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BIOSYNTHESIS OF PSEUROTIN A

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Abstract The ^{13}C-NMR spectra of pseurotin A (**1**), a secondary metabolite of *Pseudeurotium ovalis* Stolk, and of some derivatives were measured and interpreted. Incorporation experiments with [1,2-$^{13}C_2$]-propionate, [2-^{13}C]-malonate, [1-^{13}C]- and [2,3-$^{13}C_2$]-phenylalanine and [^{13}C-CH_3]-L-methionine showed that the building blocks of pseurotin A (**1**) are 1 unit of propionate (starter unit), 4 units of malonate, 1 unit of phenylalanine and 2 units of methionine. A biogenetic pathway is proposed. Experiments with [$^{13}C^2H_3$]-L-methionine demonstrated that both O- and C-methylation occur with retention of all three hydrogen atoms of the methyl group. Using [2-$^{13}C^2H_2$]-propionate as precursor the fate of the α-H atoms of the starter unit was studied. Finally, the incorporation rates with [2-^{13}C, ^{15}N]-phenylalanine were determined by mass spectrometry and ^{13}C NMR spectroscopy. By comparing the labelling pattern with the results obtained after the incorporation of [^{15}N]-phenylalanine it could be deduced that the ^{15}N atoms are not statistically distributed. Hence, a nitrogen-free intermediate can be excluded.

The pseurotins are a new class of secondary metabolites which have been isolated recently from cultures of *Pseudeurotium ovalis* Stolk.[1,2] They are characterized by an 1-oxa-7-aza-spiro[4.4]non-2-ene-4,6-dione skeleton. The individual members of the family differ from each other by the functionalization of the side chain attached to the bicyclic system. To our knowledge no other class of natural products containing this heterospirocyclic structure is known. Therefore an investigation of its biogenetic origin seemed to be of general interest. It appeared to us reasonable to assume that the skeleton of the pseurotins arises by condensation of a polyketide chain with the amino acid phenylalanine. We have tested this hypothesis in the case of pseurotin A (**1**) by incorporation experiments using radioactive as well as ^{13}C-labelled precursors. In order to distinguish between the various theoretically possible pathways, Na[1-^{14}C]- and [2-^{14}C]-acetate, Na[2-^{14}C]-malonate, Na[1-^{14}C]-propionate, [^{14}C-CH_3]-L-methionine, [U-^{14}C]- and [4′-^3H]-L-phenylalanine, [2-^{13}C]-malonic acid, [^{13}C-CH_3]-L-methionine, Na[1,2-$^{13}C_2$]-propionate, [1-^{13}C]- and [2,3-$^{13}C_2$]-D,L-phenylalanine, [$^{13}C^2H_3$]-L-methronine, Na[2-$^{13}C^2H_2$]-propronate, [^{15}N]-L-phenylalanine and [2-^{13}C, ^{15}N]-D,L-phenylalanine were administered to growing cultures of *Pseudeurotium ovalis* Stolk (strains S 2269 and S 3484†). From the signal enhancement in the ^{13}C NMR spectra of the ^{13}C-enriched pseurotin A (**1**) samples, the labelling pattern could easily be deduced. Therefore the ^{13}C NMR spectra of pseurotin A (**1**) and some of its derivatives were measured and fully interpreted.

†We thank Dr. E. Härri, Sandoz AG., Basel, for providing the strain and advice for growing the cultures.

‡According to the ^1H and ^{13}C NMR spectra, one of the two possible epimers is about 90% predominant. However, even from molecular models it is not easy to predict which side of the CO group is less hindered.

§This name proposed by Eugster *et al.*[4] does not correspond to the IUPAC-rules. Nevertheless it is preferred, since these compounds show no chemical relationship with the aromatic furanones.

^{13}C NMR *spectroscopy*

The structure of pseurotin A (**1**) was elucidated by spectroscopic and chemical methods as well as by the X-ray analysis of 12(S), 13(S)-dibromopseurotin A (**2**). Because the halogenation proceeds by *trans*-opening of the bromium ion and since the dibromo derivative obtained can be reconverted to the natural product by treatment with zinc in acetic acid, the Z-geometry of the double bond is proven. The proton noise decoupled ^{13}C NMR spectrum of pseurotin A (**1**) exhibited 19 signals for the 22 C atoms. The assignments as summarized in Table 1 are based essentially on the comparison with the off-resonance decoupled spectrum and with the chemical shifts of 12(S), 13(S)-dibromopseurotin A (**2**), 12,13,17-tetrahydropseurotin A (**3**), 12,13-dihydropseurotin A (**4**) and 17-dihydropseurotin A (**5**). While the dibromo derivative **2** and the epimeric mixture of 12,13,17-tetrahydropseurotin A (**3**) were prepared as previously described,[1] the selective reduction of the double bond of the side chain to form compound **4** was achieved by homogeneous catalytic hydrogenation with tris-(triphenylphosphine)-rhodium(I)-chloride in ethanol/benzene (1:1) (*cf* Scheme 1). Sodium cyanoborohydride attacked only the benzoyl-system and gave the 17-dihydropseurotin A (**5**) in quite a good yield.‡

The two Me groups are separated sufficiently from each other to be assigned without any uncertainty. The same is true for C(14) and the OMe signal at 52.2 ppm. The three peaks at 69.7, 72.8 and 75.3 ppm arising from the secondary carbinol C atoms were assigned to C(11), C(10) and C(9) respectively. The latter remains nearly unchanged in all the derivatives, while the former two are characteristically affected by the functionality in the side chain. C(11), as well as (14), is upfield shifted in compounds **1** and **5** owing to the γ-effect resulting from the interaction in the sterically less favourable Z-position.[3] C(2) and C(3) in the Δ^2-furenidone-(4) system§ are easy to distinguish as the β-atom is very strongly deshielded. The C atoms of the aromatic moiety absorb in all the compounds containing an intact benzoyl group at the same frequencies within the experimental error. The

Table 1. Assignments of the C-atoms in the ^{13}C NMR spectra

Compound	1	2	3	4	5
C(2)	167.3	167.8	168.6	167.5	168.2
C(3)	113.6	114.5	113.6	113.7	113.6
C(4)	197.9	197.4	198.2	197.7	198.2
C(5)	92.7	93.0[a]	93.4/93.0[b]	92.7	93.0
C(6)	187.7	186.7	187.9/187.7[b]	188.0	187.3
C(8)	92.4	92.9[a]	90.2/89.7[b]	92.4	89.6
C(9)	75.3	75.2	74.8	75.3	74.9
C(10)	72.8	70.2	73.2	73.4	72.8
C(11)	69.7	71.1	72.4	72.5	69.5
C(12)	129.0	62.5	32.9	33.1	128.7
C(13)	135.9	61.6	28.5	28.6	135.9
C(14)	21.8	29.2	23.2	23.3	21.9
C(15)	14.5	13.0	14.3	14.3	14.6
C(16)	5.8	5.7	5.7	5.7	5.8
C(17)	196.2	196.4	73.9/73.7[b]	196.3	73.9
C(18)	134.7	134.6	140.4/140.0[b]	134.7	140.2
C(19)/C(23)	131.3	131.3	128.4	131.3	128.7
C(20)/C(22)	129.2	129.2	128.7	129.2	128.7
C(21)	134.7	134.4	128.7	134.7	128.7
$-OCH_3$	52.2	52.4	52.4/52.2[b]	52.3	52.5

a) Assignments may be reversed

b) Epimeric mixture

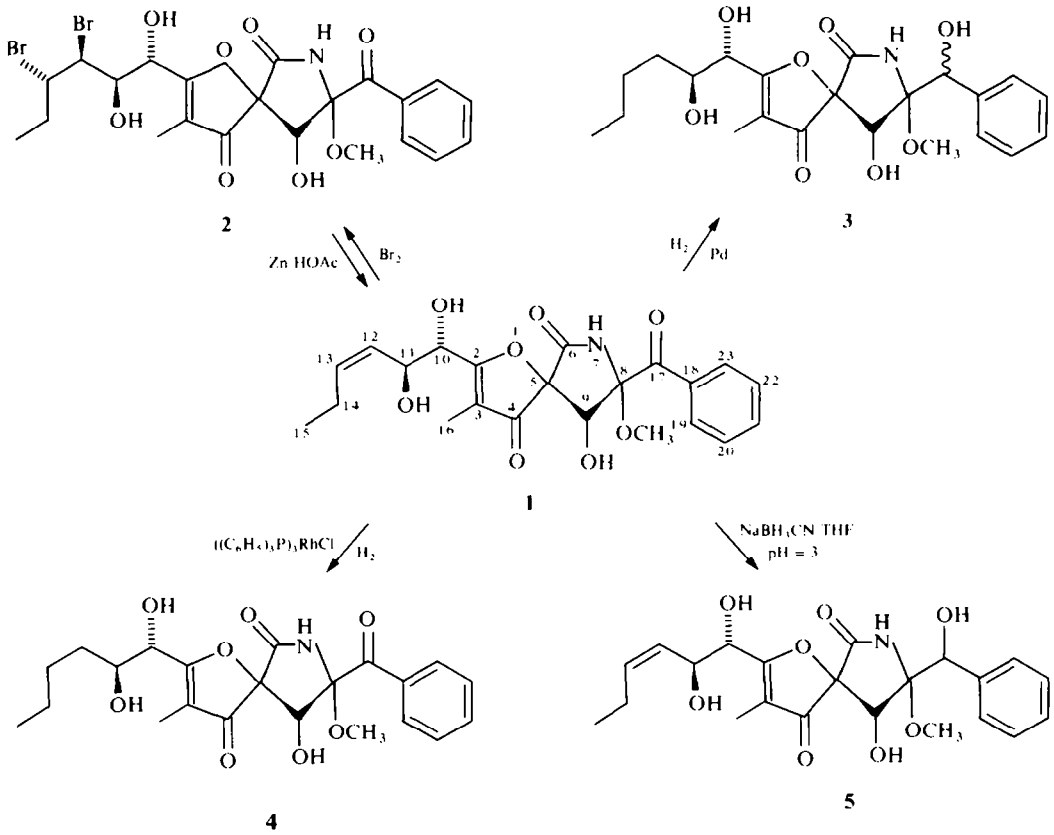
All spectra were measured in $(CD_3)_2CO$.

Table 2. Incorporation of radioactive precursors into pseurotin A (1)

Precursor	Absolute Incorporation rate (%)
Na[1-^{14}C]-acetate	0.17
Na[2-^{14}C]-acetate	0.23
Na[2-^{14}C]-acetate	0.14[a]
Na[2-^{14}C]-malonate	1.4
Na[1-^{14}C]-propionate	2.5
[^{14}C-CH_3]-L-methionine	7.2
[U-^{14}C]-L-phenylalanine	10.5[b]
[4'-^{3}H]-L-phenylalanine	8.7[b]

a) Precursors diluted with inactive material and added at the beginning.

b) The differences of the incorporation rates are probably not significant but may be due to some tritium loss in the precursor owing to its short half-life.



Scheme 1.

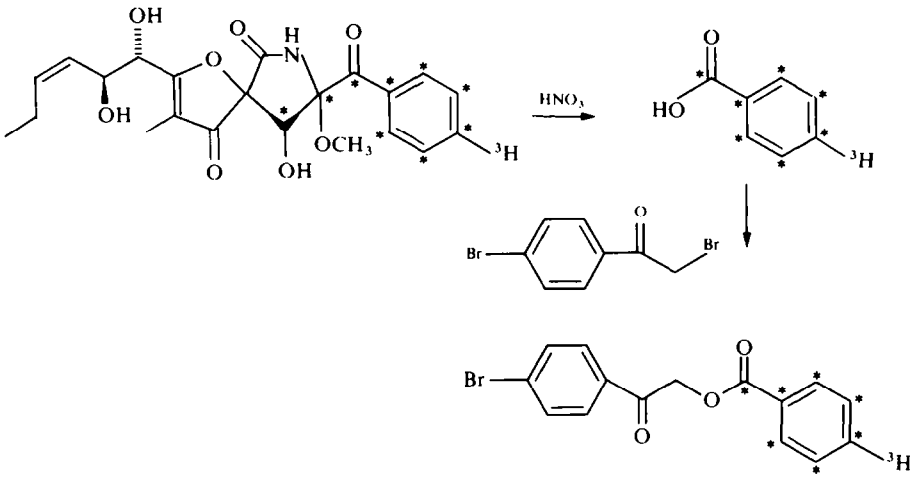
assignment of the signal at 129.0 ppm to C(12) and of 135.9 ppm to C(13) respectively is compatible with the expected values for these olefinic C atoms. This was later confirmed by the incorporation of the doubly labelled propionate. The signal for the lactam-CO-atom needs no further comment. Finally, the C(4)-atom could easily be detected, since it is little affected by the reduction of the benzoyl group. C(5) and C(8) whose resonance frequencies are separated only by 0.3 ppm could be distinguished by the incorporation of the [2,3-$^{13}C_2$]-phenylalanine. The spectra of the four other pseurotins[2] agree well with the assignments for pseurotin A (**1**).

Incorporation experiments

The strains of *Pseudeurotium ovalis* were grown on a glucose/peptone media. The production curve revealed that the pseurotin A (**1**) concentration begins to increase after two or three days. Therefore, the precursors were administered after 48 hr. The cultures were harvested after 8 days yielding 60–100 mg pseurotin A (**1**) per liter. The radioactive samples were recrystallized three times from tetrahydrofuran/-hexane in order to achieve constant radio-activity. The results obtained after addition of the potential precursors are presented in Table 2. All of them are incorporated significantly, thus confirming the origin of the pseurotins from an acetate/malonate derived polyketide and from phenylalanine. At least one of the two Me groups must be derived from methionine. Propionate could be the starter unit or- via methylmalonate—a link of the polyketide-chain. However, the incorporation rate is high enough to exclude an indirect labelling.

After the administration of [U-^{14}C]- and [4′-^{3}H]-L-phenylalanine the pseurotin A (**1**) was degraded with HNO_3 to benzoic acid whose *p*-bromo-phenacylester was analyzed for the determination of the labelling pattern (*cf* Scheme 2). The specific tritium activity was found to be the same as in the metabolite within the experimental error (Table 3). The result shows that phenylalanine is incorporated exclusively at C atoms Nr. 8, 9 and 17–23, provided that no decarboxylation has taken place. The $^{3}H/^{14}C$ ratio increased from 4.2 to 5.8 suggesting that the amino acid is incorporated intact and that two labelled atoms are lost during the degradation to benzoic acid.

Having established the basic biogenetic units of pseurotin A (**1**) the various precursors were also fed as ^{13}C-labelled molecules in order to elucidate the labelling pattern. The expected ^{13}C NMR signal enhancement can be estimated assuming that the absolute incorporation rates are the same in both experiments with ^{14}C and ^{13}C labelled precursors. Since the culture conditions always vary slightly, the calculation is on a theoretically weak ground. The proton noise decoupled ^{13}C NMR spectrum obtained after administering Na[1,2-$^{13}C_2$]-propionate showed for C(14) and C(13) a triplet. The adjoining ^{13}C-atoms of the precursor are incorporated as a unit and



Scheme 2.

Table 3. Degradation of pseurotin A (1) to benzoic acid

	$^3H/^{14}C$	^3H-Activity (dpm/mmol)
Pseurotin A (1)	4.2	$2.0 \cdot 10^7$
p-Bromophenacylester	5.8	$2.1 \cdot 10^7$
(calc.:	5.4[a])	

[a] 5.4 is the expected value if the activity is distributed equally over the nine C atoms.

Table 4. Relative intensities after the incorporation of [2-^{13}C]-malonate

C-atom	Pseurotin A (1) Natural	Enriched
16	1.01	1.07
15	1.07	0.94
14	1.36	1.24
$-OCH_3$	1.00	1.00
11	1.46	1.43
10	1.50	4.46
9	1.54	1.36
8	0.42	0.33
5	0.34	0.74
3	0.32	0.72
12	1.53	4.52
20/22	6.75	2.99
19/23	6.53	2.46
18/21	2.08	1.00
13	1.63	1.28
2	0.37	0.24
6	0.42	0.32
17	0.36	0.18
4	0.20	0.15

therefore split into a doublet (J = 43 Hz), while the singlets between them are due to the natural pool. Thus the C atoms Nr. 13, 14 and 15 are derived from propionate which serves as starter unit for building the polyketide chain.

As expected the incorporation rate of radioactive malonate has been higher than that of acetate from which it is formed in a biotin dependent enzymatic reaction (*cf* Table 2). Therefore, in the ^{13}C experiment malonate was chosen as an equivalent for acetate in the chain propagation step of the polyketide formation. [2-^{13}C]-malonic acid was administered as the monosodium salt and the resulting spectrum compared with that which was obtained from natural pseurotin A (**1**) measured under identical conditions. For the unequivocal determination of the labelled positions we applied the method of Hanson[5] by normalizing the peak heights with respect to the OMe group, a C atom which is not acetate/malonate derived. In Table 4 these relative intensities are presented. C(3), C(5), C(10) and C(12) show a 3-fold signal enhancement. This result agrees with a biosynthesis via a C_{11}-polyketide. Surprisingly the signal intensities of the phenylalanine derived atoms seem to be lowered. A part of the ^{13}C activity is obviously scrambled during the biosynthesis, e.g. via citrate-cycle (*cf*[6]).

The C_1-donor methionine which had been shown to be a further biogenetic precursor was administered with the methyl group labelled by C-13. In the resulting ^{13}C NMR spectrum the intensity of both the OMe signal and the 16-Me group are increased about 15-fold. Since the unambiguous proof 15 years ago that in the biosynthesis of ergosterol exactly two of the three hydrogens of the Me group of methionine are retained during the alkylation of the side chain[7] much attention has been paid to the problem of the stereochemistry of the reaction and the different possible pathways. In order to gain more insight into the mechanism of the methylation process in pseurotin A (**1**) a sample of [$^{13}C^2H_3$]-L-methionine was synthesized from S-benzylhomocysteine and double labelled methyliodide according to known procedures[8] and administered to growing cultures of *Pseudeurotium ovalis*. Fig. 1 shows the proton noise decoupled ^{13}C NMR spectrum in the region 0–60 ppm. Both Me signals are clearly split into a septet with a coupling constant of 19.5 Hz for C(16) and 22 Hz for the O-OMe group, respectively. The resonance frequencies are both shifted upfield by 0.7 ppm. This value is also consistent with retention of all three D atoms. The OMe signal due to the natural methionine pool is superimposed by one of the seven lines, but the corresponding C(16)-peak is clearly resolved. Owing to quadrupole broadening and lacking nuclear Overhauser effect the intensities cannot be compared with those which are obtained after incorporation of mono-labelled methionine. A ^{13}C NMR spectrum, simultaneously noise decoupled of protons and deuteriums, shows two broad singlets for $^{13}CD_3$ and two narrow singlets for $^{13}CH_3$ (Fig. 2), but no further signal corresponding to a partially exchanged Me group. Accordingly, both alkylation processes proceed with retention of all three H atoms. These findings agree with the presumption that the C-methylation takes place in an early stage of the biosynthesis when the enolic double bond of the polyketide can react with S-adenosylmethionine, while the O-alkylation is probably a secondary transformation.

During the past few years much effort has been made to explore the origin and fate of the H atoms during a biosynthesis. One of the critical problems is the detection of the incorporated label. By using $^{13}C/^2H$ double labelled precursors it has become possible to observe indirectly the directly bound hydrogens. Thus "intact incorporation" can be established. The use of [$^{13}C^2H_3$]-acetate permits to localize the starter unit of a polyketide chain and to study the rate of exchange in acidic methylene groups.[9] We have extended this method to propionate in order to explore its scope and limitations in the case of the biogenesis of pseurotin A (**1**) and E (**6**). The latter compound is a minor metabolite of the same strain. It differs from pseurotin A (**1**) only by the geometry of the double bond and the state of oxidation of C(14).† If pseurotin E (**6**) is a biogenetic precursor of pseurotin A (**1**) quantitative

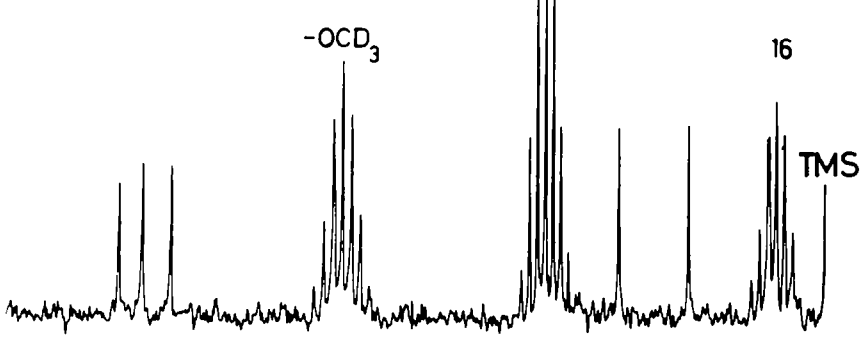


Fig. 1. Proton noise decoupled ^{13}C NMR spectrum of pseurotin A (**1**) (0–70 ppm) after incorporation of [$^{13}C^2H_3$]-L-methionine ($(CD_3)_2CO$).

†In a preliminary experiment pseurotin A (**1**) and E (**6**) gave almost the same dilution values after the incorporation of [2-^{14}C]-malonate.

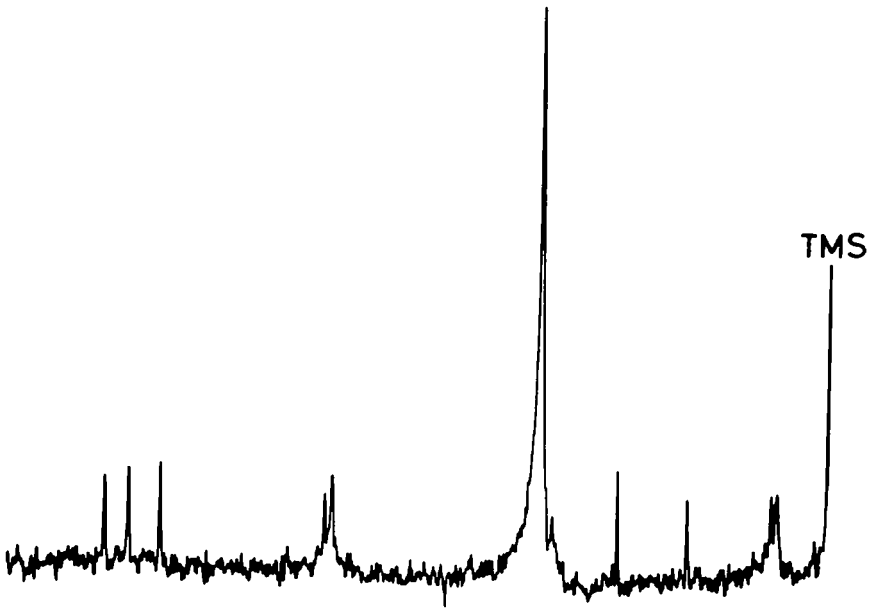


Fig. 2. Proton and deuterium noise decoupled ^{13}C NMR spectrum of pseurotin A (**1**) (0–70 ppm) after incorporation of [$^{13}C^{2}H_3$]-L-methionine ($(CD_3)_2CO$).

deuterium-loss is to be expected. [2-$^{13}C^{2}H_2$]-propionate was prepared according to Atkinson *et al.*[10] by repeated exchange of commercially available Na[2-^{13}C]-propionate in D_2O/OD^- at 160°. After six iterations a deuterium content of at least 98% was achieved. Fig. 3 shows the relevant region of the proton noise decoupled ^{13}C NMR spectrum of pseurotin A (**1**) after incorporation of this precursor. Nine clearly resolved lines appear: a quintet corresponding to the molecules which have retained both D atoms centred at 21.21 ppm; a triplet at 21.54 ppm and a singlet at 21.86 ppm for $^{13}CH_2$ whose intensity is slightly enhanced over the natural abundance. Taking into account that the fully deuterated species are lacking the nuclear Overhauser effet and that their integral is distributed over five (broadened) signals in the ratio 1:2:3:2:1 we can estimate that by far more than the half of the precursor molecules are incorporated in an intact manner. From these results it can be concluded that pseurotin E (**6**) does not act as an intermediate in the biosynthesis of pseurotin A (**1**). Either the biogenetic relationship is inverse or the pathways separate in an earlier stage. The observation that some deuterium is lost during the biosynthesis is not unexpected. Whereas it is unlikely that the propanoyl-coenzyme A or some acyl carrier derivatives are acidic enough to exchange with the hydrogens of water under physiological conditions, a rapid competitive equilibrium between propanoyl-coenzyme A and methylmalonyl-coenzyme A could explain the lower incorporation rate of deuterium as compared with ^{13}C.

For the experiments with [1-^{13}C]-D,L-phenyl-alanine a specimen was prepared using the Strecker-synthesis as described for radioactive samples.[11]

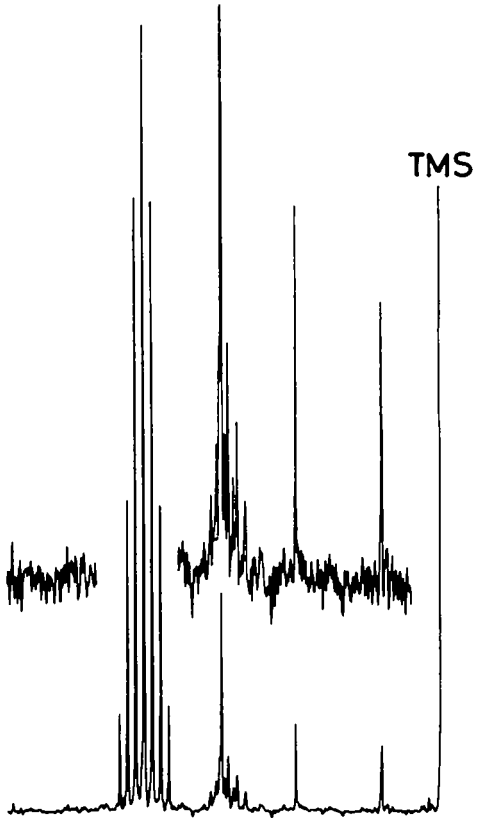
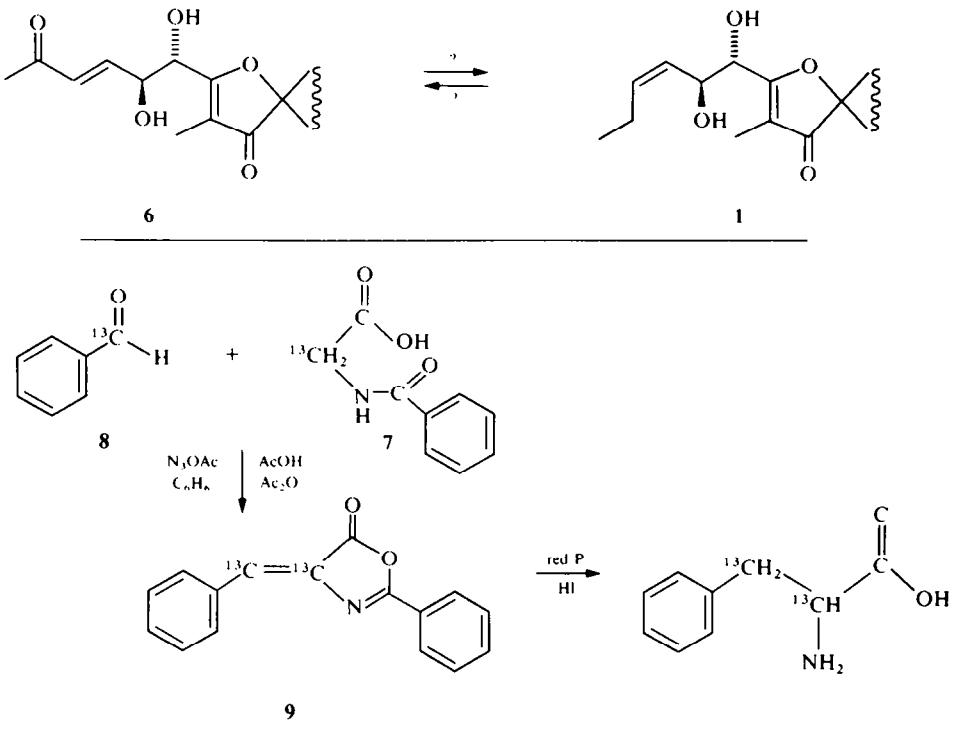


Fig. 3. Proton noise decoupled ^{13}C NMR spectrum of pseurotin A (**1**) (0–30 ppm) after incorporation of [2-$^{13}C^{2}H_2$]-propionate ($(CD_3)_2CO$).



Scheme 3.

Incorporation into pseurotin A (**1**) led to the expected results. The integral for C(9) is dramatically enhanced, showing that the amino acid is incorporated without decarboxylation or rearrangement. As mentioned above the double labelling technique was applied to distinguish the signals of C(5) and C(8) separated only by 0.3 ppm. For this purpose a synthesis for [2,3-$^{13}C_2$]-D,L-phenylalanine was carried out starting with commercially available Na[2-^{13}C]-acetate and [^{13}C]-benzoic acid. In the key step [2-^{13}C]-hippuric acid (**7**)[12] was condensed with [^{13}C]-benzaldehyde (**8**) obtained via the imidazolide.[13] Reductive opening of the resulting azlactone **9** yielded the desired double labelled amino acid (*cf* Scheme 3).[14] The ^{13}C NMR spectrum of the enriched pseurotin A (**1**) is shown in Fig. 4.

The adjacent carbons are incorporated as an intact unit and are therefore split into a doublet ($J = 51.5$ Hz). Since the resonance frequencies are separated by 2350 Hz (at 22.63 MHz) a first order spectrum is observed with the satellites lying symmetrically with respect to the middle signals. They are slightly enhanced because about 9% of the precursor molecules are each enriched with ^{13}C at C(2) and C(3), respectively. The peak at 92.4 ppm can now definitively be assigned to C(8) and the other at 92.7 ppm to C(5).

CONCLUSIONS

Using singly and doubly labelled ^{13}C precursors the biosynthetic origin of each of the 22 C atoms of pseurotin A (**1**) has been established. On the basis of these results two possible biogenetic pathways as outlined in Scheme 4 are proposed. However, neither the postulated intermediates nor the reaction sequence are based on an experimental proof. Propanoyl-coenzyme A acts as the starter unit. It is condensed in four successive cycles with malonate. The resulting C_{11}-polyketide combines with phenylalanine probably forming first the amide linkage. In the next step the lactam ring is closed to yield a tetramic acid derivative. For the construction of the spiro-center an OH group might be introduced in order to generate an electrophilic site at C(5). The C-methylation presumably occurs before the stabilization of the polyketide and its separation from the enzyme have taken place. In an alternative pathway the Δ^2-furenidone-(4) ring could be formed after the attachment of the 16-Me group at C(3). The partial reduction of the side chain is arbitrarily thought to take place in an early stage of the biosynthesis. The further reactions are likely to be secondary transformations and to occur at a late stage. They comprise oxidations at the benzylic C atom and at the nucleophilic positions C(8) and C(10) as well as the O-methylation.

The origin of the nitrogen atom

In spite of the fact that the biogenetic origin of N atoms in natural products has been studied for more than 30 years,[15] many crucial problems of the N metabolism have remained unresolved. No radioactive isotope is known which has a sufficiently long half-life suitable for biosynthetic investigations. Thus the incorporation of ^{15}N-labelled precursors is the only feasible method. The enriched metabolite can be examined either by mass spectrometry or more directly by ^{15}N NMR spectroscopy. However, the low natural abundance of ^{15}N of only 0.37% and the

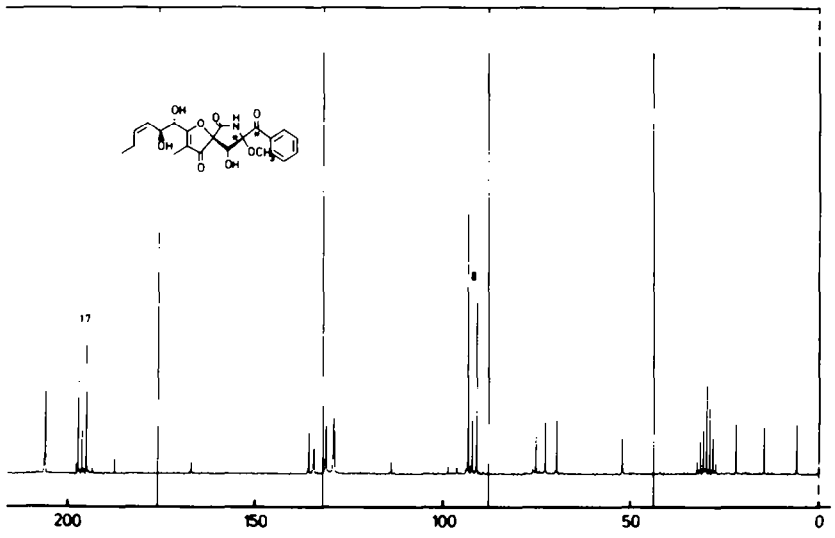
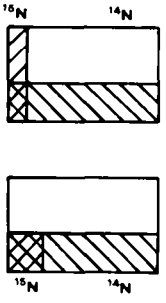


Fig. 4. Proton noise decoupled ^{13}C NMR spectrum of pseurotin A (**1**) after incorporation of [2,3-$^{13}C_2$]-D,L-phenylalanine ($(CD_3)_2CO$).

unfavourable nuclear properties are serious disadvantages. Additional difficulties for the establishment of the biogenetic network are due to the ubiquitous cellular transaminases. They convert α-amino acids reversibly into the corresponding α-keto acids. Therefore a negative incorporation experiment does not allow one to draw reliable conclusions. We now propose a novel approach to circumvent these problems to obtain some information concerning the origin of the nitrogen atoms in mould metabolites. [^{15}N]-L-phenylalanine and—in a separate experiment—equal amounts of [2-^{13}C,^{15}N]-D,L-phenylalanine, which was synthesized from commercially available double labelled glycine in a similar way as shown in Scheme 3, were administered to growing cultures of *Pseudeurotium ovalis*. From the mass spectra of both enriched specimens of pseurotin A (**1**) the rates of incorporation were determined using the fragment ion *m/z* 326 (M^+-benzoyl). The intensity of the molecular peak is too low—even after silylation with trimethylchlorosilane/hexamethyldisilazane—to supply reliable results. On the other hand both interesting atoms are certainly part of the fragment ion *m/z* 326 and since this peak is not superimposed by others this disadvantage carries little weight. The percentage of ^{15}N loss was also determined by ^{13}C NMR spectroscopy of the double labelled pseurotin A (**1**) sample.

The two experiments are based on the following reasoning. Even if transaminase activity is present in the fungal metabolism, the probabilities for the incorporation of ^{13}C and ^{15}N are not independent of each other. Although the bulk of the N isotope is certainly lost during the biosynthesis and dispersed over the whole amino acid pool, the retained ^{15}N atoms will be not exclusively but predominantly, situated next to a ^{13}C-atom unless the rate of exchange is of another order of magnitude than if the rate of the pseurotin A (**1**) biosynthesis. Only in this rather improbable case a statistical and independent incorporation of ^{13}C and ^{15}N is to be expected. This dependence should be reflected by the ratios of the isotopic peaks in the mass spectra as well as by the intensities of the ^{15}N-satellites in the ^{13}C NMR spectra. Let P13, P15 be the probabilities that atom Nr. 8 is a ^{13}C-, Nr. 7 a ^{15}N-isotope respectively. The following graphic illustration which represents the two readily calculable extremes, shows that the first isotopic peak is (relatively) smaller, the second one (relatively) higher, if the ^{15}N atoms are preferably adjacent to a ^{13}C atom.†



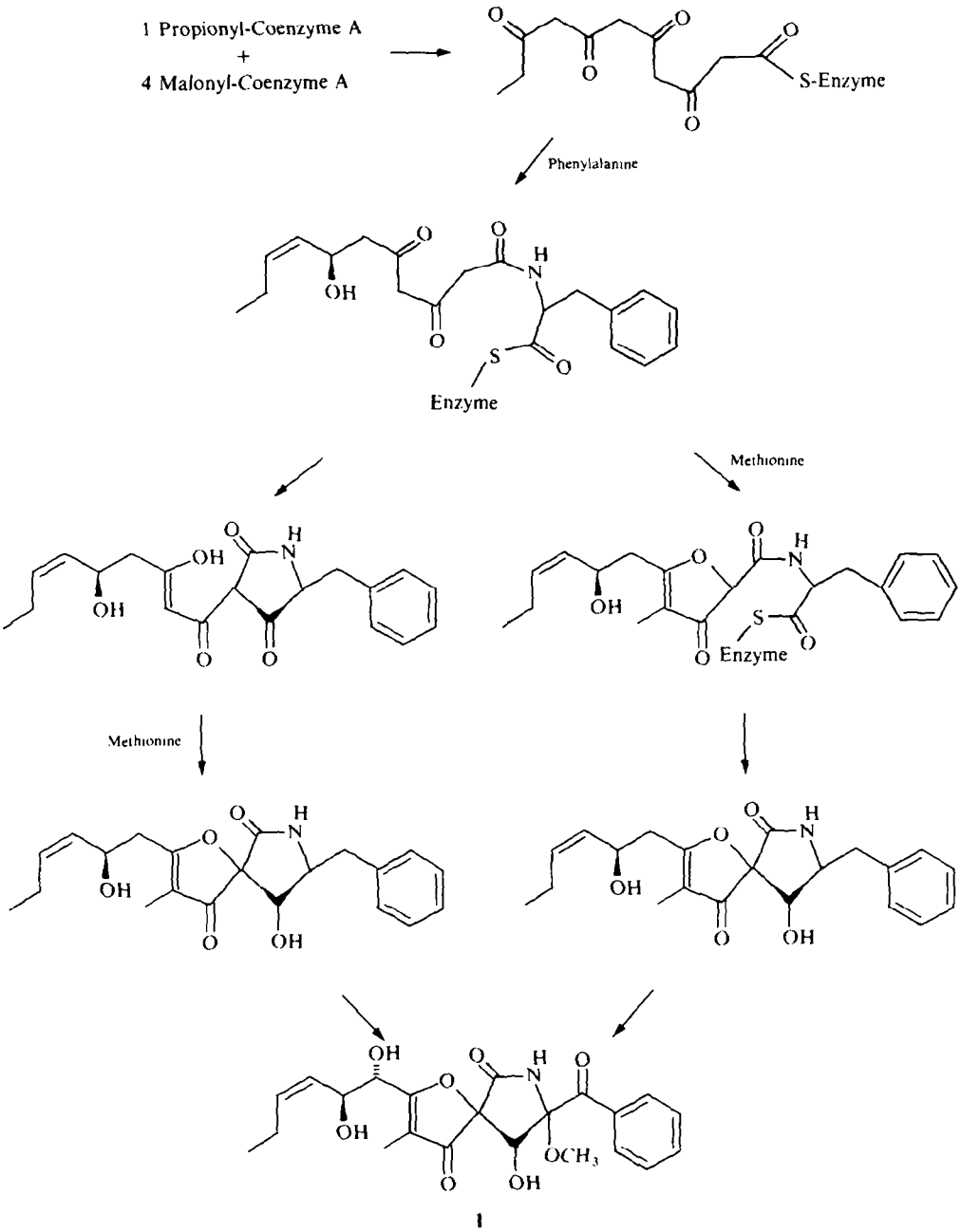
	(1-P13) × (1-P15)	M^+
	P13 × (1-P15)	$M^+ + 1$
	P15 × (1-P13)	
	P13 × P15	$M^+ + 2$
	1 − P13	M^+
	P13 − P15	$M^+ + 1$
	P15	$M^+ + 2$

In natural pseurotin A (**1**) and in the enriched specimen obtained after administering 63 mg [^{15}N]-L-phenylalanine (95 atom%)/600 ml the ratios of the isotopic peaks were determined as follows:

326	327	
100	16.7 ± 2.13 (±0.870, 95%, 7)	natural pseurotin A (**1**)
100	20.9 ± 1.24 (±0.489, 95%, 6)	[^{15}N]-enriched pseurotin A (**1**)

Taking into account that due to the low natural abundance of ^{15}N, the isotopic pattern of the labelled molecules is nearly the same as that of the unlabelled molecules, except for one atomic mass unit shift, the

†Complete independence would be necessarily observed if a nitrogen free compound like phenylpyruvic acid or a cinnamic acid derivative was a real intermediate.



1

Scheme 4.

mole fraction X of ^{15}N can be calculated with a fair accuracy:

326	327	328	
39.2	6.55	1	Natural labelling pattern
$(1-X) \times 39.2$	$(1-X) \times 6.55$	$(1-X) \times 1$	$(1-X)$ Molecules are unlabelled

$(1-X) \times 39.2 : [(1-X) \times 6.55 + X\,39.2]$ = Labelling pattern of ensemble of labelled and unlabelled molecules

$= 25.6 : 5.34$

$\Rightarrow X = 0.04$

About 4% of all molecules are labelled at position 7. The overwhelming majority of ^{15}N is lost, the dilution is much greater than in the corresponding ^{13}C-experiments. But the incorporation rate is always higher than in similar experiments in the cytochalasan series.[16]

The results have to be compared with those obtained after the incorporation of 88 mg of [2-^{13}C, ^{15}N]-D,L-phenylalanine/800 ml (91.3 atom % ^{13}C, 99.1 atom % ^{15}N). Fig. 5 shows the relevant region of the proton noise decoupled ^{13}C NMR spectrum. The peak at 92.4 ppm is strongly enhanced, thus the main part of the label is lost as in the preceding experiment. In addition, two symmetrical ^{15}N-satellites can be observed, the left one being superimposed by the signal for the spiro centre C(5). A coupling constant of 12.5 Hz is consistent with the published data for $J_{^{13}C^{15}N}$.[17]

Their intensity is nearly 10% of the middle peak and not 4% as expected. Although comparisons of integral values of digitalized NMR spectra should be done with care, a statistical incorporation seems to be improbable. With a 50-fold signal enhancement supposed for C(8), each of the satellites should show a lower intensity than an unlabelled C atom. On the other hand, the ^{13}C-satellites of C(8)—obviously 1.1% of the central resonance—should each exhibit an intensity which is 25–30% of the intensity of the ^{15}N-satellites. But this behaviour is not observed. The two symmetrical lines separated by 90 Hz are side bands, because we know from the incorporation experiments with [2,3-^{13}C-D,L-phenylalanine that the coupling constant between C(8) and C(17) is 51.5 Hz. The coupling with C(9), a sp^3-nucleus, must be still weaker.[17] Thus the ^{15}N-satellites show nearly a ten times greater intensity than the ^{13}C-satellites which are not clearly recognized in the noise.

These observations and their interpretation are in good agreement with the results of the mass spectra. Taking into account the isotopic pattern for natural pseurotin A (**1**), one can set up two equation systems; one assuming that the probabilities for incorporation of ^{13}C and ^{15}N are fully independent, the other supposing that ^{15}N is found only next to ^{13}C:

	^{13}C and ^{15}N statistically distributed			^{15}N only next to ^{13}C			
	326	327	328	326	327	328	
Natural labelling pattern	100	16.7	2.55	100	16.7	2.55	
(1 − P13) × (1 − P15)	100	16.7	2.55	100	16.7	2.55	(1-P13)
P13 × (1-P15)		100	16.7		100	16.7	(P13-P15)
P15 × (1-P13)		100	16.7				
P15 × (1-P13)		100	16.7				
P13 × P15			100			100	P15
pattern found in the enriched metabolite	100	131.8	33.5	100	131.8	33.5	
	P13 = 0.51; P15 = 0.10			P13 = 0.56; P15 = 0.05			

Fig. 5. Proton noise decoupled ^{13}C NMR spectrum of pseurotin A (**1**) (70–120 ppm) after incorporation of [2-^{13}C, ^{15}N]-D,L-phenylalanine ($(CD_3)_2CO$).

Both solutions are compatible with the ^{13}C NMR spectrum, but only the second one with the results obtained after incorporation of [^{15}N]-L-phenylalanine. It is impossible to explain why the ^{15}N-incorporation rate should now be more than doubled. Although the accuracy of our experiment is not ideal, because the metabolites were produced in two separate runs, a nitrogen free intermediate, which would necessarily require an independent incorporation of ^{13}C and ^{15}N, is highly improbable.

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EXPERIMENTAL

1. *General methods.* mps were determined on a Kofler block and are corrected. Optical rotations were measured on a Perkin-Elmer polarimeter, model 141. The 90 MHz ^1H NMR and 22.63 MHz ^{13}C spectra were recorded with a Bruker WH 90 spectrometer with Fourier transform in our institute (K. Aegerter). The mass spectra were measured in the Institute of Physical Chemistry, University of Basel, on an A.E.I. MS 30 instrument (R. Fink). We are indebted to Mr. G. Marbach, Sandoz AG., Basel, for radioactivity determinations and to Dr. J. C. Vederas, University of Alberta, Edmonton, Canada, for running the deuterium noise decoupled ^{13}C NMR spectrum.

Radioactive precursors were purchased from Amersham Radiochemical Centre (Buckinghamshire, England). ^{13}C- and ^{15}N-labelled compounds were obtained from Radium Chemie (Teufen, AR, Switzerland), Sharp & Dohme GmbH (München, BRD), KOR Isotopes (Cambridge, Mass., U.S.A.) and Prochem (London, England). Preparative tlc was carried out with silica gel PF 254 (Merck) and column chromatography with silica gel 70-325 mesh from E. Merck AG., Darmstadt, BRD, or with Florisil 100-200 mesh from Sigma, St. Louis, Mo, U.S.A.

2. *Production of pseurotin A* (**1**) *and E* (**6**). Inocula were prepared from slants of *Pseudeurotium ovalis* Stolk (strains S 2269 and S 3484) on agar (2% malt, 0.4% yeast extract). Cultures were grown in 500 ml Erlenmeyer flasks, each flask containing 200 ml of the following medium (g/l): glucose 20; pepton 2; yeast extract 2; KH_2PO_4 2; $MgSO_4 \cdot 7H_2O$ 2; pH 4.8-5.3 (not adjusted). All cultures were shaken for 8 days at 28° on a rotary shaker (220 rpm). Aqueous solns of the precursors (pH was adjusted if necessary) were sterilized and added aseptically after 48 hr. The mycelium and the medium were extracted with the same volume of EtOAc and the organic layer washed with water. The organic extract was dried with Na_2SO_4 and concentrated *in vacuo*. The residue was purified on a silica gel column (CH_2Cl_2/MeOH) to give 60-100 mg/l of **1** and ≤ 8 mg/l of **6**, respectively. The latter was isolated after tlc as unstable oil, pseurotin A (**1**) could be recrystallized from THF/hexane.

3. *Degradation to benzoic acid.* A soln of 100 mg of **1** (0.23 mmol) in 4 ml 30% HNO_3 was refluxed for 3.5 hr. The resulting benzoic acid was isolated from the mixture by steam distillation (350 ml) and neutralized with 0.05 N KOH against phenolphthalein. After evaporation the K-salt was dissolved in 2 ml H_2O and 4 ml MeOH and the pH adjusted to 6 (dil HCl). 84 mg (0.30 mmol) of *p*-bromophenacylbromide was added and the mixture boiled for 3.5 hr. The solvent was evaporated, the residue extracted with CH_2Cl_2 and the insoluble KBr filtered off. Purification by tlc (benzene/2 × development) gave 23.6 mg (0.074 mmol) of pure *p*-bromophenacylester, m.p. 116-117°, which was identical with an authentic sample.

4. 12, 13-*Dihydropseurotin A* (**4**). 144 mg (0.33 mmol) of **1** was dissolved in 20 ml benzene/EtOH (1:1) and hydrogenated in the presence of 70 mg (0.076 mmol) of tris-(triphenylphosphine)rhodium chloride for 12 hr at room temp and atmospheric pressure. The solvent was evaporated and the catalyst removed by filtering through a short column of alumina. The crude product was purified on a silica gel column (CH_2Cl_2/MeOH) to yield 83 mg (0.19 mmol) of **4**, mp. 176-179° (dec). IR (KBr): 1715, 1680, 1605; ^1H-NMR (DMSO): 0.8 (m, 3 H, C(15)); 1.2 (m, 6 H, C(14), C(13), C(12)); 1.64 (s, 3 H, C(16)); 3.25 (s, 3 H, O CH_3); 4.39 (d, J = 8.8, 1 H, C(9)); 4.3-4.6 (m, 2 H, C(10), C(11)); 4.57 (d, J = 5.9, 1 H, C(11) OH); 5.68 (d, J = 5.6, 1 H, C(10)-OH); 6.29 (d, J = 8.8, 1 H, C(9)-OH), 7.4-7.7 (m, 3 H, C(20) C(22)); 8.2-8.3 (m, 2 H, C(19), C(23)); 9.94 (s, 1 H, -NH); ^{13}C NMR ($(CD_3)_2CO$): Table 1: MS: 401 (M^+ - 32 (MeOH), 328 (M^+ - 105 (benzoyl)); 315 (base peak, M^+ - 32 - 86 (pentanal)), 105 (benzoyl), 77 ($C_6H_5^+$).

5. 17-*Dihydropseurotin A* (**5**). A few crystals of methylorange were dissolved in 10 µl H_2O and 200 µl THF. 144 mg (0.33 mmol) of **1** and 22 mg (0.35 mmol) $NaBH_3CN$ and then, drop by drop, a mixture of 30 µl H_2O and 30 µl acetylchloride in 200 µl THF were added so that the indicator kept its red colour. The reaction was followed by Hc CH_2Cl_2/MeOH). The crude product was diluted with 10 ml H_2O and extracted with three portions of 10 ml CH_2Cl_2. The organic layer was dried with Na_2SO_4 and concentrated *in vacuo*. After purification on a silica gel column (CH_2Cl_2/MeOH) 73 mg (0.17 mmol) of **5** (epimeric mixture in a ratio of 9:1) and a small amount of starting material were obtained.

^1H-NMR (DMSO): 0.92 (t, J = 7, 3 H, C(15)); 1.60 (s, 3 H, C(16)); 1.9-2.2 (m, 2 H, C(14)); 3.23 (s, 3 H OCH_3); 4.3-4.8 (m, 6 H, C(9), C(10), C(11), C(9)-OH, C(17) OH); 4.87 (br d, 1 H, C(11)-OH); 5.4 (m, 2 H, C(12), C(13)); 5.74 (d, J = 4.4, 1 H, C(10) OH); 7.25-7.44 (m, 5 H, C(19) C(23)), 8.97 (s, 1 H, NH). ^{13}C-NMR ($(CD_3)_2CO$): Table 1.

Synthesis of precursors

6. [$^{13}C^2H_3$]-L-*methionine* (*cf*.[8]). S-Benzyl-L-homocysteine was prepared from commercially available D,L-homocystine via brucine salt of the N-formyl-S-benzyl-derivative according to ref. 8. The crude product showed $[\alpha]_D^{20}$ + 17.5 (*c* 1 in 1 N HCl). 0.62 g (2.75 mmol) thereof dissolved in liquid ammonia was reduced by adding small pieces of Na (~0.2 g) until the blue colour persisted for several min. The temp of the cooling bath was raised to −50° and 170 µl (365 mg, 2.65 mmol) of [$^{13}C^2H_3$]-methyliodide (90 atom % ^{13}C, 98 atom % ^2H) was introduced by a syringe directly beneath the surface of the mixture. After evaporation of the ammonia the residue was dissolved in water and treated with HI to pH 5. The filtered soln was concentrated to *ca* 2 ml, 50 ml hot EtOH was added and the mixture kept at 0° to yield 344 mg (2.25 mmol) of [$^{13}C^2H_3$]-L-methionine, which was checked on HC (n-butanol: acetic acid: water = 8:2:2). Runs with unlabelled material showed $[\alpha]_D^{20}$ − 4.5° (*c* 0.96 in H_2O).

^1H-NMR (Dioxane): 3.8 (m, 1 H, C(2)); 2.0-2.7 (m, 4 H, 2 × CH_2); no aromatic protons and no signal for $^{13}CHD_2$.

7. [1-^{13}C]-D,L-*phenylalanine* (*cf*.[11]). 5.8 g (35 mmol) of 1,1-dimethoxy-2-phenylethane was refluxed in 2 ml MeOH and 2 ml 6 N HCl for 10 min. After removing the solvent carefully and quantitatively (otherwise the yields are very low) under reduced pressure the crude phenylacetaldehyde was added to a soln of 323 mg (6.03 mmol) ammonium chloride in 1 ml H_2O and cooled to 0°. This was immediately followed by the methanolic soln (10 ml) of 50 mg of labelled sodium cyanide (90 atom % ^{13}C). The residual 279 mg of $Na^{13}CN$ (total amount 329 mg (6.6 mmol) were dissolved in 4 ml 50% MeOH and added drop by drop. After standing overnight 0.7 ml 50% NaOH was added to the yellow mixture and the alcohol removed by distillation. The α-aminonitril and the polymerized aldehyde were separated from the inorganic salts by extraction with ether (2 ×). The combined ether solns were acidified with 7 ml 4 N HCl and distilled into a soln of NaOH (HCN!). The gummy residue containing the amino acid was heated with three 10 ml portions 3 N HCl. The combined extracts were evaporated to dryness and then treated with

conc HCl. After filtration the soln was concentrated to a volume of *ca* 1.5 ml; *ca* EtOH was added and the pH adjusted to 6 with alcoholic ammonia. On cooling, 330 mg (2 mmol) of [1-^{13}C]-D,L-phenylalanine separated out. The product was checked on Hc (n-butanol: acetic acid: water = 8:2:2) and used without further purification.

8. [2,3-$^{13}C_2$]-D,L-*Phenylalanine* (*cf* [12,13,14]). Starting from Na[2-^{13}C]-acetate (90 atom% ^{13}C), [2-^{13}C]-hippuric acid (**7**) was prepared according to known procedures.[12] The latter was condensed with [^{13}C]-benzaldehyde (**8**), which had been synthesized from [^{13}C]-benzoic acid (90 atom% ^{13}C) via the imidazolide,[13] in the following manner: 1.88 g (8.9 mmol) of $C_6H_5{}^{13}CHO \cdot NaHSO_3$ and 1.61 g (8.9 mmol) of 7 were dissolved in 5.1 ml Ac_2O, 0.55 ml AcOH and 11 ml benzene. 750 mg NaOAc was added and the mixture refluxed for 5 hr. After cooling the cake was transferred to a Büchner funnel, washed twice with 2 ml cold H_2O and dried. The mother liquor was chromatographed on a florisil column (CH_2Cl_2). The combined crops of **9** (1500 mg, 5.58 mmol) were directly used for the next step. 325 mg (1.2 mmol) of **9** was treated with 275 mg (7.3 mmol) red P and 1.7 ml Ac_2O. During a period of about 1 hr 1.6 ml of 50% HI was added (exothermic reaction! If the mixture solidified the cake was stirred with a glass rod). The suspension was then refluxed for 3 hr and, after cooling, filtered with suction. The filtrate and the washings (2 × 2 ml glacial AcOH) were evaporated *in vacuo*, dissolved in 20 ml H_2O and evaporated again to dryness. 20 ml ether and 20 ml H_2O were added to the residue and the mixture vigorously shaken. The aqueous layer was separated and extracted with two 10 ml portions ether. The water soln was heated on a steam bath with a trace of Na_2SO_3 to remove the dissolved ether. The volume was reduced to *ca* 2 ml and the pH adjusted to *ca* 6 with alcoholic ammonia. 150 mg (0.90 mmol) of [2,3-$^{13}C_2$]-D,L-phenylalanine could be isolated which was checked on Hc (n-butanol: acetic acid:water = 8:2:2) and incorporated without further purification. Phenylalanine from runs with unlabelled material was also examined by ^{1}H NMR.

9. [2-^{13}C,^{15}N]-D,L-*Phenylalanine* (*cf* [12,14]). According to ref. 12 210 mg (2.73 mmol) of commercially available [2-^{13}C,^{15}N]-glycine (91.3 atom% ^{13}C, 99.1 atom% ^{15}N) was treated with 422 mg (3.0 mmol) benzoyl chloride to yield 482 mg (2.66 mmol) double labelled hippuric acid (mp. 182–183°). Condensation with benzaldehyde and reductive opening of the azlactone were carried out following the procedure for [2,3-$^{13}C_2$]-D,L-phenylalanine. The resulting amino acid (159 mg, 0.95 mmol) was used without further purification.

^{13}C-NMR (D_2O/OD$^-$, chemical shifts refer to the signal at 131.1): 41.1 (d, $J_{^{13}C,^{13}C}$ *ca* 35, C(3)); 58.8 (d, $J_{^{13}C,^{15}N}$ 3, C(2) ninety-fold increased); 128.7 (*para*); 130.5 (*meta*); 131.1 (*ortho*), 139.3; C(1) (d) could not be detected.

10. *Sodium* [2-$^{13}C^2H_2$]-*propionate* (*cf* [10]). 1.45 g (15 mmol) of sodium [2-^{13}C]-propionate (91.8 atom% ^{13}C) was dissolved in 20 ml of D_2O (99.7 atom% ^{2}H) containing 5 mmol of NaOD and kept in a sealed tube at 160° for 48 hr. This process was repeated six times yielding a precursor containing at least 98 atom% ^{2}H in α-position. MS (free acid): 77 (M^+ Calc. for $CH_3{}^{13}CD_2COOH$), 60, 45. ^{13}C-NMR (D_2O/OD$^-$, chemical shifts refer to the signal at 30.2): 10.1 (d, $^2J_{^{13}C,^{13}C}$ 35, C(3)); 30.2 (qui, $^2J_{^2H,^{13}C}$ 19, C(2)D_2); 30.5 (t, $^2J_{^2H,^{13}C}$ 19, C(2)HD, the intensity is 4.8% of that for $^{13}CD_2$); the signal for C(1) (d) was too weak to be detected.

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