86. Studies on the Biosynthesis of Spirostaphylotrichin A

by **Peter Sandmeier** and **Christoph Tamm***

Institut für Organische Chemie der Universität, St. Johanns-Ring 19, CH-4056 Basel

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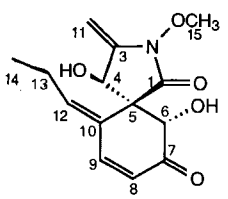
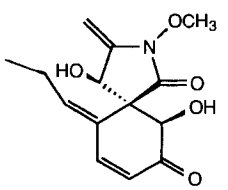
Incorporation of ^{14}C-labelled acetate and amino acids as well as of [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetate, L-[*methyl*-^{13}C]methionine, [2,3-$^{13}C_2$]succinate, and L-[2,3-$^{13}C_2$]aspartate into spirostaphylotrichin A (**1**) by *Staphylotrichum coccosporum* demonstrates that the building blocks of **1** are 5 units of acetate/malonate, 1 unit of methionine, and a C_4-dicarboxylic acid. The latter is most likely aspartate and derived from the citric-acid cycle. Using [2-^{13}C,2-$^{2}H_3$]acetate as a precursor, the starter unit of the polyketide chain was identified.

Introduction. – From a strain of *Staphylotrichum coccosporum, Peter* and *Auden* [1] isolated spirostaphylotrichin which we call in the following spirostaphylotrichin A (**1**). It



1



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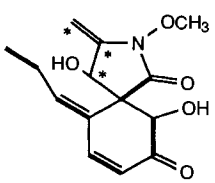
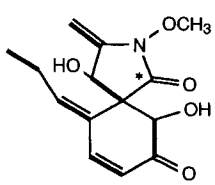
possesses lipid-lowering activity. Its unusual spirocyclic structure and relative configuration have been established by an X-ray analysis. With its γ-lactam moiety, spirostaphylotrichin A (**1**) is structurally related to the tetramic acids, *e.g.* α-cyclopiazonic acid [2], erythroskyrine [3], and tenuazonic acid [4], to the cytochalasans [5], and to the pseurotins [6]. In these secondary metabolites, the biogenetic origin of the γ-lactam ring is mixed, *i.e.* it is derived from an amino acid and acetate. It appeared reasonable to us to assume that the skeleton of **1** is formed in a similar way by condensation of a pentaketide chain with an amino acid such as, *e.g.* alanine or serine (*Fig. 1,* **A**). However, a different



A



B

* amino acid

— acetate / malonate

Fig. 1. *Hypothesis for the biosynthesis of* **1**

pathway might also be possible where condensation occurs between a heptaketide and an amino acid, *e.g.* glycine, with the loss of C(1) (*Fig. 1*, **B**). In the following, we report on our studies on the biosynthesis of spirostaphylotrichin A (**1**) using radioactive and ^{13}C-labelled precursors.

Results. – The strain *Staphylotrichum coccosporum* DSM 2601[1]) was grown in two different media, a complex one on the basis of soya meal and D-mannitol and a minimal medium containing NH_4NO_3, $MgHPO_4$, $MgSO_4$, NaCl, $CaCl_2$, a phosphate buffer, some vitamins, trace elements, and D-glucose. The latter medium was used to prevent dilution of labelled amino acids by amino acids from the medium. The production curve revealed that the concentration of spirostaphylotrichin A (**1**) began to increase after one day and reached the maximum after 4 or 5 days. The concentration of **1** was followed by HPLC analysis of the culture broth on a reversed-phase column. The cultures were harvested, when the concentration of **1** no longer increased, usually yielding 800–1600 mg of **1** per litre.

To elucidate potential precursors, we first carried out incorporation experiments with [1-^{14}C]- and [2-^{14}C]acetate, L-[*methyl*-^{14}C]methionine, DL-[1-^{14}C]alanine, and DL-[1-^{14}C]serine. Based on these results and on those of feeding experiments with ^{13}C-labelled acetates, we chose L-[U-^{14}C]aspartic acid for a further incorporation experiment.

The radioactive samples of **1** were recrystallized three times from CH_2Cl_2/Et_2O in order to achieve constant radioactivity. The results obtained after addition of the ^{14}C-pre-

Table 1. *Incorporation Experiments with ^{14}C-Labelled Precursors*

Precursor			Time[a]) [h]	Medium [b])	Spirostaphylotrichin A (**1**)		
Compound	Activity				Activity [dpm/mg]	Incorporation rate	
	total [μCi]	specific [mCi/mmol]				absolute [%]	specific [%]
sodium [1-^{14}C]acetate	14.3	59	0	S	5100	2.2	$1.1 \cdot 10^{-3}$
sodium [1-^{14}C]acetate	14.3	59	0	S	8200	2.8	$1.7 \cdot 10^{-3}$
sodium [1-^{14}C]acetate	14.3	59	24	M	26800	14.8	$5.7 \cdot 10^{-3}$
sodium [1-^{14}C]acetate	14.3	59	24	M	27000	13.0	$5.8 \cdot 10^{-3}$
sodium [2-^{14}C]acetate	17.2	55	0	S	6500	2.6	$1.5 \cdot 10^{-3}$
sodium [2-^{14}C]acetate	17.2	55	0	S	10500	2.9	$2.4 \cdot 10^{-3}$
L-[*methyl*-^{14}C]methionine	13.1	51	0	S	32500	7.6	$8.0 \cdot 10^{-3}$
L-[*methyl*-^{14}C]methionine	13.1	51	24	S	29400	21.1	$7.3 \cdot 10^{-3}$
DL-[1-^{14}C]alanine	14.1	47	0	S	320	0.24	$8.6 \cdot 10^{-5}$
DL-[1-^{14}C]alanine	14.1	47	24	S	820	0.52	$2.2 \cdot 10^{-4}$
DL-[1-^{14}C]alanine	14.1	47	24	M	1230	0.58	$3.3 \cdot 10^{-4}$
DL-[1-^{14}C]serine	6.1	51	24	S	310	0.36	$7.7 \cdot 10^{-5}$
DL-[1-^{14}C]serine	6.1	51	24	M	200	0.12	$4.9 \cdot 10^{-5}$
L-[U-^{14}C]aspartate	6.4	200	24	S	1150	1.2	$7.2 \cdot 10^{-5}$
L-[U-^{14}C]aspartate	6.4	200	24	M	5200	4.0	$3.3 \cdot 10^{-4}$
L-[U-^{14}C]aspartate[c])	6.4	$8.5 \cdot 10^{-3}$	24–48	M	5500	5.6	8.1

[a]) Addition of the precursors to the cultures.
[b]) S: soya; M: minimal medium.
[c]) L-[U-^{14}C]aspartate diluted with 100 mg of unlabelled material and added in 5 portions between 24 and 48 h.

[1]) We thank Dr. *H. Peter* and *J. A. L. Auden, Ciba-Geigy AG*, Basel, for providing the strain, a reference sample of **1**, and advice for growing the culture.

cursors are represented in *Table 1*. The incorporation rate of acetate is rather high for such an unspecific precursor. Methionine shows very high incorporation, an observation that was also made in other cases [7] [8]. On the other hand, the incorporation of alanine and serine is quite poor as compared, *e.g.*, with the rates observed in the case of 19-*O*-acetylchaetoglobosin A for DL-tryptophan (11.7%) [7] and pseurotin A for L-phenylalanine (10%) [8]. Significantly higher incorporation is found for aspartic acid using the minimal medium. The reason for its low incorporation rate in the soya medium is not yet clear. Soya meal contains a relative large amount of protein leading to a considerable dilution of the precursor. In addition, the enzymes for aminoacid catabolism are activated which may result in a less specific incorporation.

In some experiments using the minimal medium, a new metabolite, spirostaphylotrichin B, was isolated; its structure was proven to be **2** [9]. Although it is reasonable to assume that for the two epimeric metabolites **1** and **2** the same reaction sequence would occur, significant differences in the incorporation rates of radioactive labelled precursors were observed *(Table 2)*. When the precursor was added to the culture after 24 h of incubation (*Exper. 1, 2,* and *4*), **1** had *ca.* the 5-fold activity of **2**. By using pulse-feeding between 24 and 48 h of incubation (*Exper. 3, 5,* and *6*), the differences became smaller. These results may be explained by the observation, that the concentration of **2** began to increase slightly later than that of **1**. Thus, the precursor is already partially used when no pulse-feeding is applied. However, using pulse-feeding, the concentration of the precursor remained more constant over a longer period of time.

In order to elucidate the labelling pattern of **1** derived from acetic acid, ^{13}C-labelled molecules were fed. Administration of [1-^{13}C]acetate to a culture of *S. coccosporum* in the soya medium led to enhanced signals in the ^{13}C-NMR of **1** for C(1), C(4), C(6), C(8), C(10), and C(13) with an average enrichment of *ca.* 3.7%. Administration of [2-^{13}C]-acetate in a similar manner led to enhanced signals for C(3), C(5), C(7), C(9), C(11), C(12), and C(14) with an average enrichment of *ca.* 3.3% and for C(4) and C(15) of *ca.* 0.9%. In the ^{13}C-NMR of **1**, coupling signals between C(3) and C(11) with a coupling constant of 85 Hz and to a lower extent between C(3) and C(4) (J(C,C) = 49 Hz) were

Table 2. *Activity of* **1** *and* **2** *after Incorporation of* ^{14}C*-Labelled Precursors* (minimal medium)

Exper.	Precursor	Spirostaphylotrichin A (**1**) [dpm/mg]	Spirostaphylotrichin B (**2**) [dpm/mg]
1	sodium [1-^{14}C]acetate	26800	4300
2	sodium [1-^{14}C]acetate	27000	5200
3	sodium [2-^{14}C]acetate	26400	20400
4	L-[U-^{14}C]aspartate	5200	1040
5	L-[U-^{14}C]aspartate	5500	3900
6	L-[U-^{14}C]aspartate	5500	5600

Table 3. ^{13}C*-NMR Data* ($CDCl_3$) *of Spirostaphylotrichin A* (**1**) *after Incorporation of Sodium [1,2-*$^{13}C_2$*]Acetate* (soya medium)

C-Atom	δ [ppm]	J(C,C) [Hz]
C(14)	13.3	34.6
C(13)	23.1	33.8
C(12)	151.0	72.8
C(6)	128.3	72.8
C(7)	152.4	64.7
C(8)	120.7	62.5
C(9)	197.0	39.0
C(10)	73.8	39.0
C(5)	57.3	50.0
C(1)	167.4	50.0
C(4)	64.8	49.3
C(3)	143.9	49.3
C(11)	86.2	83.8
C(15)	62.2	–

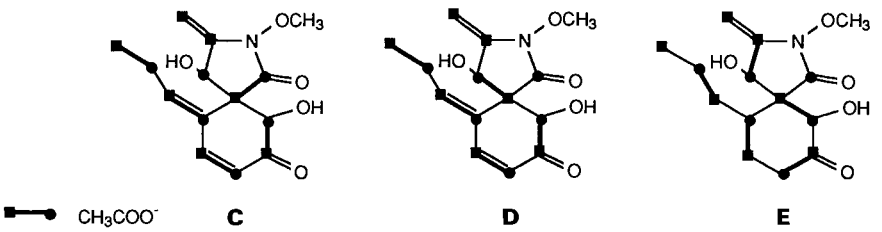


Fig. 2. *Possible routes to* **1** *based on incorporation experiments with [1-^{13}C]- and [2-^{13}C]acetate*

observed. Repeating this experiment in the minimal medium led to a similar result. In the latter case, no significant incorporation into C(4) and C(15) was observed. Based on these results, different routes for building up **1** from acetate are still possible. Three of such possibilities are shown in *Fig. 2* (see **C**–**E**). Using doubly ^{13}C-labelled acetate, it should be possible to distinguish unequivocally between these cases. *Table 3* shows the observed coupling constants between linked C-atoms in **1** after administration of [1,2-$^{13}C_2$]acetate to a growing culture of *S. coccosporum* in soya medium. This result clearly precludes the arrangements **D** and **E** and confirms distribution **C** of *Fig. 2*.

A further question was, whether the acetate moiety building up C(13) and C(14) in **1** is the starter unit of the polyketide chain or if initially a longer chain was formed which was degraded with the loss of the starter unit to the observed chain length. In the first case, it should be possible to observe retention of all three ^{2}H-atoms after feeding of [2-^{13}C,$^2H_3$]acetate. In *Fig. 3*, part of the ^{1}H-noise decoupled ^{13}C-NMR of **1** obtained after administration of [2-^{13}C,$^2H_3$]acetate is shown. Clearly visible, besides the natural abundance signal of C(14), is a *t* of a CDH_2 group with an isotopic shift of 0.28 ppm, a *quint.* of

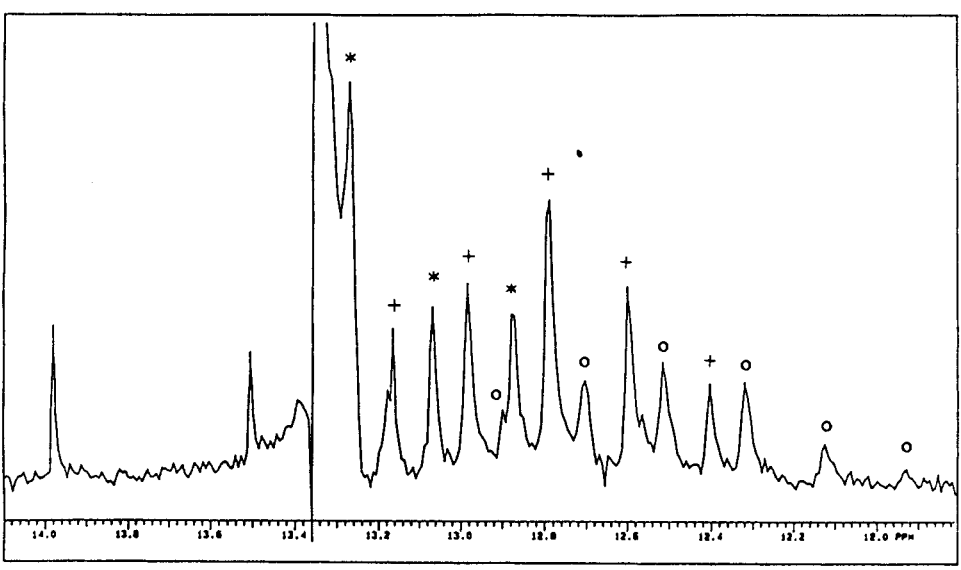


Fig. 3. *Part of the ^{1}H-noise-decoupled ^{13}C-NMR* ($(^2H_6)$acetone) *of* **1** *after incorporation of [2-^{13}C,$^2H_3$]acetate* (minimal medium). ○ = CD_3; + = CD_2H; * = CDH_2.

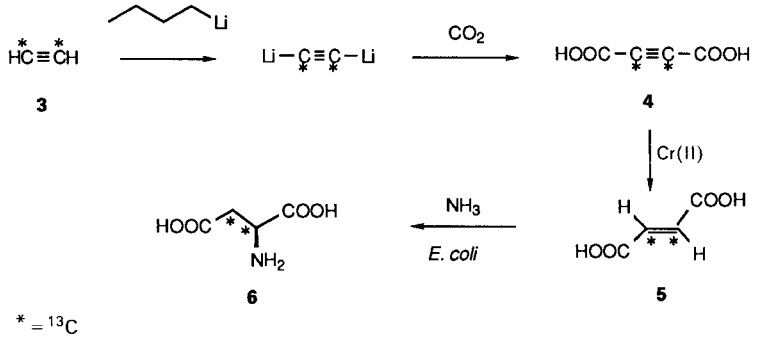
a CD_2H group with a shift of 0.55 ppm, and part of a *sept.* of a CD_3 group shifted by 0.83 ppm. Thus, it is obvious that C(13) and C(14) of **1** really are the starter unit. It is not astonishing that part of the acetate has lost one or more ^2H-atoms because of the fast equilibrium between acetate and malonate. For all the other positions, it was not unequivocally possible to detect incorporation of ^2H linked to ^{13}C. This is an observation that was already made in many other polyketide biosyntheses.

We confirmed the high incorporation rate found in the experiment with the radioactive precursor L-[*methyl*-^{14}C]methionine by the administration of L-[*methyl*-^{13}C]-methionine to *S. coccosporum* and showed that the methoxy C-atom C(15) of **1** is derived from the Me group of methionine. C(15) of the enriched metabolite **1** showed a 26-fold intensity increase of the corresponding ^{13}C-NMR signal.

Yet unclear is the origin of the three atoms C(3), C(4), and C(11). Only low incorporation was observed after feeding of ^{14}C-labelled alanine and serine. Incorporation of acetate into these positions occurs to a similar extent than incorporation into other acetate-derived positions. In principle, it could be possible that first a polyketide is formed from at least 7 acetate/malonate units. The latter is then transformed by rearrangements to the spirostaphylotrichin skeleton. Such rearrangements leading to two adjacent C-atoms which originate from the methyl group of acetate occur, *e.g.*, in the biosynthesis of cryptosporiopsin [10], 2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran [11], aspyrone, and asperlactone [12]. However, such a rearrangement would be difficult to accommodate in the case of spirostaphylotrichin A (**1**). A more plausible explanation is that an intermediate of the citric-acid cycle is involved in the formation of C(3), C(4), and C(11). Incorporation of labelled acetate into fumarate and oxaloacetate formed by the citric-acid cycle leads to labelled C_4 units where the carboxyl groups are labelled by C(1) of acetate. C(2) and C(3) of the C_4 units are labelled by the methyl group of acetate, which also labels – to a lesser extent – the carboxyl groups. This observation was made after feeding of [2-^{13}C]acetate using the soya medium. No labelling by [2-^{13}C]acetate of C(4) in **1** using the minimal medium for a culture of *S. coccosporum* was detected. C(4) would be derived from a carboxyl group of a C_4-dicarboxylic-acid unit. This result may be explained by the glyoxylate cycle as an anaplerotic reaction. It would lead to C_4-dicarboxylic acids in which the methyl group of acetate is only incorporated into C(2) and C(3). A preliminary experiment with L-[U-^{14}C]aspartate *(Table 1)* showed a rather good incorporation using the minimal medium for spirostaphylotrichin A (**1**) production. Therefore, incorporation experiments with ^{13}C-labelled aspartate and succinate were carried out.

L-[2,3-$^{13}C_2$]Aspartic acid (**6**) was synthesized using [1,2-$^{13}C_2$]acetylene (**3**) as starting material *(Scheme 1)*. The latter was converted to [2,3-$^{13}C_2$]acetylenedicarboxylic acid (**4**) and reduced to [2,3-$^{13}C_2$]fumaric acid (**5**). Addition of NH_3 by aspartase which is present in immobilized cells of *E. coli* ATCC 11303 [13] led to the desired labelled L-[2,3-$^{13}C_2$]-aspartic acid (**6**). The ^{13}C-NMR of the enriched spirostaphylotrichin-A (**1**) sample isolated from the minimal medium after incorporation of **6** is shown in *Fig. 4*. The observed ^{13}C,^{13}C-coupling pattern is consistent with the assumption that the atoms C(3), C(4), and C(11) are derived from aspartic acid. Beside the high incorporation of doubly labelled precursor into C(3) and C(11) of **1**, coupling signals of a lower extent are also observed for all other C-atoms – excluding the methoxy group – as it is already known from the incorporation experiment with [1,2-$^{13}C_2$]acetate. This observation is easily ex-

Scheme 1



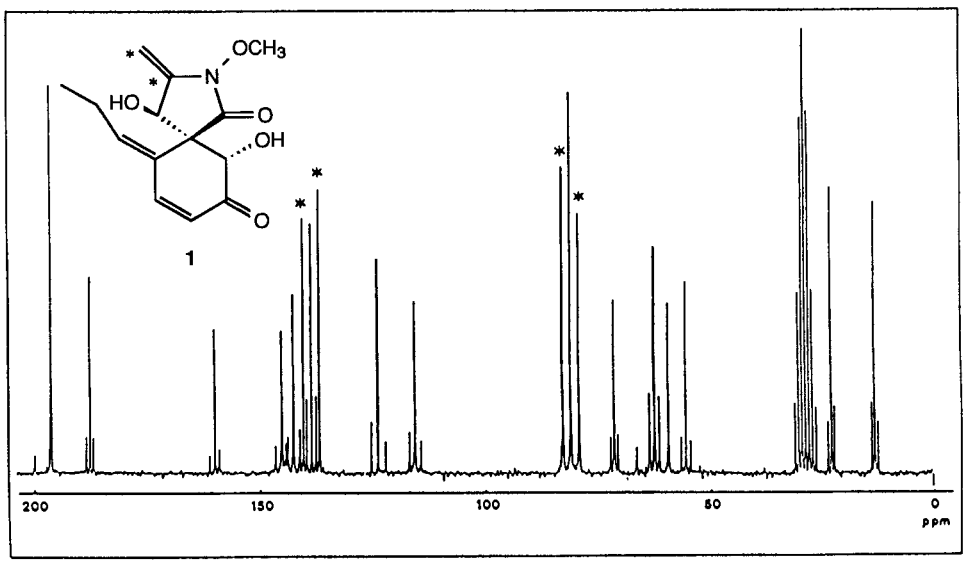


Fig. 4. *Noise-decoupled ^{13}C-NMR* (($^2H_6$)acetone) *of* **1** *after incorporation of* L-*[2,3-$^{13}C_2$]aspartate* (minimal medium)

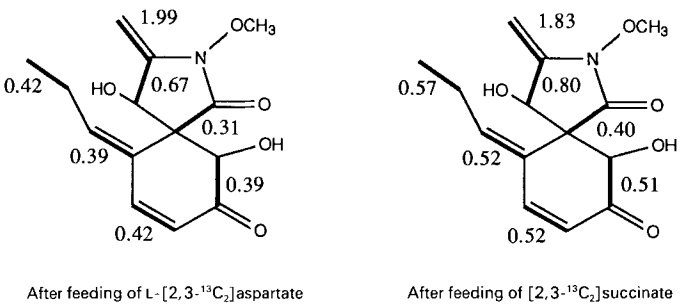
Table 4. *^{13}C-NMR Data* ($C^2H_3O^2H$/($^2H_6$)DMSO) *of Spirostaphylotrichin B* (**2**) *after Incorporation of* L-*[2,3-$^{13}C_2$]Aspartate* (minimal medium)

C-Atom	δ [ppm]	J(C,C) [Hz]	C-Atom	δ [ppm]	J(C,C) [Hz]
C(14)	13.4	33.8	C(10)	74.8	41.9
C(13)	25.5	33.8	C(5)	58.2	47.1
C(12)	151.8	72.1	C(1)	167.7	[a])
C(6)	129.3	73.5	C(4)	69.6	49.3
C(7)	153.4	61.8	C(3)	143.5	83.8 and 49.3
C(8)	121.4	61.8	C(11)	86.0	83.8
C(9)	197.3	42.7	C(15)	62.8	–

[a]) Not observed (below noise level).

plained by the fact that L-[2,3-$^{13}C_2$]aspartic acid (**6**) forms [1,2-$^{13}C_2$]acetate by deamination and subsequent decarboxylation. The same result was obtained for spirostaphylotrichin B (**2**) isolated from the same experiment. *Table 4* shows the spectral data of **2** after incorporation of L-[2,3-$^{13}C_2$]aspartate (**6**).

A result very similar to that with aspartate was obtained for **1** after administration of [2,3-$^{13}C_2$]succinate to a culture of *S. coccosporum* in the minimal medium. One might expect a better incorporation rate for L-aspartic acid which is considered to be the more specific precursor than succinic acid. In our experiments, this was in fact not the case. *Fig. 5* shows incorporation into **1** after feeding of $^{13}C_2$-labelled L-aspartate and succinate.



After feeding of L-[2,3-$^{13}C_2$]aspartate

After feeding of [2,3-$^{13}C_2$]succinate

Fig. 5. *Incorporation* [%] *of doubly labelled molecules in* **1** (minimal medium)

It can be seen that aspartate was incorporated to a somewhat lower extent after degradation to acetate corresponding to intact incorporation as compared with succinate. In the case of aspartate, for each intact aspartate molecule incorporated, 1.3 molecules of doubly labelled acetate derived from the precursor were incorporated into **1**. For succinate, the ratio was 1:1.8. This may be considered as an indication that L-aspartate is the more direct precursor than succinate. But the differences are too small to allow us to draw this conclusion with certainty. The observed differences may also reflect slight variations in the fermentation process, *e.g.* in the growth speed.

Conclusion. – Using singly and doubly ^{13}C-labelled precursors, the biosynthetic origin of each of the 14 C-atoms of spirostaphylotrichin A (**1**) has been established. The results are summarized in *Fig. 6*. Acetyl-coenzyme A acts as the starter unit. It is condensed in four successive cycles with malonyl-coenzyme A. The resulting C_{10}-polyketide **7** *(Scheme 2)* combines at a yet undefined stage with a C_4 unit derived from the citric-acid cycle. Such a mixed biosynthetic origin of the C-skeleton is also established in the case of the tetronic acids formed by cultures of *Penicillium charlesii* [14]. In our case, the C_4 unit most

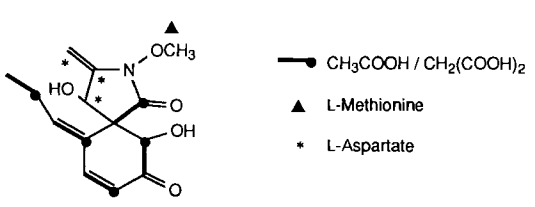
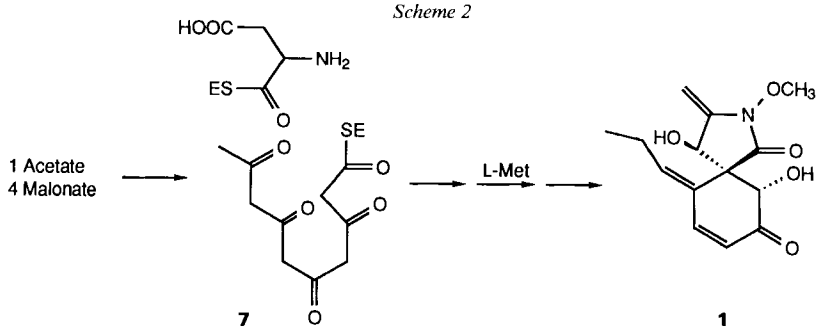


Fig. 6. *Biogenetic building blocks of spirostaphylotrichin A* (**1**)

Scheme 2



probably is aspartic acid. This assumption would also explain the origin of the N-atom. However, other C_4 units cannot strictly be ruled out. A definitive proof of L-aspartate as direct precursor for **1** would be an incorporation experiment with L-[2-^{13}C,^{15}N]aspartate. This doubly labelled precursor should lead to doubly labelled **1** which could be detected in the ^{13}C- and ^{15}N-NMR of **1**. Nevertheless, such an experiment probably has only a poor chance of being successful because, like glutamate, aspartate occupies a central place in transamination reactions. Therefore, a rapid equilibrium between aspartate and oxaloacetate is probable and would lead to the loss of the labelled N-atom. In the case of cytochalasin D, it was not possible to measure an intact incorporation of L-[2-^{13}C,^{15}N]phenylalanine, even with expensive analytical work [15].

Already some biosynthetic paths to metabolites are known in which a polyketide is condensed to an amino acid leading ultimately to a γ-lactam moiety. This is the case with the tetramic acids tenuazonic acid [16], α-cyclopiazonic acid [17], and erythroskyrine [18], and also with pseurotin A [8] and the cytochalasans [5] [15] [19]. Probably, the fusarins [20] also belong to this class of compounds.

Introduction of the MeO group whose C-atom is derived from the Me group of methionine is probably a late step in the biosynthesis of **1**.

A more detailed proposal for the pathway leading from the basic precursors to the spirostaphylotrichin skeleton and further to **1** cannot be presented on the basis of the previous results. Further work including the search of biosynthetic intermediates is necessary. We will report on our corresponding studies with mutants blocked in spirostaphylotrichin A (**1**) biosynthesis in a subsequent paper.

The financial support of these investigations by the *Swiss National Science Foundation* and *Ciba-Geigy AG*, Basel, is gratefully acknowledged.

Experimental Part

General. ^{14}C- and ^{13}C-Labelled compounds were purchased from *Medipro AG*, Teufen, *Amersham International plc*, England, and *New England Nuclear*, USA. Stock cultures of *S. coccosporum*, strain DSM 2601, were maintained on *Mycophil Agar® (Baltimore Biological Laboratories)* or *Mycological Agar® (Difco)* and stored at 4°. The org. extracts were dried (Na_2SO_4) and evaporated under reduced pressure at 40°. Column chromatography: silica gel *60* (63–200 μm, *Merck*). TLC: silica gel *60* F_{254} *(Merck)*, detection with UV, I_2, $KMnO_4$, or H_2SO_4. HPLC (pump *Pye Unicam LC-XPD;* UV-VIS detector *Uvikon 722 LC, Kontron;* integrator *HP 3380 A*): *Nucleosil-C_8* (10 μm, 4.5 × 250 mm, *Macherey-Nagel*) using H_2O/CH_3CN 7:3; *μBondapak C_{18}* (5 μm, 3.9 × 300

mm, *Waters*) using H_2O/CH_3CN 85:15. M.p.: *Kofler* block; corrected. UV: *Beckman* spectrophotometer, model *25*. IR: *Perkin-Elmer-781* spectrometer. NMR: *Bruker-WH-90* spectrometer with *Fourier* transform (^1H, 90 MHz; ^{13}C, 22.63 MHz). MS: *VG-70-250* instrument. Radioactivity: *Nuclear Chicago* scintillation counter.

Production of Spirostaphylotrichin A (**1**) *and B* (**2**). To a preculture on 25 ml agar medium in a 100-ml *Erlenmeyer* flask, after 5–7 days of incubation, 25 ml of *NI 153* medium [1] (10 g of yeast extract *(Difco)*, 20 g of peptone *(Difco)*, 20 g of D-glucose per l H_2O; pH 6.0, adjusted before sterilization) were added, and the mycelium was roughened. This culture was incubated further 24 h at 28° on a rotary shaker (200 rpm) in the dark. Then, this culture was used to inoculate a further preculture of 180 ml *NI 153* medium in a 500-ml *Erlenmeyer* flask (2 flutes). After incubation for 36–48 h, the preculture was ready for inoculation of the production media. The soya medium (120 ml; 40 g soya meal (*ca.* 15% fat), 40 g of D-mannitol per l H_2O; pH 5.5, adjusted before sterilization) or the minimal medium (150 ml; 40 g of D-glucose, 6.8 g of NH_4NO_3, 1.7 g of yeast nitrogen base w/o amino acids and ammonium sulfate *(Difco)*, 8 g of $MgHPO_4$ per l H_2O, pH *ca.* 6, not adjusted) in a 500-ml *Erlenmeyer* flask (1 flute) was inoculated with 10–12 g of preculture and further incubated at 28° and 200 rpm until the maximum concentration of **1** was reached. Aq. soln. of radioactive precursors were aseptically added to the cultures before inoculation or after 24 h. Sterile soln. of ^{13}C-labelled compounds were administered to the growing cultures in 5 portions between 24 and 48 h. Amounts of ^{13}C-labelled precursors used per incorporation experiment (one flask): 300 or 200 mg of sodium acetate (soya or minimal medium, resp.); 100 mg of sodium [2-^{13}C,$^2H_3$]acetate; 102 mg of L-[*methyl*-^{13}C]methionine, 108 mg of L-[2,3-$^{13}C_2$]aspartic acid, or 100 mg of [2,3-$^{13}C_2$]succinic acid. The culture was freed of cells by filtration through *Celite* and extracted with CH_2Cl_2 (4 times) or with Et_2O in a *Kutscher-Steudel* apparatus for 2 h. The org. extracts were dried and evaporated yielding *ca.* 200–500 mg crude red-brown extract which was chromatographed on silica gel with a pentane/Et_2O gradient. The fractions containing pure (by TLC) **1** were pooled. Crystallization from CH_2Cl_2/Et_2O yielded *ca.* 100–200 mg pure **1** from the soya medium. Crystals from the minimal medium contained **1** and **2** which were separated further by prep. HPLC on a *Lichrosorb-Si60* column (10 μm, 32 × 500 mm, *Knauer*) with 1–2% MeOH in CH_2Cl_2 (**1**: 100–180 mg; **2**: 0–70 mg). Radioactive samples of **1** were further recrystallized twice from CH_2Cl_2/Et_2O.

Spirostaphylotrichin A (= *(4R*,5S*,6S*)-4,6-Dihydroxy-2-methoxy-3-methylidene-10-[(Z)-propylidene]-2-azaspiro[4.5]dec-8-en-1,7-dione;* **1**). M.p. 106–111° (from CH_2Cl_2/Et_2O). UV (EtOH): 289 (15400), 224 (19300). CD (qual./EtOH): 218 (–), 232 (0), 239 (+), 247 (0), 273 (–). IR (KBr): 3460 (br., OH), 3390 (br., OH), 1720*s*, 1680*s*, 1660*s*, 1615*m*, 1260*m*, 1140*m*, 1110*m*, 1070*m*. ^1H-NMR ($CDCl_3$): 1.05 (*t*, *J* = 7.3, CH_3(14)); 2.1 (*m*, CH_2(13)); 2.63 (*d*, *J* = 6.2, OH–C(4), exchangeable with D_2O); 3.80 (*d*, *J* = 2.1, OH–C(6), exchangeable with D_2O); 3.92 (*s*, CH_3(15)); 4.56 (*t*, *J* = 1.7, 1 H–C(11)); 4.64 (*dt*, *J* = 6.2, 1.5, H–C(4); with D_2O, *t*); 4.72 (*t*, *J* = 1.8, 1 H–C(11)); 4.76 (*d*, *J* = 2.1, H–C(6); with D_2O, *s*); 5.93 (*d*, *J* = 9.8, H–C(8)); 6.27 (*t*, *J* = 7.4, H–C(12)); 7.07 (*d*, *J* = 10.1, H–C(9)). ^{13}C-NMR (C^2DCl_3): *Table 3*. ^{13}C-NMR ($(^2H_6)$acetone): 13.3 (C(14)); 23.5 (C(13)); 57.8 (C(5)); 62.0 (C(15)); 65.3 (C(4)); 74.6 (C(6)); 84.9 (C(11)); 121.3 (C(8)); 129.9 (C(10)); 145.6 (C(3)); 149.8 (C(12)); 152.4 (C(9)); 168.1 (C(1)); 197.2 (C(7)). EI-MS (70 eV, 140°): 279 (M^+), 248 ([M – CH_3O]$^+$), 230 ([M – H_2O–CH_3O]$^+$), 204, 177 (100), 161. Anal. calc. for $C_{14}H_{17}NO_5$ (279.30): C 60.20, H 6.14, N 5.02; found: C 60.19, H 6.12, N 5.11.

[2,3-$^{13}C_2$]Acetylenedicarboxylic Acid (**4**). A mixture of dry Et_2O (200 ml) and 1.55M BuLi in hexane (50 ml) was degassed [21], and [1,2-$^{13}C_2$]acetylene (99% ^{13}C; 500 ml) was added. Then, the mixture was given to an excess of dry ice. After 4 h, the product was extracted from the mixture with NaOH soln. and the aq. phase adjusted to pH 1. The yield of crude **4** was determined by HPLC: 50%. After extraction of the 1-pentanoic acid by hexane, **4** was extracted with Et_2O in a *Kutscher-Steudel* apparatus. Drying and evaporation yielded 1.6 g of crude **4** which was directly used for further conversion.

[2,3-$^{13}C_2$]Fumaric Acid (**5**). A soln. of crude **4** (720 mg) in H_2O (4 ml) was reduced according to [22] under Ar with a 0.278M Cr(II) soln. (13.6 ml): 396 mg of **5** containing 37% of [2,3-$^{13}C_2$]succinic acid.

L-*[2,3-$^{13}C_2$]Aspartic Acid* (**6**). Conversion of **5** to **6** by immobilized cells of *E. coli* and purification of **6** was run according to [13]. The sample purity of **6** was > 99% ee and > 90% content of L-aspartic acid, whereof *ca.* 97% of $^{13}C_2$-labelled and 3% of unlabelled molecules (MS). ^{13}C-NMR (D_2O/NaOD): 37.1 (*d*, *J* = 36.8, C(3)); 52.6 (*d*, *J* = 35.3, C(2)). CI-MS (NH_3): 136 (100, M^+), 120, 92, 90.

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