189. Biosynthesis of Mikrolin

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Summary

Mikrolin (**8**) and dechloromikrolin (**9**) have been shown to exist as tautomeric mixtures in solution. The structures of mono-*O*-trifluoroacetyl mikrolin (**10**) and di-*O*-acetyl mikrolin (**11**) have been elucidated. The products **15** to **23** from reduction of the metabolites **8** and **9** with Pd/C and Zn in aqueous acetic acid have been identified. The ^{13}C-NMR. spectra of mikrolin (**8**) and dechloromikrolin (**9**) and their derivatives have been completely assigned. Based on the results of incorporation experiments with sodium [1-^{13}C]-, [2-^{13}C]- and [1,2-^{13}C]-acetate, a biosynthetic pathway is proposed for mikrolin (**8**).

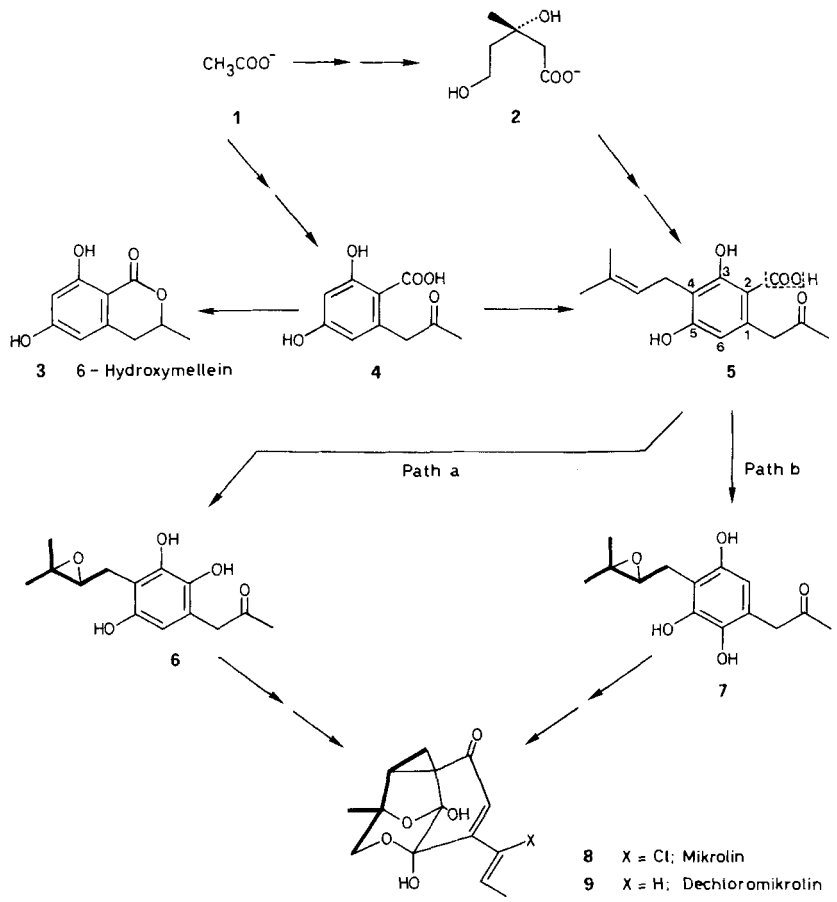
1. Introduction. – The isolation and structure elucidation of two novel compounds, mikrolin (**8**) and dechloromikrolin (**9**), isolated from culture filtrates of *Gilmaniella humicola Barron,* were reported recently [1] [2]. These compounds may formally arise through the intermediacy of a polyketide chain and an isoprene unit *(Scheme 1)*. The simultaneous occurrence of 6-hydroxymellein (**3**) [3] and several other biogenetically related metabolites [4] tend to support this hypothesis. 2,4-Dihydroxy-6-acetonyl-benzoic acid (**4**) is anticipated to be an important intermediate in the biogenetic pathway. Isoprenylation of **4** at C(4) and decarboxylation at C(2) followed by hydroxylation either at C(2) (pathway a) or at C(6) (pathway b) would lead to the further intermediates **6** and **7** respectively. Various transformations of the latter would yield mikrolin (**8**) and dechloromikrolin (**9**) respectively. In order to test the validity of this hypothesis, and especially to distinguish between pathways a and b and to establish the origin of C(13) derived either from C(2) or from the methyl group at C(3) of mevalonate (**2**), incorporation experiments using radioactive and [^{13}C]-labelled precursors have been carried out. Sodium [1-^{14}C]- and [2-^{14}C]-acetate, [2-^{14}C]-malonate, [2-^{14}C]-mevalonate, [1-^{14}C]-propionate and [U-^{14}C]-phenylalanine as well as sodium [1-^{13}C]- and [2-^{13}C]-acetate and doubly labelled sodium [1,2-^{13}C]-acetate were administered to growing cultures of the original strain of *Gilmaniella humicola*[1]). For this purpose the ^{13}C-NMR. spectra of mikrolin (**8**) and dechloromikrolin (**9**) and many of their derivatives have been fully assigned.

[1]) We thank Dr. *E. Härri*, Sandoz AG., Basel, for providing the strain and advice for growing the cultures.

2. ^{13}C-NMR. spectra and chemical transformations. – For the elucidation of the structure of mikrolin (**8**) and dechloromikrolin (**9**) spectroscopic and chemical methods [1] as well as X-ray analysis [2] of mikrolin (**8**) were used. The metabolites **8** and **9** were interrelated by chemical transformations and shown to exist in tetracyclic form **8a** and **9a**, possessing two hemiacetal groups, both in solid state and in solution. In the course of our biosynthetic studies it became apparent that, at least in solution, the molecules are always present as a tautomeric mixture of the tetracyclic **8a** and **9a** and the tricyclic structures **8b** and **9b**.

Based on structure **8a** as postulated by *Bollinger & Zardin-Tartaglia* [1] only 14 signals were expected in the ^{13}C-NMR. spectrum of mikrolin[2]). However, the spectrum exhibited 27 signals (*cf. Fig. 1)* indicating two forms in a ratio of 3:1. The signals corresponding to the major component agree well with structure **8a**. The assignments of the ^{13}C-signals *(Table 1)* are based essentially on the comparison

Scheme 1



[2]) We are grateful to Dr. *P. Bollinger*, Sandoz AG, Basel, for helpful discussions and for the gift of authentic specimens.

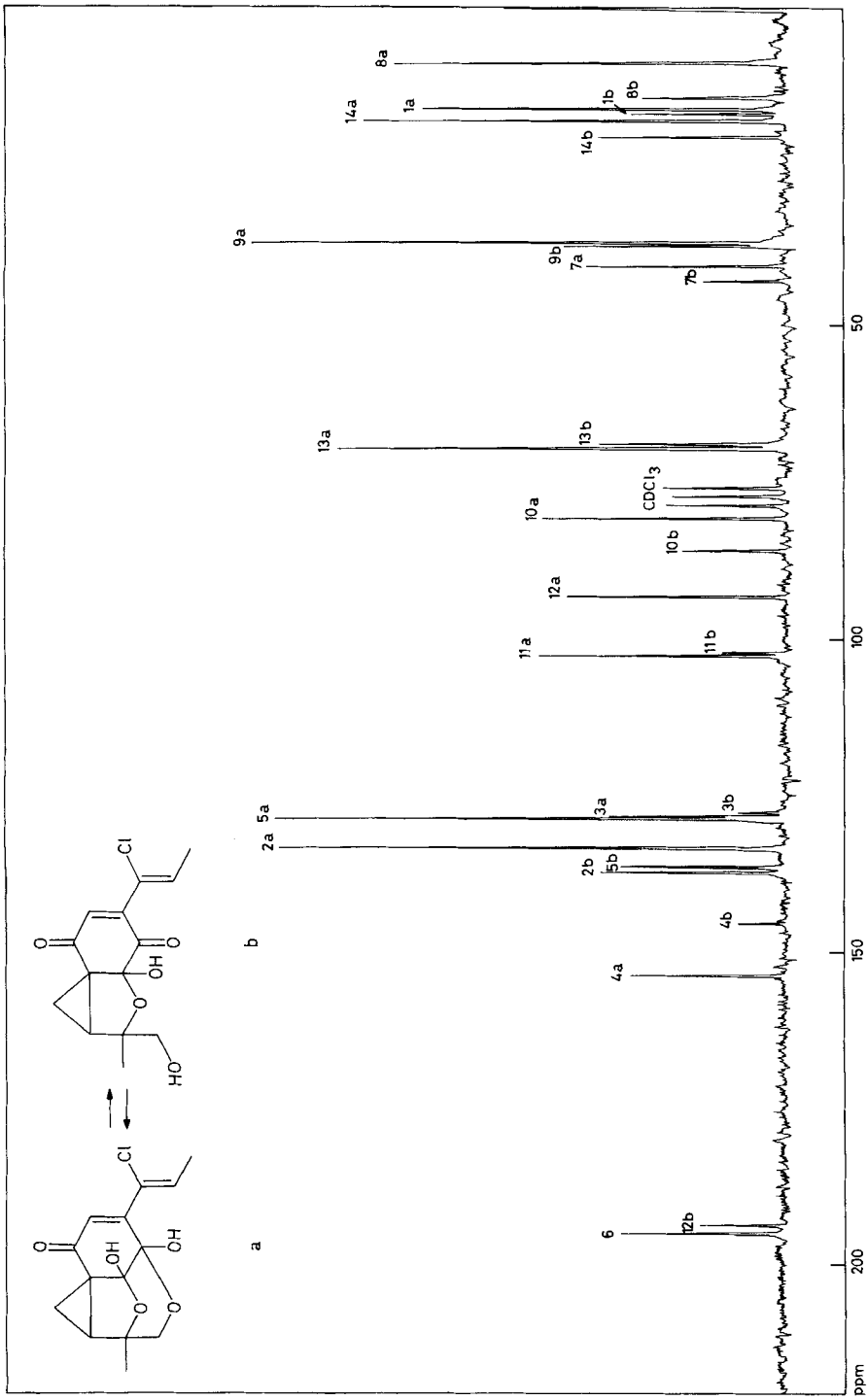


Fig. 1. *^{13}C-NMR.-spectrum of mikrolin* (**8**) ($CDCl_3$)

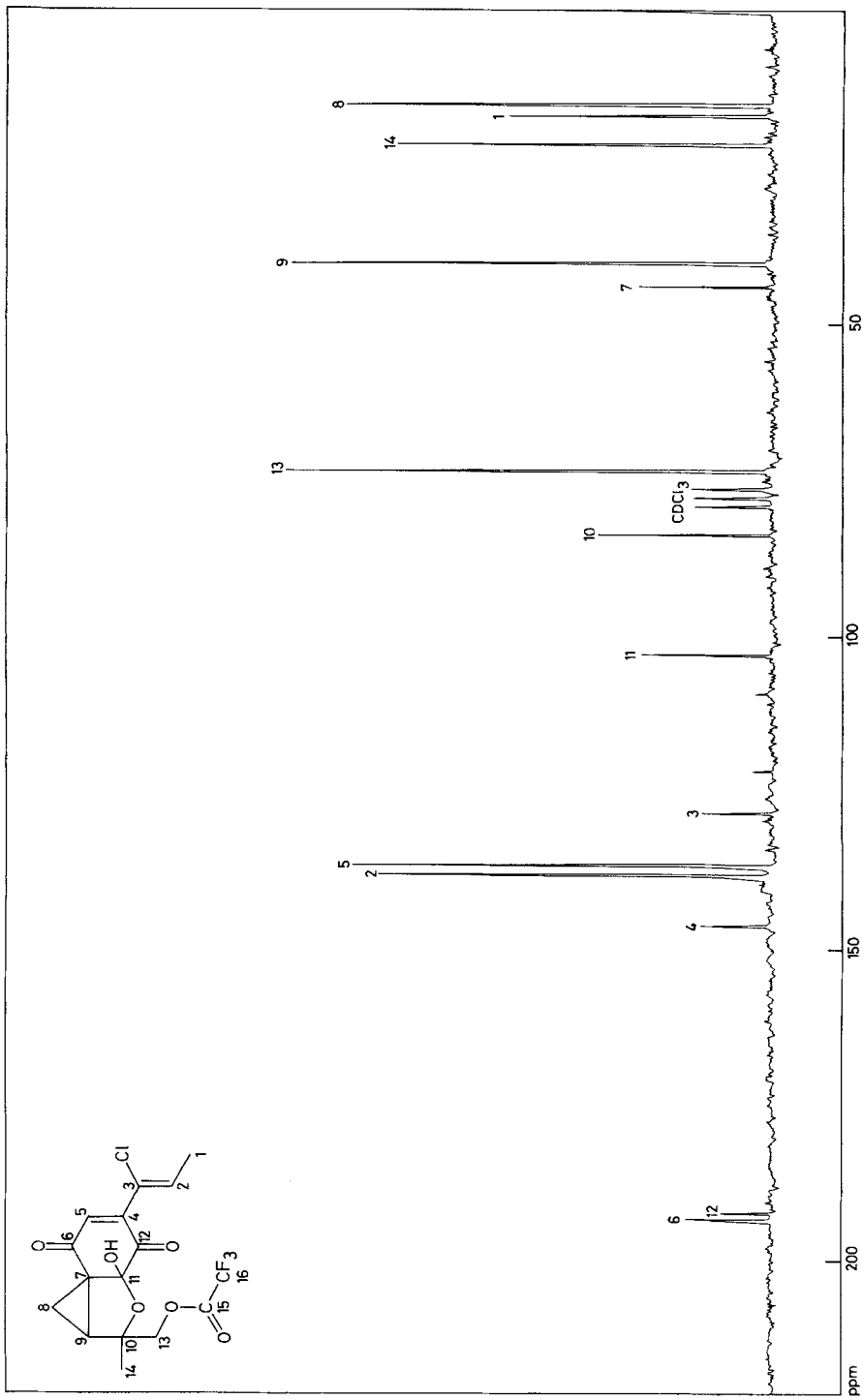


Fig. 2. *^{13}C-NMR.-spectrum of* O-*trifluoroacetylmikrolin* (**10**) ($CDCl_3$; signals for CF_3CO group are not shown)

of partially decoupled *Fourier* transform spectra with the spectra obtained by proton noise decoupling [5]. The two methyl signals at 15.66 and 17.48 ppm were distinguished by their intensities. The greater mobility of C(1) as compared to C(14) produces less pronounced relaxation, visible as a decrease in the intensity of the former carbon atom signal *(Fig. 1)*. Three sp^3-signals at 80.57, 92.85 and 102.13 ppm arising from quarternary carbon atoms were assigned to C(10), C(12) and C(11) respectively by comparing their chemical shifts to those exhibited by the anomeric carbon atoms of β-glucose (92.8 ppm), and of furanoses (101 ppm). The rule of additivity of olefins permits the assignment of vinylic carbon atoms. The signal at 153.15 ppm, although appearing at relatively low field, must be assigned to C(4). This carbon atom being the only vinylic carbon atom substituted by highly branched C(12), a downfield shift of up to 10 ppm from the expected value is quite compatible. The signals due to the minor component were also assigned *(Table 1)* on similar grounds and are in agreement with **8b**. The notable difference in the ^{13}C-NMR. spectrum of two forms is the replacement of the signal at 92.85 ppm (C(12)) in **8a** by a signal at 192.15 ppm, indicating an α,β-unsaturated carbonyl carbon atom in the spectrum of **8b**. The vinylic carbon atoms C(4) and C(5) show a marked change in their chemical shifts as expected owing to change in the hybridization character at C(12), *i.e.* an upfield shift (from 163.15 to 144.77 ppm) for C(4) and a downfield shift (from 128.13 to 136.91 ppm) for C(5). All other signals are also affected to a minor degree but do not warrant critical comment.

The ^{1}H-NMR. spectrum of mikrolin (**8**) also exhibited two sets of signals for a total of two olefinic protons at C(5) *(d)* and C(2) *(qa)* in a ratio of 3:1. Two sets of signals were also observed for the protons at C(13), although the spectrum was complex because a part of the *AB*-system centered at 3.76 ppm arising from the major form **8a** overlapped the peaks centered at 3.3 ppm from the minor form **8b**. In the other parts of the spectrum the difference was not so apparent.

Similarly the ^{1}H- and ^{13}C-NMR. spectra (*Tables 1* and *2*) of dechloromikrolin (**9**) also revealed the presence of two tautomers **9a** and **9b** in solution in a ratio of 3:1. The recyclizations of **8b** and **9b** are stereospecific processes, since no isomers of **8a** and **9a** possessing reversed configuration at C(12) have been found. The configurations of the substituents at C(10) and especially at C(11) may account for this observation.

Preparation of the monotrifluoroacetate **10** and of the diacetate **11** of mikrolin (**8**), without assigning a definite structure, was reported [1]. It became necessary to know whether these derivatives also exist in two forms as **8** or only in one form. If the latter is the case, it may be the tetracyclic or the tricyclic tautomer. Both derivatives were prepared according to the published procedure [1]. In the ^{1}H-NMR. spectra of **10** and **11** the olefinic protons appeared at 6.96 ppm *(qa)*, 7.1 ppm *(s)* and at 7.08 ppm *(qa)*, 7.18 ppm *(s)*. These chemical shifts seem to be more compatible with a tricyclic rather than with a tetracyclic structure as originally proposed [1]. The appearance of the signals of the C(13)-protons at 4.25 ppm *(s)* in **10**, and 4.05 ppm *(s)* in **11** further suggested that the hydroxyl group at C(13) must be esterified in both the cases, thereby causing a downfield shift of *ca.* 0.6–0.8 ppm for

these protons *(Table 2)*. The chemical shifts of C(4), C(5) and particularly of C(12) in the ^{13}C-NMR. spectra (*cf. Table 1* and *Fig. 2*) of both derivatives are in better agreement *(vide supra)* with the tricyclic structure. Structures **10** and **11** for mono-trifluoroacetate and diacetate of mikrolin (**8**) respectively are quite definite.

Mono-*O*-trifluoroacetyl-mikrolin (**10**) could be deacetylated either by treatment with Zn in ethanol or by passing through a silica gel column (chloroform/methanol 95:5). The ^{1}H- and ^{13}C-NMR. spectra of the recovered mikrolin were identical with those obtained from the natural sources, *i.e.* both tautomeric forms were present again in a ratio of 3:1. Therefore in solution mikrolin is always present in two tautomeric forms, and that it crystallizes as a tetracyclic tautomer is only a matter of coincidence.

Scheme 2

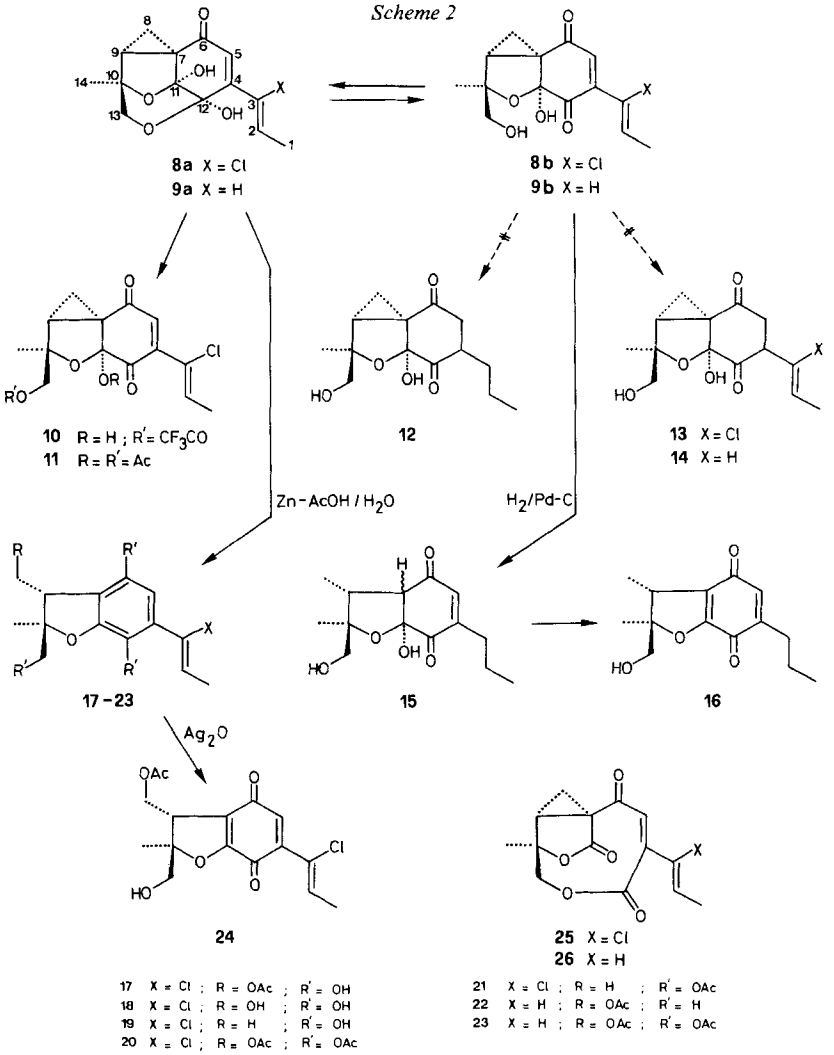


Table 1. *Assignments of the*

Compound	**8a**	**9a**	**8b**	**9b**	**10**	**11**
C(1)	15.66*qa*	15.53*qa*	16.37*qa*	16.37*qa*	16.37*qa*	16.44*qa*
C(2)	133.07*d*	135.08*d*	136.80*d*	137.10*d*	137.36*d*	137.65*d*
C(3)	127.81*s*	123.26*d*	127.16*s*	121.57*d*	127.17*s*	126.77*s*
C(4)	153.15*s*	153.73*s*	144.77*s*	144.96*s*	145.28*s*	144.51*s*
C(5)	128.13*d*	127.74*d*	136.10*d*	136.79*d*	135.73*d*	135.47*d*
C(6)	194.15*s*	194.21*s*	194.15*s*	194.73*s*	193.96*s*	190.7*s*
C(7)	40.61*s*	40.60*s*	42.95*s*	42.88*s*	43.53*s*	44.90*s*
C(8)	8.45*t*	8.12*t*	13.77*t*	15.79*t*	14.68*t*	14.55*t*
C(9)	36.97*d*	36.58*d*	37.49*d*	37.62*d*	39.96*d*	41.71*d*
C(10)	80.57*s*	80.63*s*	85.64*s*	85.64*s*	83.04*s*	85.51*s*
C(11)	102.14*s*	102.27*s*	101.62*s*	100.45*s*	101.88*s*	104.55*s*
C(12)	92.85*s*	92.39*s*	192.85*s*	192.98*s*	192.00*s*	187.50*s*
C(13)	69.39*t*	69.52*t*	68.61*t*	68.87*t*	72.84*t*	69.72*t*
C(14)	17.48*qa*	17.48*qa*	20.01*qa*	19.48*qa*	20.86*qa*	20.92*qa*
other C-Atoms					150.90*qa*	20.53*qa*
					114.12*qa*	170.17*s*
						169.78*s*

*)+) = Assignments may be reversed in a particular column.

All spectra were measured in $CDCl_3$. *s* = singlet, *d* = doublet, *t* = triplet, *qa* = quartet.

In order to study the influence of the Δ^4 double bond on the equilibrium between the two tautomeric forms of the mikrolins, the tetrahydro **12** and the dihydroderivatives **13** and **14** were examined. Dechloromikrolin (**9**) was hydrogenated over 5% Pd/C. By preparative thin layer chromatography, two products were separated, one (Rf 0.3) being a precursor of the other (Rf 0.6). Structure **15** was assigned to the compound of Rf 0.3 and **16** to that of Rf 0.6, compound **15** undergoing dehydration on silica gel. The ^{1}H-NMR. spectrum of **15** supports this conclusion. The UV. spectrum of **16** ($\lambda_{max} = 274$; log $\varepsilon = 4.20$ in ethanol) indicated the presence of a 1,4-quinoid moiety in the molecule [7]. In the ^{1}H-NMR. spectrum of **16**, the presence of a signal at 6.33 ppm (*t*, 1 H; $J = 1.6$ Hz) showing allylic coupling to a methylene group (*m*, centered at 2.45 ppm) indicated that the addition of 1 equivalent of H_2 has occurred at the Δ^2 double bond. Appearance of one CH_3 group (*t*, 0.96 ppm) also supports this conclusion. The absence of signals attributable to cyclopropane ring protons, *i.e.* to the sequence $CH_2(8)$-$CH(9)$, and the appearance of signals for a new methyl group at 1.28 ppm (*d*, $J = 7$ Hz) coupled to one proton at 3.38 ppm (*q*, $J = 7$ Hz) implies the opening of the cyclopropane ring during hydrogenation. The ^{13}C-NMR. spectrum *(Table 1)* is in good agreement with **16**. 1,4-Addition of 1 equivalent of H_2 at C(8), results in formation of **15** by cyclopropane ring fission at the less substituted 7,8 bond, in preference to the 7,9 bond. During purification on silica gel **15** undergoes dehydration to yield the observed product, **16**. A very similar reductive opening of the cyclopropane ring in the case of barbatusin was reported very recently [8]. Although the original authors have claimed the formation of compound **12** under these conditions [1], in our experiments no trace of this compound was observed. In order to find out whether saturation of Δ^2 double bond in dechloromikrolin (**9**) rather than rupture

C-atoms in the ^{13}C-NMR. spectra

16	17	20	22	25	26	Compound
13.71*qa*	14.94*qa*	14.75*qa*	14.75*qa*	15.73*qa*	18.65*qa*	C(1)
21.25*t*	126.96*d*	127.03*d*	129.17*d*	128.55*d*	127.49$^+$*d*	C(2)
30.21*t*	127.88$^+$*s*	127.67*s*	123.17*d*	127.70*s*	126.77$^+$*d*	C(3)
146.07*s*	128.46$^+$*s*	133.5*s*	126.18*s*	140.27*s*	143.08*s*	C(4)
133.40*d*	109.29*d*	115.40*d*	109.03*d*	133.02*d*	137.89*d*	C(5)
180.76*s*	146.20**s*	144.25*s*	145.94**s*	190.05*s*	190.25*s*	C(6)
124.82*s*	114.23*s*	120.73*s*	110.15*s*	39.49*s*	39.12*s*	C(7)
13.71*qa*	63.16*t*	60.72*t*	63.55*t*	11.65*t*	11.25*t*	C(8)
39.31*d*	44.83*d*	45.29*d*	44.76*d*	33.72*d*	32.56*d*	C(9)
95.71*s*	91.81*s*	90.64*s*	91.36*s*	82.85*s*	83.17*s*	C(10)
156.40*s*	146.91**s*	151.33*s*	146.26**s*	170.56*s*	171.28*s*	C(11)
185.31*s*	131.84*s*	129.30*s*	131.77*s*	166.55*s*	167.90*s*	C(12)
68.03*t*	68.22*t*	68.03*t*	68.16*t*	69.26*t*	69.01*t*	C(13)
17.35*qa*	17.48*qa*	17.87*qa*	17.48*qa*	17.41*qa*	17.22*qa*	C(14)
	20.86*qa*	20.40 } 4 CH_3				other C-Atoms
	170.08*s*	20.66 }				
		167.05 }				
		168.03 } 4 CO				
		169.72 }				

of the cyclopropane ring in the first step, the metabolite was subjected to hydrogenation under similar conditions for 5 min and the reaction worked up in the same manner. Thin layer chromatogrammes of the crude product showed only the presence of the products **15** and **16** (~40%) along with starting material suggesting that both processes occur simultaneously.

Attempted reduction of the α,β-unsaturated carbonyl groups of mikrolin (**8**) with Zn in acetic acid/water 9:1, gave three compounds **17**, **18** and **19** (84:12:4; overall yield 40% based on starting material) along with unreacted starting material. Close Rf values of **17** and **19** and very small amounts of the latter made separation of these compounds difficult, so on the preparative scale, they were separated as their acetyl derivatives **20** and **21**. The structures were assigned on the basis of the following. The IR. spectrum of **17** indicated hydroxyl groups and ester functions (3400–3600, 1740 cm^{-1}). The band at 1740 cm^{-1} was absent in the IR. spectra of both **18** and **19**. As both **17** and **18** on acetylation yield the same acetyl derivative **20**, **17** must be a mono-*O*-acetate of **18**, a fact also supported by the mass and ^{1}H-NMR. spectra. The UV. spectra of all these products were almost identical and show maxima at 258 and 306 nm (log $\varepsilon = 4.26$ and 3.87 respectively). In the ^{1}H-NMR. spectrum of **20** *(Table 1)* the protons attributable to the cyclopropane ring were again missing, and instead an *ABX*-system, assigned to the O–CH_2–CH-group, was observed. The chemical shifts of the $H_2C(8)$ showed that the hydroxyl group on this carbon atom in compound **18** is acetylated in **17**. The ^{13}C-NMR. spectra of these transformation products *(Table 1)* are compatible with the proposed structures. The formation from **17** by Ag_2O oxidation of the 1,4-benzoquinoid compound **24** with a typical UV. and IR. spectra (^{1}H-NMR. spectrum, see *Table 2*), also corroborates the assigned structure. The ^{1}H-NMR. spectrum of **21**

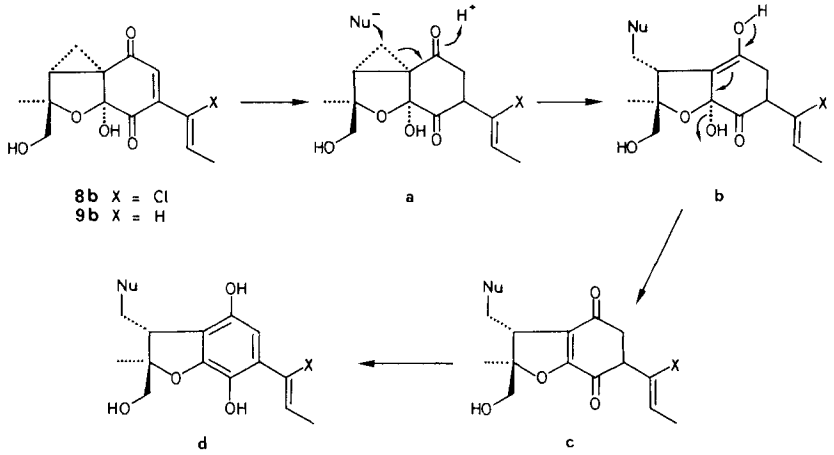
Table 2. *Assignments of H-atoms in the ^{1}H-NMR. spectra*

Compound	C(1)	C(2)	C(3)	C(5)	C(8)	C(9)	C(13)	C(14)	Others
8a	1.98*d* *J* = 6.5	6.68*qa* *J* = 6.5	–	6.38*s*	1.45*t* *J* = 4.5 1.8 →	 2.1 (2H)*m*	3.60/3.90 *J* = 11	1.22*s*	3.5 2 OH
9a	1.92*d* *J* = 6.7	complex pattern	6.1 →	6.4	1.45 →	2.1 (3H)*m*	3.62/3.88 *J* = 11	1.22*s*	4.5 br. OH
8b	1.98*d* *J* = 6.5	6.96*qa* *J* = 6.5	–	7.06*s*	1.55*t* *J* = 4.5 1.8 →	 2.15 (2H)*m*	3.3br.	1.28*s*	3.5 2 OH
9b	1.93*d* *J* = 6.7	complex pattern	6.1 →	6.6	1.45 →	2.1 (3H)*m*	3.42br.	1.39*s*	4.5br. OH
10	2.02*d* *J* = 7	6.96*qa* *J* = 7	–	7.1*s*	1.7 (1H)*t* *J* = 5 ~ 1.95 (1H) *d* × *d* *J* = 5, 8	2.35*d* × *d* *J* = 5, 8	4.2*s*	1.40*s*	6.0 1 OH
11	2.05*d* *J* = 7	7.05*qa* *J* = 7	–	7.18*s*	1.68 (1H)*t* *J* = 5 ~ 1.92 (1H) *d* × *d*, *J* = 5, 8	2.36*d* × *d* *J* = 5, 8	4.1*s*	1.40*s*	1.92; 2.1 2 CH_3–CO–
15[a])	0.98*t* *J* = 6.8	1.6*m*	2.34*m*	6.02*s*	1.29*d* *J* = 8	3.34*qa* *J* = 8	3.58*s*	1.40*s*	
16	0.97*t* *J* = 7	1.52*m*	2.34*m* *J* = 5, 1.55	6.33*t* *J* = 1.5	1.28*d* *J* = 7.3	3.38*qa* *J* = 7.3	3.62*s*	1.4*s*	
17	1.93*d* *J* = 7	6.08*qa* *J* = 7	–	6.39*s*	4.41*d* *J* = 6	~ 3.62*t* *J* = 6	3.61*s*	1.48*s*	2.12*s* CH_3–C(=O)– 5.15br. OH

18	1.86*d* $J = 6.7$	6.09*qa* $J = 6.7$	–	6.46*s*	3.86*d* $J = 8$	~ 3.70	3.65*s*	1.41*s*	~ 3.9br. OH
20	1.86*d* $J = 6.7$	5.84*qa* $J = 6.7$	–	6.64*s*	4.5 1H $d \times d$, $J = 11, 4$ 4.06 1H $d \times d$, $J = 11, 9$	3.6 $d \times d$, $J = 4, 9$	4.1*s*	1.49*d*	2.0; 2.05; 2.24; 2.30 4 $CH_3-\overset{\overset{O}{\|}}{C}-$
22	1.77$d \times d$ $J = 6.7, 1.4$	5.92*m* $J = 6.7, 12$	6.35*m* $J = 12, 1.4$	6.25*s*	4.41*d* $J = 8$	3.61*t* $J = 8$	3.62*s*	1.48*s*	2.12*s* $CH_3-\overset{\overset{O}{\|}}{C}-$ 4.90br. OH
23	1.76$d \times d$ $J = 5.7, 1.4$	5.88*m* $J = 6.7, 12$	6.23*m* $J = 12, 1.4$	6.53*s*	4.08 1H $d \times d$, $J = 11.5, 9$ 4.54 1H $d \times d$, $J = 11.5, 4$	3.58 $d \times d$, $J = 4, 9$	4.13*s*	1.5*s*	2.0; 2.04; 2.28; 2.31 4 $CH_3-\overset{\overset{O}{\|}}{C}-$
21	1.87 *d*, $J = 6.8$	5.91 *d*, $J = 6.8$	–	6.64*s*	1.21*d* $J = 7.3$	3.39 *qa*, $J = 7.3$	4.13*s*	1.35*s*	2.04, 2.27; 2.29 3 $CH_3-\overset{\overset{O}{\|}}{C}-$
24	1.98*d* $J = 7$	6.86*qa* $J = 7$	–	6.72*s*	4.22 1H $d \times d$, $J = 11, 9$ 4.49 1H $d \times d$, $J = 11, 4$	3.6 $d \times d$, $J = 9, 4$	3.62*s*	1.48*s*	2.04*s* CH_3-CO 2.4br. OH

All spectra were measured in $CDCl_3$. *s* = singlet, *d* = doublet, *t* = triplet, *qa* = quartet, *m* = multiplet, *br.* = broad.
All chemical shift values are given in (ppm) relative to TMS. Value of spinspin coupling constant (*J*) in Hz.
[a]) This spectrum was measured as a mixture of **9** and **10** as **9** could not be obtained in pure form.

Scheme 3



(Table 2) is similar to that of compound **20**, except that the signals due to the O–CH_2–CH moiety derived from the cyclopropane ring in the former have been replaced by the signals due to a CH_3–CH group in the latter.

The formation of these products from mikrolin **8** and dechloromikrolin **9** can be explained by a path shown in *Scheme 3*. The observation that no reaction occurs in the absence of Zn powder leads to the conclusion that a 1,4 reduction of the Δ^4 double bond is the first step towards the intermediate **a**. Acid-catalyzed 1,4-addition of a nucleophile (CH_3COO^-, OH^-) at C(8) with fission across the least substituted cyclopropane bond C(7)–C(8) followed by elimination of water leads, *via* the intermediate **b**, to compound **c**, which finally is transformed to **d**. The formation of **19** is an example of a 1,4-reduction of a cyclopropyl ketone[3]).

Dechloromikrolin (**9**) underwent similar transformation with Zn in aqueous acetic acid and structures **22** and **23** were assigned to the products on similar grounds.

The ^{13}C-NMR. spectra of the products obtained by oxidation of mikrolin (**8**) and dechloromikrolin (**9**) respectively with CrO_3 in H_2SO_4/acetone, are in agreement with structures **25** and **26** [1]. Interestingly, both **25** and **26** underwent normal 1,4-reductions with Zn in aqueous acetic acid and opening of the cyclopropane ring was not observed.

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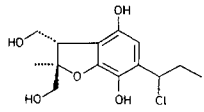
[3]) In some experiments, addition of 1 equiv. of H_2 to the double bond at C(2) of mikrolin (**8**) to give **27** (^{1}H-NMR.) in very small amounts was also observed. This compound was eluted with the tetrahydro derivative **18** on the silica gel plates.



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Attention was now turned to the tracer experiments with ^{14}C- and ^{13}C-labelled potential precursors as the next step in elucidation of the biosynthetic pathway of the mould metabolites.

3. Incorporation Experiments. – The strain of *Gilmaniella humicola* was grown on peptone media [1]. Production curve studies revealed that the production of the mikrolins commences after 3–4 days, reaching a maximum after 6 days. Best incorporation rates were observed when the precursor was added after 4 days and the cultures were harvested after 6 days. Administration of the precursor at the beginning of the fermentation did not lead to good incorporation. The isolated mikrolin (**8**) or dechloromikrolin (**9**) were usually oxidized with CrO_3 in H_2SO_4/acetone to **25** and **26** which were recrystallized to constant radioactivity if radioactive precursors had been used. The results with various potential precursors are given in *Table 3*, from which the origin of the mikrolins from acetate (**1**) and mevalonate (**2**) precursors is clear. As the incorporation of phenylalanine and propionate is insignificant, the possible involvement of prephenic acid/shikimate intermediates in the biosynthesis is unequivocally ruled out.

After the administration of [^{14}C]-acetate it was deduced on the basis of a calculation scheme by *Graf et al.* [9] that an increase of 300% per labelled carbon atom in the molecule would be achieved if 1 g of precursor per liter of culture fluid is added. In order to check whether the biological system in question would accept such a large amount of precursor and how the yield mikrolin (**8**) would be affected, an experiment with 1 g of [1-^{14}C]-sodium acetate was performed. The yields of mikrolin (**8**) and dechloromikrolin (**9**) were affected drastically. Only 30–50 mg/l of the metabolites were obtained compared with 150–200 mg/l without acetate precursor. However, when the precursor was supplied as acetate buffer of pH 5.5[4]) the yields improved to 100–120 mg/l. Mikrolin (**8**) was the only product under these conditions. To simplify the pattern of the ^{13}C-NMR. spectra, the mikrolin isolated from feeding experiments with [^{13}C]-labelled precursors was always acetylated to the di-*O*-acetyl derivative. The labelling pattern observed on supplementing the media with sodium [1-^{13}C], [2-^{13}C]- and [1,2-^{13}C]-acetates is shown in *Scheme 4*.

Table 3. *Incorporation of potential precursors into dechloromikrolin* (**9**)[a])

Precursor	Time at which precursor was added to culture (h)	Incorporation rate (%)	
		Absolute	Specific
Na[1-^{14}C]-Acetate	0	0.0395	0.531
Na[1-^{14}C]-Acetate	96	0.217	37.04
Na[2-^{14}C]-Acetate	0	0.115	1.165
Na[2-^{14}C]-Acetate	96	0.184	33.04
Na[2-^{14}C]-Malonate	96	0.776	0.0133
Na[2-^{14}C]-*D,L*-Mevalonate	96	2.78	0.013
Na[1-^{14}C]-Propionate	96	0.007	0.000004
[U-^{14}C]-*D,L*-Phenylalanine	96	0.054	0.0000036

[a]) Radioactivity was measured after oxidizing dechloromikrolin (**9**) to **26**.

[4]) 1 g of sodium acetate dissolved in 5 ml of 1N HCl.

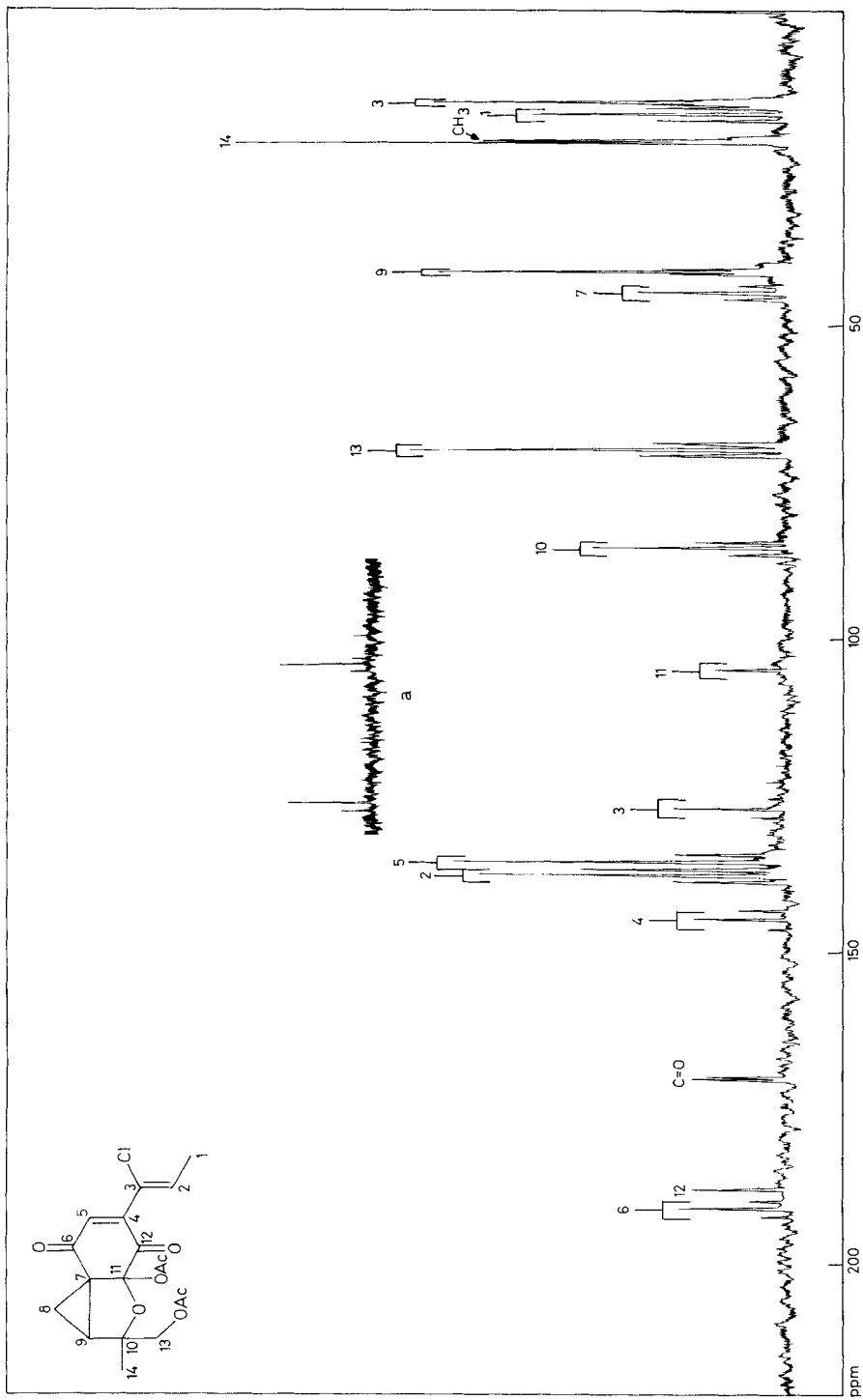
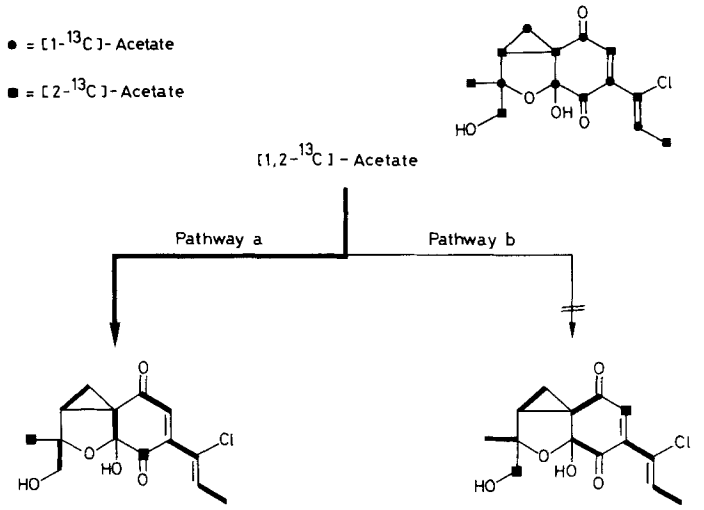


Fig. 3. *^{13}C-NMR. spectrum of di-O-acetylmikrolin* (**11**) *after incorporation of sodium [1,2-^{13}C]-acetate* ($CDCl_3$)[5])

[5]) Insertion a shows the corresponding part of the ^{13}C-NMR. spectrum of **11** after addition of Cr(AcAc)

Scheme 4



The alternate labelling of carbon atoms with [1-^{13}C]- and [2-^{13}C]-acetates supports the polyketide mode of biogenesis. The origin of C(8), C(9), C(10), C(13) and C(14) from an isoprene unit is also confirmed. In the ^{13}C-NMR. spectrum of di-*O*-acetyl-mikrolin (**11**) labelled by [1,2-^{13}C]-acetate *(Fig. 3)*, 12 of the 14 signals showed – in addition to the usual singlet arising for ^{13}C present at natural abundance – a doublet, attributed to ^{13}C-^{13}C coupling in the doubly labelled acetate unit. Thus C(1)–C(2), C(3)–C(4), C(5)–C(6), C(7)–C(11), C(8)–C(9) and C(10)–C(13) show coupling of 44, 65, 54, 47, 16 and 44 Hz respectively. The signals for C(12) and C(14) appeared as singlets in the spectrum.

The appearance of C(12) as a singlet and C(5)–C(6) as a pair (J_{CC} = 54 Hz) in the ^{13}C-NMR. spectrum can only be accommodated if the biosynthesis of mikrolin (**8**) proceeds according to pathway **a** *(Scheme 4)*. If pathway **b** were correct then C(5) should have appeared as a singlet and C(11)–C(12) should have exhibited C–C-couplings. Similarly the appearance of C(14) as a singlet and C(10)–C(13) as a pair clearly defined the origin of C(13) from the methyl group attached to C(3) in the mevalonate precursor.

4. Conclusion. – Mikrolin and dechloromikrolin exist, in solution, as equilibrium mixtures in closed (**8a** and **9a**) and open (**8b** and **9b**) tautomeric forms in a ratio of 3:1.

Using singly and doubly labelled ^{13}C-acetate precursors the biosynthetic pathway of mikrolin (**8**) has been elucidated, although the nature of the intermediates remains to be proven.

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Experimental Part

1. *General methods.* The melting points were determined on a *Kofler* block and are uncorrected. IR. (cm^{-1}), UV. [λ_{max} nm (log ε)] and optical rotations were measured on a *Perkin-Elmer* model 125 grating spectrometer, *Beckman* D.K.2 spectrophotometer and *Perkin-Elmer* model 141 polarimeter respectively. The 90 MHz ^{1}H-NMR. and 22.63 MHz ^{13}C-NMR. spectra were recorded with a *Bruker*-WH-90 spectrometer with *Fourier* transform in our institute *(K. Aegerter)*. The mass spectra were measured in the Physical Chemistry Institute, University of Basel, on an A.E.I. MS-30 instrument *(A. Raas)*. We are indebted to Mr. *G. Marbach, Sandoz AG.*, Basel, for radioactivity determinations.

Radioactive precursors were purchased from *Amersham Radiochemical Center* (Buckinghamshire, England) and from *Radium Chemie* (9053 Teufen, Switzerland). ^{13}C-labelled acetates were obtained from *Merck, Sharp and Dohme* (Montreal, Canada). Preparative thin layer chromatography (TLC.) was carried out with silica gel PF 254 *(Merck)* and column chromatography, with silica gel 0.05–0.2 mm from *E. Merck AG.*, Darmstadt.

2. *Mikrolin* (**8**). M.p. 112–113°; $[\alpha]_D^{20} = +135° \pm 2°$ ($c = 0.91$, $CHCl_3$). – ^{1}H-NMR. spectra ($CDCl_3$): *Table 2.* – ^{13}C-NMR. spectra ($CDCl_3$): *Table 1.* – MS.: 298 (M^+ calc. for $C_{14}H_{15}ClO_5$: 298).

3. *Di-O-acetylmikrolin* (**11**) *from* **8**. A solution of 150 mg of mikrolin (**8**) in 5 ml of abs. pyridine and 3 ml of acetic anhydride was stirred at RT. for 24 h. The solvent was evaporated under N_2 and then in high vacuum. The residue was purified by preparative TLC. (benzene/ethyl acetate 9:1), to yield 122 mg of **9** as oil. – IR. ($CHCl_3$): 1740, 1708, 1670, 1610. – ^{1}H-NMR. ($CDCl_3$): *Table 2.* – ^{13}C-NMR. ($CDCl_3$): *Table 1.* – MS.: 382 (M^+ calc. for $C_{18}H_{19}ClO_7$: 382).

4. *O-Trifluoroacetylmikrolin* (**10**) *from* **8**. A solution of 200 mg of mikrolin (**8**) in 3 ml of a 1:1 mixture of trifluoroacetic acid and trifluoroacetic anhydride was stirred at RT. for 4 h. After removal of the solvent in high vacuum, the residue yielded crystals, m.p. 140° (CH_2Cl_2/heptane). – IR. ($CHCl_3$): 3500, 1780, 1720, 1615, 1580. – ^{1}H-NMR. ($CDCl_3$): *Table 2.* – ^{13}C-NMR. ($CDCl_3$): *Table 1.* – MS.: 394 (M^+ calc. for $C_{16}H_{14}ClF_3O_6$: 394).

5. *Mikrolin* (**8**) *from* **10**. 5.1. A solution of 50 mg of *O*-trifluoroacetylmikrolin (**10**) in 20 ml of ethanol was stirred with 30 mg of Zn dust at RT. for 1 h. After filtration the solution was dried, the residue taken up in CH_2Cl_2 and washed with water, dried (Na_2SO_4) and the residue crystallized from ether/pentane to give 10 mg of mikrolin (**8**), identical with natural material (IR., ^{1}H-NMR.).

5.2. A solution of 30 mg of **10** in 1 ml of CH_2Cl_2 was chromatographed on silica gel column (10 g) in CH_2Cl_2 methanol 95:5 to yield 16 mg of crystalline mikrolin (**8**), identical with the authentic sample.

6. *Dechloromikrolin* (**9**). Oil. – IR. ($CHCl_3$): 3500, 1670. – ^{1}H-NMR. ($CDCl_3$): *Table 2.* – ^{13}C-NMR. ($CDCl_3$): *Table 1.* – MS.: 264 (M^+ calc. for $C_{14}H_{16}O_5$: 264).

7. *Hydrogenation of dechloromikrolin* (**9**). A solution of 100 mg of **9** in 10 ml of ethanol was hydrogenated in the presence of 50 mg of Pd/C (5%) for 2 h at RT. and atmospheric pressure. The solution was filtered and the filtrate evaporated *in vacuo*. On preparative TLC. (CH_2Cl_2/methanol 95:5) two bands were separated to give **15** (contaminated with **16**) and 32 mg of **16**. The crude product **15** on rechromatography was converted to **16** (18 mg). Compound **15**: ^{1}H-NMR.: *Table 2.* Compound **16**: orange crystals, m.p. 72°; $[\alpha]_D^{20} + 39 \pm 2°$ ($c = 0.31$, $CHCl_3$). – UV. (ethanol): 274 (4.2). – IR. ($CHCl_3$): 3500, 1680. – ^{1}H-NMR. ($CDCl_3$): *Table 2.* – ^{13}C-NMR. ($CDCl_3$): *Table 1.* – MS.: 250 (M^+ calc. for $C_{14}H_{18}O_4$: 250).

8. *Reduction of mikrolin* (**8**) *with zinc in acetic acid.* A solution of 300 mg of **8** in 6 ml 90% aqueous acetic acid was stirred with 40 mg of Zn dust at RT. After 2 h the reaction mixture was diluted with 100 ml of ethyl acetate and neutralized with 1N $NaHCO_3$. The organic layer was washed with water (2×50 ml), dried (Na_2SO_4) and evaporated *in vacuo*. Preparative TLC. (CH_2Cl_2/methanol 95:5) of the residue yielded 152 mg of unreacted mikrolin (**8**) (Rf 0.6), 100 mg of mono-*O*-acetyl-trihydroxy derivative **17** (Rf 0.5) contaminated with 4 mg of compound **19** and 14 mg of compound **18** (Rf 0.38). *Compound* **17**: m.p. 116° (CH_2Cl_2/hexane). – UV. (ethanol): 258, 306 (4.25, 3.86). – IR. ($CHCl_3$): 3400–3600, 1740, 1602. – ^{1}H-NMR. ($CDCl_3$): *Table 2.* – ^{13}C-NMR. ($CDCl_3$): *Table 1.* – MS.: 342 (M^+ calc. for $C_{16}H_{19}ClO_6$: 342).

Tetrahydro derivative **18**: m.p. 122°. – (CH_2Cl_2/hexane) – UV. (ethanol): 260, 305 (4.23, 3.85). – IR. ($CHCl_3$): 3500, 1605. – ^{1}H-NMR. ($CDCl_3$): *Table 2.* – ^{13}C-NMR. ($CDCl_3$): *Table 1.* – MS.: 300 (M^+ calc. for $C_{14}H_{17}ClO_5$: 300).

9. *Acetylation of mono-O-acetyl-trihydroxy derivative* **17** *to tetra-O-acetyl derivative* **20**. A solution of 60 mg of **17** in 3 ml of abs. pyridine and 1.5 ml of acetic anhydride was stirred at RT. for 18 h. The solution was then evaporated in high vacuum and the residue purified by preparative TLC. (CH_2Cl_2/methanol 97:3) to yield 64 mg of compound **20**, m.p. 101° (acetone/hexane); $[\alpha]_D^{20} - 11 \pm 2°$ ($c = 0.64$, $CHCl_3$). – IR. ($CHCl_3$): 1769, 1749. – ^{1}H-NMR. ($CDCl_3$): *Table 2*. – ^{13}C-NMR. ($CDCl_3$): *Table 1*. – MS.: 468 (M^+ calc. for $C_{22}H_{25}ClO_9$: 468).

10. *Acetylation of tetrahydroxy derivative* **18** *to tetra-O-acetyl derivative* **20**. Acetylation of 26 mg of **18** with pyridine and acetic anhydride yielded 17 mg of **20**, identical with the sample described above.

11. *Isolation of compound* **21**. After the crystallizations of **17**, 62 mg of the mother liquor were treated with 2 ml of pyridine and 1 ml of acetic anhydride for 18 h at RT. The solution was evaporated *in vacuo* and the residue purified by preparative TLC. to yield 28 mg of tetra-*O*-acetyl derivative **20** and 20 mg of tri-*O*-acetyl derivative **21**. The latter gave crystals m.p. 97° (acetone/hexane); $[\alpha]_D^{20} - 7 \pm 2°$ ($c = 0.064$, $CHCl_3$). – IR. ($CHCl_3$): 1768, 1750. – ^{1}H-NMR. ($CDCl_3$): *Table 2*. – MS.: 410 (M^+ calc. for $C_{20}H_{23}ClO_7$: 410).

12. *Reduction of dechloromikrolin* (**9**) *with zinc and acetic acid*. A solution of 100 mg of **9** in 3 ml of 90% aqueous acetic acid was stirred with 18 mg of Zn dust at RT. After 2 h it was worked up as described for **8**. Preparative TLC. (CH_2Cl_2/methanol 95:5) of the residue yielded 54 mg of unreacted **9**, 24 mg of mono-*O*-acetyl derivative **22** as gum. – UV. (ethanol): 256, 303 (4.2; 3.84). – IR. ($CDCl_3$): 3500, 1744, 1600. – ^{1}H-NMR. ($CDCl_3$): *Table 2*. – ^{13}C-NMR. ($CDCl_3$): *Table 1*. – MS.: 308 (M^+ calc. for $C_{16}H_{20}O_6$: 308).

13. *Acetylation of mono-O-acetyl derivative* **22** *to tetra-O-acetyl derivative* **23**. A solution of 52 mg of **22** in 3 ml of pyridine and 2 ml of acetic anhydride was stirred at RT. for 18 h. The solution was evaporated *in vacuo* and the residue purified by preparative TLC. to yield 48 mg of tetra-*O*-acetyl derivative **23**, m.p. 104° (acetone/hexane); $[\alpha]_D^{20} - 10°$ (c = 0.79, $CHCl_3$). – IR. ($CHCl_3$): 1768, 1744. – ^{1}H-NMR. ($CDCl_3$): *Table 2*. – MS.: 434 (M^+ calc. for $C_{22}H_{26}O_9$: 434).

14. *Oxidation of the mono-O-acetyl-trihydroxy derivative* **17** *to the quinone* **24**. A solution of 60 mg of **17** in 6 ml of abs. ether was stirred with 80 mg of K_2CO_3 and 100 mg of freshly prepared Ag_2O. After 30 min the solution was filtered, the residue washed with ether and the organic filtrate evaporated. Purification by preparative TLC. yielded 31 mg of quinone **24** as orange gum. – UV. (ethanol): 274 (4.2). – IR. ($CHCl_3$): 3450, 1746, 1680. – ^{1}H-NMR. ($CDCl_3$): *Table 2*. – MS.: 340 (M^+ calc. for $C_{16}H_{17}ClO_6$: 340).

15. *Oxidation of mikrolin* (**8**) *to the dilactone* **25**. A cold solution of 110 mg of **8** in 5 ml of acetone was treated with 0.5 ml of CrO_3/H_2SO_4 (*Jones*'s Reagent) for 5 min. Then 2 ml of ethanol was added to destroy excess of the reagent. After 10 min the reaction mixture was diluted with 100 ml of CH_2Cl_2, washed with water, and the organic layer dried (Na_2SO_4). The solvent was removed *in vacuo* and the residue purified by preparative TLC. (benzene/ethyl acetate 9:1) to yield 42 mg crystalline **25**, m.p. 118° (ethyl acetate/heptane). – IR. ($CHCl_3$): 1750, 1740, 1680. – ^{13}C-NMR. ($CDCl_3$): *Table 1*. – MS.: 296 (M^+ calc. for $C_{14}H_{13}ClO_5$: 296).

16. *Oxidation of dechloromikrolin* (**9**) *to the dilactone* **26**. A cold solution of 60 mg of **9** in 3 ml of acetone was treated with 0.3 ml of CrO_3/H_2SO_4 (*Jones*'s reagent) and worked up as described for **8**. Purification by preparative TLC. (benzene/ethyl acetate 9:1) yielded 22 mg of **26**, m.p. 152–158° (acetone/hexane). – IR. ($CHCl_3$): 1780, 1740, 1682. – ^{13}C-NMR. ($CDCl_3$): *Table 1*. – MS.: 260 (M^+ calc. for $C_{14}H_{14}O_5$: 260).

17. *Growth curve of the microorganism*. Spores of *Gilmaniella humicola Barron* NRNL 5487 were grown on a medium consisting of glucose · H_2O; 20 g, malt extract 2 g; yeast extract 2 g, peptone 2 g, KH_2PO_4 2 g and $MgSO_4 \cdot 7\ H_2O$ 2 g per liter distilled water. Erlenmeyer flasks (500 ml) containing 150 ml of the sterile medium were inoculated with 1 ml of spore suspension and shaken on a rotary shaker (200 rev./min.) at 27°. After 6 days the mycelium and the medium were extracted with ethyl acetate and the organic layer washed with water. The organic extract was dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified on a silica gel column to give mikrolin (**8**) and dechloromikrolin (**9**). Yields of **8** and **9** were 50–60 mg and 70–75 mg/l respectively. Presence of **8** and **9** in the culture extracts was observed after 3 days and the maximum yield was obtained after 6 days.

18. *Incorporation experiments*. – 18.1. *^{14}C-labelled precursors*. ^{14}C-labelled precursors were added to the growing cultures of *G. humicola* either at the beginning or after 4 days. The fermentation was terminated after 6 days. Isolated mikrolin (**8**) or dechloromikrolin (**9**) was oxidized with CrO_3/H_2SO_4

(*Jones*'s reagent) to give dilactone **25** or **26**. These dilactones were recrystallized three times to give constant radioactivity. Radioactivity measurements for dilactone **26** with various precursors are given below.

a) Sodium[1-^{14}C]-acetate; precursor (40 μCi/160 mg) added at 0.00 h; crystallization 2: 842 dpm/mg; crystallization 3: 922 dpm/mg.

b) Sodium[2-^{14}C]-acetate; precursor (40 μCi/160 mg) added at 0.00 h; crystallization 2: 1,488 dpm/mg; crystallization 3: 1,478 dpm/mg.

c) Sodium[1-^{14}C]-acetate; precursor (40 μCi/800 mg) added after 4 days; crystallization 2: 11,610 dpm/mg; crystallization 3: 11,780 dpm/mg.

d) Sodium[2-^{14}C]-acetate; precursor (40 μCi/800 mg) added after 4 days; crystallization 2: 12,770 dpm/mg; crystallization 3: 12,840 dpm/mg.

e) Sodium[2-^{14}C]-malonate; precursor (100 μCi/0.87 mg) added after 4 days; crystallization 2: 19,120 dpm/mg; crystallization 3: 19,190 dpm/mg.

f) Sodium[2-^{14}C]-mevalonate; precursor (40 μCi/0.53 mg) added after 4 days; crystallization 2: 35,480 dpm/mg; crystallization 3: 35,280 dpm/mg.

g) Sodium[1-^{14}C]-propionate; precursor (8 μCi/0.015 mg) added after 4 days; crystallization 2: 17 dpm/mg; crystallization 3: 18 dpm/mg.

h) [U-^{14}C]-phenylalanine; precursor (8 μCi/0.0027 mg) added after 4 days; crystallization 2: 124 dpm/mg; crystallization 3: 180 dpm/mg.

18.2. *^{13}C-labelled precursors.* Buffered solutions of sodium [1-^{13}C], [2-^{13}C], and [1,2-^{13}C]-acetates were prepared by dissolving 1 g of sodium acetate in 5 ml of 1N HCl. These solutions were added to the growing cultures through the sterile filter after 3–4 days. The fermentation was stopped after 6 days in each case. Both the mycelium and the medium were worked up as described in section 17. Isolated mikrolin (**8**), 78, 84 and 76 mg from sodium [1-^{13}C]-, [2-^{13}C]- and [1,2-^{13}C]-acetate feeding experiments was acetylated with pyridine/acetic anhydride to yield enriched di-*O*-acetylmikrolin (**11**). The ^{13}C-NMR. spectra were recorded for these samples.

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