[9] to [4-^{13}C]3-bromo-5-methyltetronic acid (3 % label), which upon hydrogenolysis afforded the title compound (yield: 0.37 g) specifically labelled and with a ^1H NMR spectrum in accordance with that published.[10]

[1′-^{14}C](R,S)-Carlosic acid. Sodium [1′-^{14}C]-butyrate (8.6 MB_q/mg, 1.08 mg) was reacted with 5 % HCl in abs. EtOH. Butyric acid (7.7 mg, 0.0875 mmol) was added and the reaction mixture was evaporated *in vacuo* at room temperature. After reaction with excess of $SOCl_2$ and evaporation 5-carboxymethyltetronic acid (16 mg, 0.1 mmol) and 0.3 mol of tin(IV) chloride were added and the acylation was carried out at 110 °C for 3 h.[11] Upon cooling, ice-cold 4 N HCl (20 ml) and (*R,S*)-carlosic acid (200 mg)[12] were added. The reaction mixture was extracted repeatedly with chloroform and the pooled organic phase was shaken with conc. aqueous sodium hydrogen carbonate. The aqueous layer was acidified with conc. HCl and extracted with chloroform. Upon evaporation of the dried chloroform phase and repeated recrystallization from ethyl acetate 146 mg of [1′-^{14}C](*R,S*)-carlosic acid of constant specific radioactivity was obtained (M.p. and NMR in agreement with the published values).[19]

Enzyme catalyzed conversion of dehydrocarolic acid to carolic acid. The washed mycelium from one flask cultivated for 10 days was frozen in liquid N_2 and passed through an Eaton press.[13] To 2 ml of the cell free extract was added 2 mg of dehydrocarolic acid and after 3 h the aqueous phase was extracted with ether. High performance liquid chromatography in ether revealed the results given in Table 5. The identity of the two compounds were secured by mass spectrometry. By total evaporation of the sample mixture the ratio of the

Table 5. The possible role of [1′-^{14}C](*R,S*)-carlosic acid as a precursor of carolic acid.

Experiment No.	[1′-^{14}C](*R,S*)-Carlosic acid[a] mg fed	dpm/mg	Carolic acid[b] mg isolated	dpm/mg
22	70	103924	160	178; 237
23	70	103924	96	193; 230

[a] The experiments were performed analogously to the experiments with ^{13}C-labelled precursors. [b] The isolated carolic acid was crystallized to constant activity.

molecular ion of dehydrocarolic and carolic acids changed from 1:9 to 13:1 by gradually increasing the sample temperature from 30 to 220 °C.

RESULTS AND DISCUSSION

As can be seen from Table 1 carolic acid could be isolated in all experiments and the labelling patterns found (Tables 2 to 4) were generally consistent with the incorporation patterns reported.[2,3] However, the detailed information obtained from the ^{13}C NMR spectra permits us to draw conclusions concerning questions which up to now have not been fully answered.

From the tabulated enrichments it can be seen that the general mode of formation of carolic acid involves the construction of a C_6-unit (carbon 2, 3, 2′, 3′, 4′ and 5′) having the anticipated labelling pattern for incorporation of intact C_2-units (*cf.* experiments 3, 4, 5, 6, 7, 8 and 9 in Tables 2 and 3). Experiments 1 and 2 are also in support of this mode, but the enrichment in carbon 3′ can only be explained by accepting another mechanism operating additionally, presumably fixation of labelled CO_2, formed by decarboxylation of carboxyl labelled acetate, into position 2 of an acetate unit.

In experiment 10 pyruvate labelled at C-2 was fed to the mould. This experiment exhibited clearly the same incorporation features as the experiments with carboxyl labelled acetate and led us to conclude that pyruvate is not incorporated into carolic acid as an intact entity, but after decarboxylation of the pyruvate the labelled acetate formed is incorporated in agreement with the experiments mentioned above.

The remaining part of the molecule (C-4, C-5, and C-6) is clearly derived from species closely related to succinic acid. This is suggested by the incorporation at C-4 in expeiment 11, 15 and 16 (Table 2) in which 1,4-doubly labelled succinic acid was fed to the mould, and it is confirmed by the observed incorporation of 2,3-doubly labelled succinic acid in experiments 12, 13 and 14 (Table 3) in which the signals from the only slightly enriched C-5 and C-6 are observable as doublets arising from the ^{13}C–^{13}C spin-spin coupling.

These results are consistent with those of Bentley *et al.*[3] and of Lybing and Reio.[2] The latter authors suggested that C-4, C-5, and C-6 in carolic acid were derived from a C_4-component of the citric acid cycle, a suggestion which does not seem fully justified on the basis of their experiments carried out only with [1-^{14}C]acetate. Bentley *et al.*[3] on the other hand, find that on adding [2,3-^{14}C]succinate as a precursor, 12.9 % of the added radioactivity was incorporated into carolic acid with most of the activity equally divided between C-5 and C-6. They state that their feeding experiments clearly demonstrate that whereas C-5 and C-6 indeed can be derived from a C_4-dicarboxylic acid, such an acid is only a poor source of the other carbon atoms of carolic and carlosic acids. The same problem arises on considering carbon atoms 2, 3, 2′, 3′, 4′ and 5′ as a subunit bearing the normal polyketide labelling pattern. A consequence of accepting this hypothesis is that metabolism of labelled acetate through the citric cycle should give rise to a labelled C_4-dicarboxylic acid with a consequent marked incorporation of activity from acetate into C-4, C-5 and C-6. This is not observed in the earlier studies.[2,3]

The use of ^{13}C-labelled acetate makes it possible to observe a slight, but specific enrichment into the C_4-derived moiety of the molecule (experiments 2, 3, 6, 7, 8 and 9), which could be arrived at through the glyoxylic bypass of the citric acid cycle. Analogously a likewise slight, but rather unspecific incorporation into the polyketide derived part of the molecule is observed on feeding labelled succinate (experiments 11, 15 and 16).

Consideration of the level of incorporation of acetate into the two parts of the molecule reveals that acetate incorporation into carbons 2, 3, 2', 3', 4' and 5' is generally much larger than the incorporation into the C_4-derived part of carolic acid. This difference in the magnitude of the incorporation can be very clearly observed in the experiments involving doubly labelled acetate.

The observed homogenity of the acetate incorporation into the C_6-carbon chain precludes the biogenetic pathway proposed [3,4] which involves an acylation reaction of 5-methyltetronic acid, since it is unlikely that the same level of incorporation of acetate could be obtained for acetate incorporated through different routes.

In the experiments, in which succinic acid was fed to the mould, lack of incorporation of the labelled acetate formed from succinate was observed in the CH_3-terminal end of the polyacetyl chain. This feature has been remarked earlier [3] and could be ascribed to the fact that metabolism is rapidly ceasing. We therefore suggest that the degradation of succinate to acetate occurs too late on the time scale to enable acetate to reach the centres of the enzymes on which the polyacetyl chain is initiated by the starter unit, but acetate is able through malonate to be incorporated in the polyacetyl chain.

Experiment 8, the only experiment from which labelled carlosic acid could be isolated in pure state, revealed that the doubly labelled acetate administered to the mould was incorporated specifically into carbon atoms 2, 3, 1', 2', 3' and 4' (Table 4) and the $^{13}C-^{13}C$ couplings observed demonstrate, in agreement with the findings of Lybing and Reio [2] that a C_6-chain formed from three intact units of acetate gives rise to C-2 and C-3 together with the butyroyl side chain. From the ^{13}C-NMR spectra it is apparent that no intact acetate unit can be observed incorporated in the remaining part of the carlosic acid molecule.

Bloomer *et al.*[4] from their replacement culture experiments give carlosic acid a role as a precursor of carolic acid. We have not been able to observe such a relationship between carlosic and carolic acids (*cf.* Table 5).

Additionally we have proved that dehydrocarolic acid (*4*: (*E*)-5-methylene-3-(2'-tetrahydrofurylidene)-tetrahydrofuran-2,4-dione) in a cell-free extract of *P. charlesii* can be converted to carolic acid (*5*) (for details *cf.* Experimental and Table 6).

The occurrence of 5-methyltetronic acid as a genuine metabolite of *P. charlesii* can be questioned. The compound has been detected in a culture filtrate (by paper chromatography [3] and not specified [4]) and has been isolated from *P. charlesii* [14] and *P. fellutanum*,[15] but it was obtained after incubation periods of 4–8 weeks and upon concentration of either 15 l or 30 l of medium *in vacuo*. From a chemical point of view such a treatment could lead to deacylation of the 3-acyltetronic acids, since they all contain a crossed β-triketone system. On deacylation 5-methyl- and 5-carboxymethyltetronic acids will be formed. In fact, on keeping carolic acid in water at 60 °C overnight 5-methyltetronic acid was detected by paper chromatography.

Our work supports the earlier proposals [2,3] that biosynthesis of carolic and carlosic acids involves the condensation of a C_4-dicarboxylic acid and a C_6-compound such as β-ketocaproyl CoA. However, the alternative mechanism that a tetronic acid lacking the 3-acyl group is first formed from the C_4-dicarboxylic acid and malonyl CoA (or acetyl CoA) [3,4] and then upon decarboxylation (or directly) acylated by a C_4-unit, is not considered valid for two reasons: (1) the observed bilateral labelling pattern observed upon feeding with different pre-

Table 6. Enzyme catalyzed conversion of dehydrocarolic acid to carolic acid.

Experiment	Carolic acid (%)	Dehydrocarolic acid (%)
a	10	90
b	17	83
c	9	91
d[a]	0	0
e[a]	0	0

[a] Control experiments without addition of dehydrocarolic acid. The extraction of the aqueous phase was done with either ether (Exp. d) or chloroform (Exp. e).

cursors and (2) the readily *in vitro* deacylation of carolic acid to 5-methyltetronic acid suggesting that the compound is an artefact.

CONCLUSIONS

The above evidence together with the presence of 3-acylated 5-carboxymethyl- as well as 5-methyltetronic acids in the same culture medium suggests a close correspondence between the biosynthesis of carolic and carlosic acids; but to accept malate as a possible representative for the C_4-dicarboxylic acid and in this way inferring that carlosic acid is decarboxylated as part of the biosynthesis of carolic acid,[3,4] is not very probable from a chemical point of view. Chemically it seems more reasonable to suggest (see Fig. 1) that the C_4-dicarboxylic acid of the citric acid cycle is oxalacetate, which upon reaction with β-ketocaproyl CoA forms α-(α-keto-succinyl)-β-ketocaproyl CoA (*1*). (*1*) can as a β-keto acid very easily decarboxylate and upon hydroxylation at the CH_3-terminal form dehydrocarolic acid (*4*), which again can be hydrogenated to carolic acid (*5*). If *1* instead is hydrogenated, carlosic acid (*2*) is formed upon lactonization, or upon hydroxylation of the CH_3-terminal carlic acid (*3*) is the product. If carlosic acid in this way is to be considered a precursor of carolic acid it has to be dehydrogenated to the key intermediate (*1*).

The reasons for suggesting this biosynthetic scheme and especially *1* as a pivotal intermediate are the following: (1) The configuration of the chiral atom in the two series of 3-acyltetronic acids is opposite (*S* in the 5-carboxymethyl series[16] and *R* in the 5-methyl series),[17] (2) by accepting *1* as a key intermediate we have a compound which easily decarboxylates and which upon hydrogenation gives the 5-carboxymethyl series with configuration identical to that of naturally occurring malate, (3) the proposal of dehydrocarolic acid as a precursor is to be seen in light of the co-occurrence of dehydrocarolic, carolic, and carlosic acids in *P. cinerascens*,[18] (4) the proved conversion of dehydrocarolic acid to carolic acid in a cell free system of *P. charlesii*, (5) the failure of demonstrating carlosic acid as a direct precursor of carolic acid, and (6) carolic, carlosic, carlic, and carolinic (= 3-succinoyl-5-methyltetronic acid) are all isolated from *P. charlesii* together with 5-methyltetronic acid.[1,14]

An alternative biosynthetic pathway not excluded by this work will be to consider the 5-carboxymethyl series as derived from fumaroyl CoA which upon condensation with β-ketocaproyl-SEnz is transformed to α-fumaroyl-β-ketocaproyl SEnz. The latter can probably cyclize spontaneously to carlosic acid. The reason for taking such a biosynthetic possibility into consideration is that α-fumaroyl-β-keto esters, *via* spontaneously formed 3(2*H*)-furanones, upon alkali treatment have been transformed to the naturally occurring tetronic acid metabolites carlosic and viridicatic acids.[5,12]

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