Biosynthesis of Cytochalasans. XI. New Results on the Incorporation of Phenylalanine into Cytochalasin D by *Zygosporium masonii* [1]

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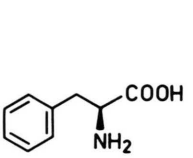
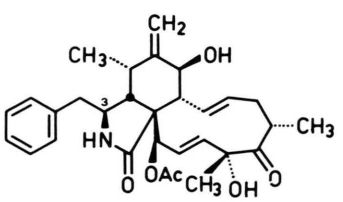
Incorporation of L-[2-^{2}H]phenyl-[2-^{2}H]alanine and L-phenyl-[2-^{13}C, ^{15}N]alanine into cytochalasin D by *Zygosporium masonii* involved the complete loss of both the α-^{2}H- and the α-^{15}N-atom. Incorporation of a mixture of L-phenyl-[^{15}N]alanine and L-[U-^{14}C]phenylalanine into cytochalasin D and protein amino acids (phenylalanine, leucine, isoleucine) was accompanied by a substantial loss of ^{15}N with respect to ^{14}C. These effects are attributed to rapid exchange reactions taking place while L-phenylalanine is part of the intracellular pool of amino acids. In addition, the medium- and concentration-dependent incorporation of the carbon skeleton of exogeneous D-phenylalanine into cytochalasin D is reported. In a peptone-based complex medium, D-phenylalanine is poorly incorporated. Throughout the whole concentration range (0–250 mg/l), the incorporation rates are less than 10% of those of L-phenylalanine. In a minimal medium containing NH_4NO_3 as nitrogen source however, D-phenylalanine is preferred over the natural enantiomer by a factor of 1.28 up to 6.78, depending on the concentrations of exogeneous D- and L-phenylalanine. These effects are attributed to the medium-dependent activities of different amino acid transport systems responsible for the uptake of D- and L-phenylalanine in *Z. masonii*.

Cytochalasin D is the major secondary metabolite of five zygosporins produced by *Zygosporium masonii* all belonging to the class of cytochalasans [2–4]. It has previously been shown by Lebet and Tamm that the carbon skeleton of L-phenylalanine is incorporated into cytochalasin D by *Z. masonii* as an intact unit [5]. Further investigations on the utilization of both phenylalanine enantiomers carried out by Vederas and Tamm [6] seemed to indicate that (1) both D- and L-phenylalanine are incorporated equally well into cytochalasin D, and (2) the aliphatic H-atoms and the amino group of both phenylalanine enantiomers are partially lost before or during the biosynthesis of cytochalasin D. However, a concise analysis of the data presented by these authors suggests that their set of experiments is actually incomplete. In this paper we first present the reinterpretation of their data and then describe additional experiments specifically designed to shed light on the remaining problems.



L-Phenylalanine



Cytochalasin D

Analysis of Previous Results

In a preliminary experiment Vederas and Tamm administered a mixture of DL-phenyl-[2-^{14}C]alanine and L-[4-^{3}H]phenylalanine to *Z. masonii*, using a complex medium based on Bacto-Peptone. An unchanged ^{3}H/^{14}C ratio in cytochalasin D suggested that both phenylalanine enantiomers are equally good precursors. To elucidate the mechanism of utilization of both enantiomers, four incorporation experiments with stereospecifically labelled phenylalanines were carried out (Fig. 1). Labels (^{3}H) were located at C(3) of both enantiomers, and at C(2) of L-phenylalanine. The ^{3}H-retention values were measured using ^{14}C-standards of L- or DL-phenylalanine, respectively. In addition, the authors describe a fifth experiment, which is however not independent from the experiments (3) and (4): Administration of a 1:1-mixture of the precursors of (3) and (4) lead of a ^{3}H-retention of

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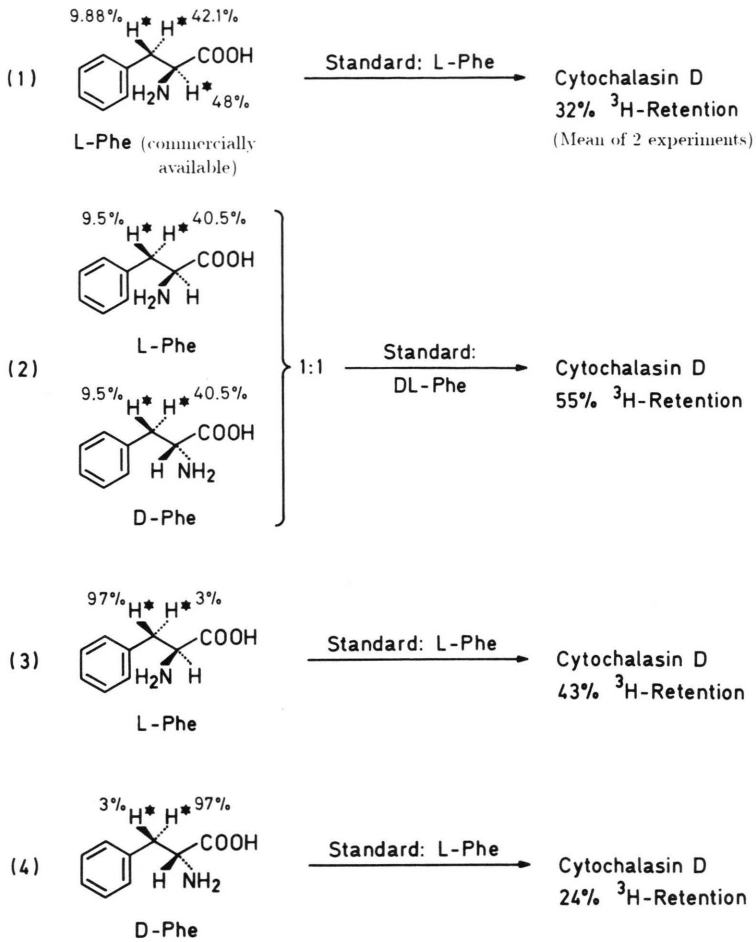


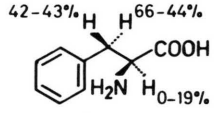
Fig. 1. Incorporation of stereospecifically labelled phenylalanines into cytochalasin D by *Z. masonii*. The data have been determined by Vederas and Tamm [6].

34%. This value is in good agreement with the mean of 43% and 24%.

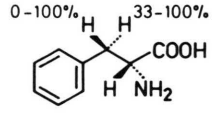
In principle, the data shown in Fig. 1 contain some information on the ^{3}H-retention of each of the aliphatic ^{3}H-atoms which were labelled in the precursor samples. The aim of the following examination is the determination of each of these retentions. Let them be *a*, *b*, *c*, *d*, *e*, each of these quantities being assigned to one of the aliphatic H-atoms of L- and D-phenylalanine as follows:

It is reasonable to define *a*, *b*, *c* in relation to the carbon skeleton of L-phenylalanine and *d*, *e* in relation to that of D-phenylalanine. As to the solution of the problem, a fundamental difficulty is encountered: Only four experiments are available for the determination of five unknowns. Hence the true values of *a*, *b*, *c*, *d*, *e* cannot be calculated. Only upper and lower limits for each of the values may be determined using the additional requirement that all unknowns are restricted to take only chemically reasonable values between 0 and 100%. An approach of this kind first leads to four (linear) equations corresponding to the four experiments. However, the system may not be solved without an unexpected inconsistency: It is not possible to place *c* and *d* between 0 and 100% at the same time. Either $-13\% > c >$

-31% or $172\% < d < 494\%$ is obtained. This difficulty may be avoided by the introduction of an additional variable $x = I_{abs.}(\text{D-Phe})/I_{abs.}(\text{L-Phe})$, where $I_{abs.}$(D-Phe) and $I_{abs.}$(L-Phe) denote the absolute incorporation rates for the carbon skeletons of D- and L-phenylalanine, respectively. Although $x = 1$ seems to be true due to the incorporation experiment mentioned first, the introduction of this variable may be rationalized as follows. The equally efficient incorporation of D- and L-phenylalanine had been determined using commercially available tracers with high specific activity, *i.e.* using minute amounts of substance. On the other hand, the specifically labelled precursors of the experiments (2) to (4) had to be prepared and administered on a 100 mg scale with lower specific activity. Since the enzyme systems for the uptake and utilization of the unnatural D-phenylalanine might depend on its concentration in the medium, it is reasonable to allow for these effects in the experiments (2) and (4). With this extension, the solution of the system is possible and leads to the following upper and lower limits for all unknowns:



L-Phenylalanine



D-Phenylalanine

$$x = \frac{I_{D\text{-Phe}}}{I_{L\text{-Phe}}} = 69\text{-}24\%$$

In summary, the experiments (1) to (4) may be consistently analysed with the assumption that D-phenylalanine, being present in the nutrient medium in larger amounts, is less efficiently incorporated into cytochalasin D than L-phenylalanine. Furthermore it is obvious that the determination of the true values of *a, b, c, d, e, x* requires two additional experiments. We have decided to directly determine the values of *c* and *x*, respectively. The retention of H−C(2) in L-phenylalanine is of special interest in this case, since a significant retention would prove the role of L-phenylalanine as an intact precursor in cytochalasin D biosynthesis. On the other hand, *x* was selected because its value might vary with the concentration of D-phenylalanine.

Incorporation of L-[2-^{2}H]Phenyl-[2-^{2}H]alanine

Instead of a ^{3}H-labelled sample a specifically deuterated precursor was used to determine the retention of H−C(2) of L-phenylalanine. Optically pure L-[2-^{2}H]phenyl-[2-^{2}H]alanine was prepared from [2-^{2}H]benzaldehyde, [$^2H_4$]malonic acid and ammonia according to a general method for the synthesis of specifically labelled L-phenylalanines [7]. The ^{2}H-atom on the aromatic ring acts as an internal reference label permitting the determination of the α-^{2}H-retention in the ^{2}H NMR spectrum.

The ^{2}H NMR spectrum of the doubly labelled precursor and the results obtained after its incorporation into cytochalasin D are shown in Fig. 2. Whereas the aromatic deuteron, representative of the L-phenylalanine carbon skeleton, was incorporated as expected, a resonance which unambiguously can be assigned to ^{2}H−C(3) of cytochalasin D is clearly missing. However, at 3.4 ppm a very weak signal may be discerned, having an intensity of not more than 1% with respect to the reference label. Though this signal might be due to ^{2}H−C(3), the assignment is rather tentative, since deuterons of natural abundance will exhibit their resonances at the same intensity.

Hence, the α-H-atom of L-phenylalanine is lost before or during the biosynthesis of cytochalasin D takes place, and a value of less than 1% may be assigned to the corresponding variable *c*.

Incorporation of the Carbon Skeleton of D-Phenylalanine

The incorporation of D-phenylalanine with respect to L-phenylalanine was measured using ^{14}C-labelled DL-phenylalanine and an internal reference of ^{3}H-labelled L-phenylalanine, both of high specific activity. Assays were based on the simultaneous counting of ^{14}C- and ^{3}H-activities in a liquid scintillation counter. Since the unknown *x* mentioned above was expected to exhibit a concentration dependence, several experiments were carried out at different D-phenylalanine concentrations (0−250 mg/l). D-Phenylalanine was added either in pure form or as a component of DL-phenylalanine. The experiment at negligible concentration was included in this series as a control, since the initial experiment of Vederas and Tamm had suggested an equally efficient incorporation, *i.e.* $x = 1$, at these conditions.

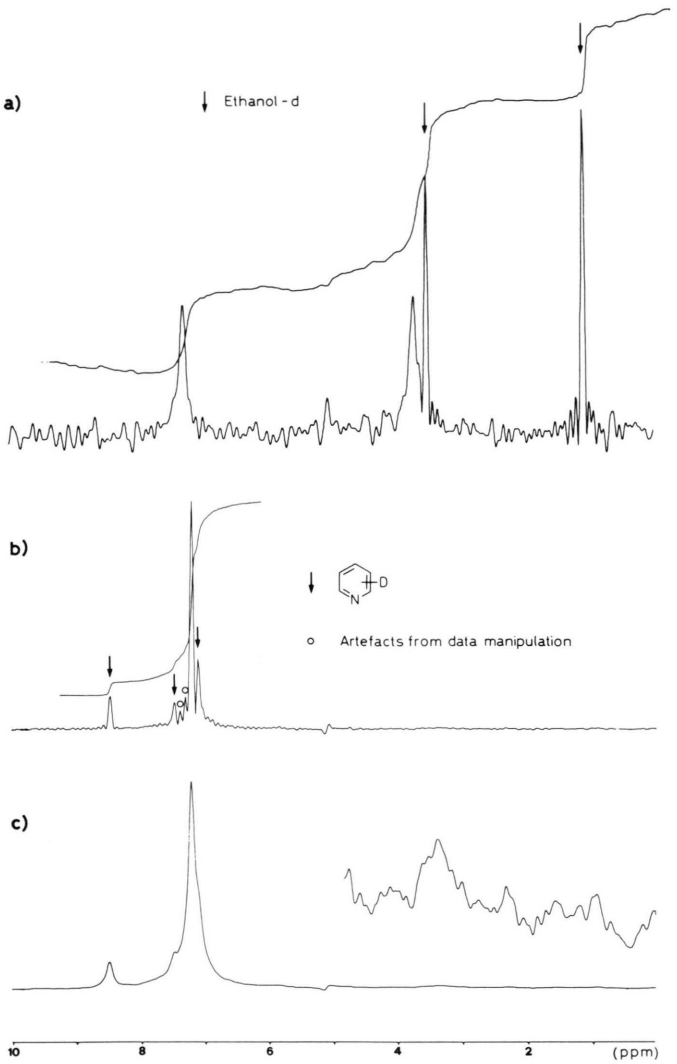


Fig. 2. Incorporation of L-[2-^{2}H]phenyl-[2-^{2}H]alanine (250 mg/l) into cytochalasin D, using the same complex nutrient medium as employed by Vederas and Tamm [6]. ^{2}H NMR spectra of a) L-[2-^{2}H]phenyl-[2-^{2}H]alanine (84% ^{2}H-ar, 96% α-^{2}H), b) cytochalasin D after incorporation of the doubly labelled precursor, c) like b) but with enhanced signal/noise ratio at the expense of signal width. We thank Prof. D. H. Günther, Siegen, F.R.G., for the measurement of these spectra.

The results which were obtained using the complex medium are shown in Fig. 3 (left-hand section). A completely unexpected value of $x = 0.1$ or less is observed throughout the whole concentration range. These findings not only contrast with the previous result $x = 1$ at negligible D-phenylalanine concentration, but are also incompatible with a range of $0.24 < x < 0.69$, which was deduced from the experiments shown in Fig. 1*. To check the significance of our results we have performed the critical experiment at negligible D-phenylalanine concentration repeatedly, using different pairs of labelled phenylalanines purchased from different manufactures each time. We obtained the weighted mean $x = 0.0896 \pm 0.0100$ showing that D-phenylalanine is practically not incorporated. Part of the corresponding low incorporation rates may even be attributed to a contamination of ^{3}H-labelled L-phenylalanine, used as an internal reference, by the ^{3}H-labelled D-enantiomer. Assuming this contamination to be 4% and $I_{abs.}$(D-Phe) = 0, an apparent value x of *ca.* 0.0417 would be observed.

* The permitted range for x even reduces to $0.48 < x < 0.69$, if the condition $c = 0$ is included.

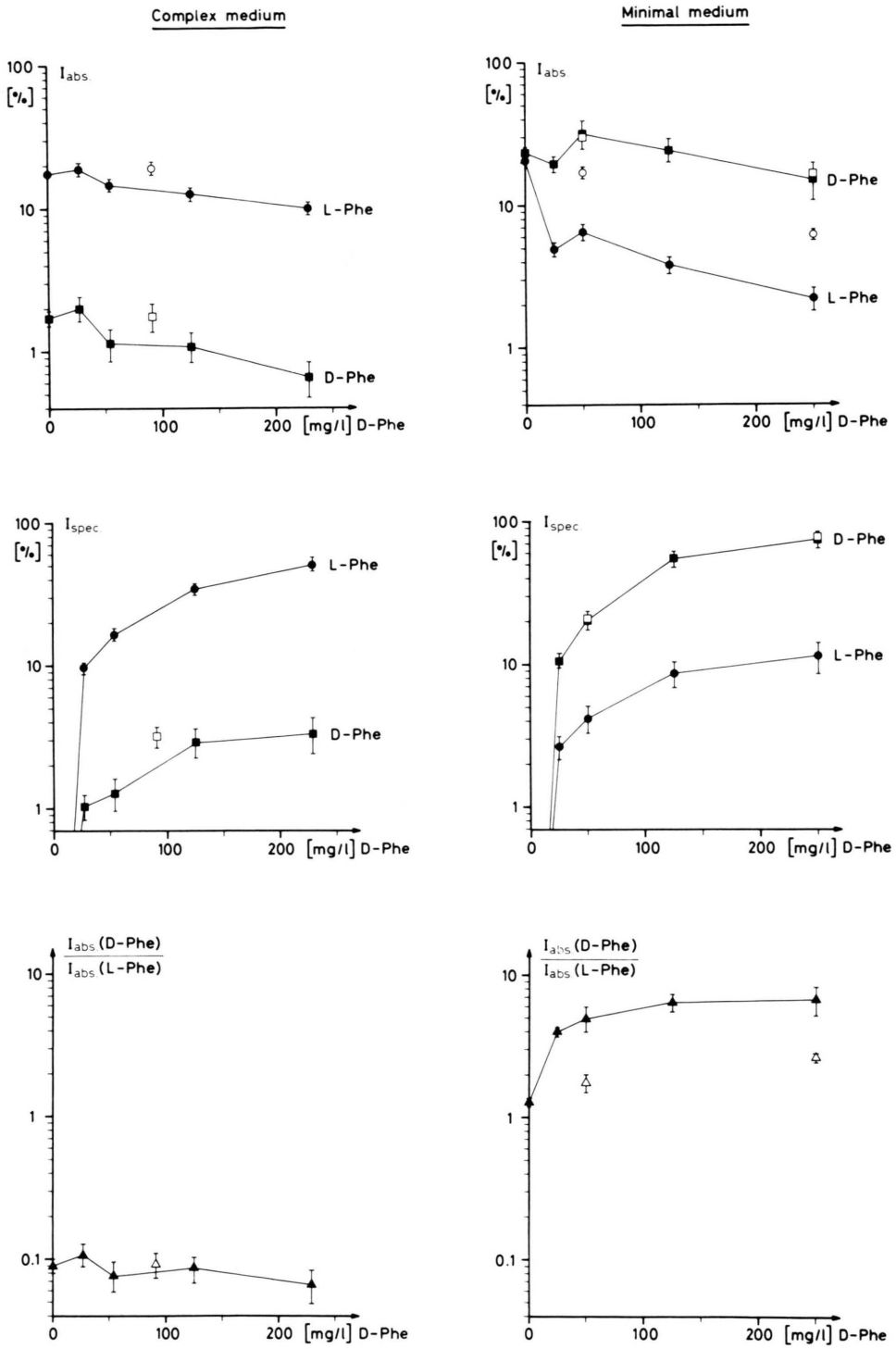


Fig. 3. Incorporation of D- and L-phenylalanine into cytochalasin D as a function of the initial concentration of D-phenylalanine in the nutrient medium. Apart from the radiolabelled tracers, inactive D-phenylalanine was added to the media either as a component of DL-phenylalanine (filled symbols and traced lines) or in optically pure form (empty symbols). $I_{abs.}$: Absolute incorporation rate; $I_{spec.}$: Specific incorporation rate. Vertical bars denote estimated standard deviations.

The fact that x is independent of the D-phenylalanine-concentration, whether or not L-phenylalanine had been added, led us to the suspicion that the complexity of the nutrient medium might be responsible for this result. Therefore we have repeated the series of experiments using a minimal medium containing D-glucose, NH_4NO_3, $MgSO_4$, NaCl, $CaCl_2$, a phosphate buffer and some vitamins and trace elements. The results are shown in Fig. 3 (right-hand section). Now, completely different incorporation rates of D-phenylalanine are observed. At negligible substrate concentration, $I_{abs.}$(D-Phe) approximately equals $I_{abs.}$(L-Phe), their ratio being 1.28. With increasing concentration of D-phenylalanine in the medium, either alone or accompanied by equal amounts of L-phenylalanine, $I_{abs.}$(D-Phe) exhibits only a slight tendency towards lower values. Together with the marked decrease of $I_{abs.}$(L-Phe) within the same range, this behaviour gives rise to a considerably increased ratio $I_{abs.}$(D-Phe)/$I_{abs.}$(L-Phe). Its value reaches 6.78 at a DL-phenylalanine-concentration of 500 mg/l. When inactive D-phenylalanine was added at the same level, leaving L-phenylalanine highly diluted, the D-enantiomer is still preferred over the natural one by a factor of 2.66. The plot of $I_{spec.}$(D-Phe) *versus* the concentration of D-phenylalanine completes the picture, although this information is redundant in cases where the concentrations of both phenylalanine enantiomers are equal, since then $I_{abs.}$(D-Phe)/$I_{abs.}$(L-Phe) = $I_{spec.}$(D-Phe)/$I_{spec.}$(L-Phe) is valid. Nevertheless it is interesting to note that at a DL-phenylalanine-concentration of 500 mg/l, 75.2% of the cytochalasin D molecules are derived from the carbon skeleton of exogeneous D-phenylalanine. Together with 11.4% derived from L-phenylalanine, a total of 86.6% of cytochalasin D material is synthesized from the exogeneous phenylalanine pool. Finally, $I_{spec.}$(D-Phe) is not affected by the presence of L-phenylalanine (*cf.* the empty squares).

With respect to the incorporation rates of L-phenylalanine, they exhibit a normal dependence on the concentration of L-phenylalanine in both the complex and the minimal medium. Thus, $I_{abs.}$ decreases with increasing concentration, whereas $I_{spec.}$ increases. In cases where no inactive L-phenylalanine had been added to the medium, $I_{abs.}$ (L-Phe) is obviously not affected by the sole addition of inactive D-phenylalanine to the complex medium. In the minimal medium however, $I_{abs.}$ (L-Phe) drops from an initial value of 20.6% down to 6.1% when 250 mg/l of exogeneous D-phenylalanine in present (*cf.* the empty circles).

In summary, our determination of x is inconsistent with previous data [6]. The equally efficient incorporation of both phenylalanine enantiomers in the complex medium is disproved, and the results summarized in Fig. 1 cannot be interpreted.

Incorporation of ^{15}N-Labelled L-Phenylalanines

Since in a complex nutrient medium the incorporation of L-phenylalanine into cytochalasin D by *Z. masonii* involves the complete loss of the α-H-atom, the α-amino group is also expected to be lost under the same conditions. Using DL-phenyl-[^{15}N]alanine and L-[U-^{14}C]phenylalanine as an internal standard, Vederas and Tamm [6] had found that only 2% of ^{15}N (with respect to the carbon skeleton of L-phenylalanine) is incorporated into cytochalasin D. To observe an enhanced ^{15}N-retention we repeated this experiment using L-phenyl-[^{15}N]alanine in place of the racemate and carrying out the fermentation in the minimal medium mentioned above supplemented with DL-glutamate. In addition to cytochalasin D, three protein amino acids, *i.e.* phenylalanine, leucine and isoleucine, were isolated from the biomass as N-[(2-nitrophenyl)thio]-amino acid ethyl esters.

The results are summarized in Table I. The specific ^{14}C-incorporation rates show that under these conditions 35–41% of the protein-phenylalanine and 42–46% of cytochalasin D are derived from exogeneous phenylalanine. The small but presumably significant difference in favour of cytochalasin D may be explained by the fact that its biosynthesis is slightly delayed with respect to cell growth (Fig. 4). Thus, while the biosynthesis of the amino acids already begins to decline due to a general stagnation of protein synthesis, phenylalanine is still being required for the synthesis of cytochalasin D. Therefore *Z. masonii* may increasingly use phenylalanine from the exogeneous pool.

Concerning the incorporation of ^{15}N in the first experiment, it is interesting to note that the ^{15}N-retention in cytochalasin D is significantly higher as compared to the retention in the earlier experiment (2% [6]). However a considerable loss of ^{15}N not only in cytochalasin D, but also in protein-phenylalanine is still observed. Hence there is no doubt that nitrogen exchange reactions occur whilst L-phenylalanine is part of the amino acid pool, presumably

Table I. Incorporation of a mixture of L-[U-^{14}C]phenylalanine and L-phenyl-[^{15}N]alanine (95% ^{15}N, 0.5 g/l) into cytochalasin D and protein amino acids. DL-Glutamic acid (2.2 g/l) was supplemented to the liquid minimal medium either unlabelled or ^{15}N-labelled (96% ^{15}N). Data in parentheses denote estimated standard deviations.

Supplement	Data [%]	Nutrient medium[1]	Cyto-chalasin D	Protein amino acids Phenylalanine	Leucine	Isoleucine
DL-Glu	$I_{spec.}$(^{14}C)	–	46.2(0.8)	41.4(0.8)	–	–
	^{15}N-content	1.5	8.0(0.7)	12.1(0.4)	9.1(0.3)	10.1(0.7)
	$I_{spec.}$(^{15}N)	–	8.4(0.7)	12.8(0.4)	9.5(0.4)	10.7(0.7)
	^{15}N-retention	–	18.2(1.5)	30.8(1.2)	–	–
DL-[^{15}N]Glu	$I_{spec.}$(^{14}C)	–	42.8(0.7)	35.5(1.8)	–	–
	Total ^{15}N-content	7.4	37.3(0.6)	43.4(0.6)	41.8(0.7)	45.0(1.6)
	From DL-[^{15}N]glu:					
	^{15}N-content[2]	5.9	29.3(0.9)	31.3(0.7)	32.8(0.8)	34.9(1.8)
	$I_{spec.}$(^{15}N)	–	30.5(1.0)	32.6(0.8)	34.2(0.9)	36.3(1.9)

[1] ^{15}N-contents shown correspond to the statistical average (see text).
[2] Total ^{15}N-content *minus* contribution from L-phenyl-[^{15}N]alanine.

due to the action of aminotransferases or amino acid oxidases, before the incorporation into cell proteins. In view of this fact the loss of ^{15}N observed for cytochalasin D is readily explained. It is clear that such processes will randomize the ^{15}N-activity over other N-containing substrates. Assuming a statistical distribution of the ^{15}N-isotopes within all nitrogen atoms present in the nutrient medium, a ^{15}N-content of 1.5% is obtained for any N-containing compound. The relatively high ^{15}N-contents found in protein-leucine and -isoleucine are in contrast with this low value. It is reasonable to assume a preferred spreading of the ^{15}N-activity merely within the pool of amino acids. On the other hand, it is possible that just leucine and isoleucine or their α-oxo analogues are differentiated from other amino acids by their co-participation with phenylalanine and phenylpyruvate in aminotransferase reactions. Interrelationships of this kind have been shown to exist in *E. coli* [8].

The introduction of an additional ^{15}N-label in DL-glutamic acid, as demonstrated in the second experiment, confirms the predominant role of this amino

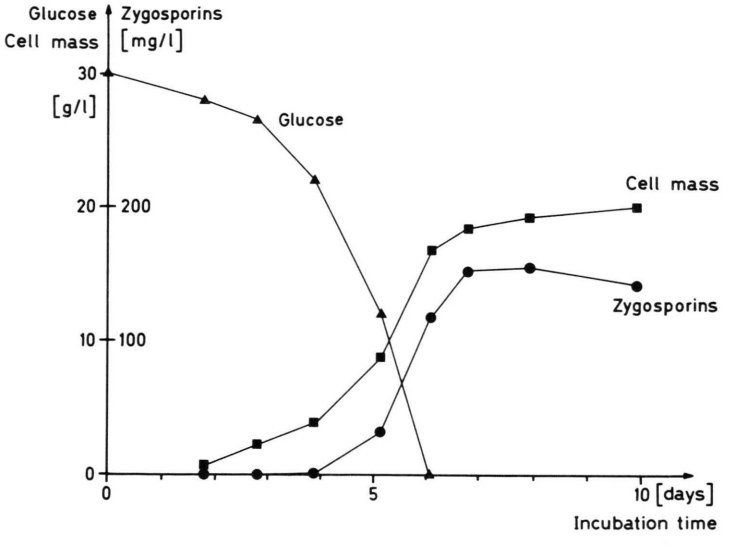


Fig. 4. Growth, glucose consumption and production of zygosporins. *Z. masonii* was cultivated in a minimal medium supplemented with 0.5 g/l L-phenylalanine and 2.2 g/l DL-glutamic acid.

acid as a source of nitrogen in amino acid biosynthesis. Thus, ^{15}N of [^{15}N]glutamic acid is introduced into unlabelled phenylpyruvate which is synthesized *via* the shikimate pathway. Phenylalanine molecules produced in this way carry a ^{15}N-, but not a ^{14}C-label. The overall incorporation of ^{15}N from exogeneous DL-[^{15}N]glutamic acid into cytochalasin D and protein-phenylalanine by this type of processes in remarkably high, as compared to the portion of these materials derived from phenylalanine which is provided by *de novo* biosynthesis (*ca.* 57% and 65%, respectively).

Although the incorporation of ^{15}N from L-phenyl-[^{15}N]alanine into cytochalasin D seems to be significant, it is not yet clear whether this incorporation is independent of the incorporation of the L-phenylalanine carbon skeleton or not. To answer this question, a specific precursor with an internal standard directly linked to ^{15}N, *i.e.* L-phenyl-[2-^{13}C,^{15}N]alanine is required. A predominant retention of ^{15}N-atoms next to ^{13}C-atoms would prove the role of L-phenylalanine as an intact precursor in cytochalasin D biosynthesis. A similar approach has been used by Mohr and Tamm for the investigation of the biosynthesis of Pseurotin A by *Pseudeurotium ovalis* [9]. Instead of racemic phenyl-[2-^{13}C,^{15}N]alanine, which has been synthesized by Mohr and Tamm from doubly labelled glycine following a classical procedure *via* the corresponding 2-phenyloxazol-5-one derivative, we synthesized the optically pure L-enantiomer in a two step reaction sequence from [2-^{13}C]malonic acid and $^{15}NH_4Cl$, using the phenylalanine ammonia lyase activity of the yeast *Rhodosporidium toruloides* ATCC 10788 [7].

The sample of cytochalasin D obtained after incorporation of L-phenyl[2-^{13}C,^{15}N]alanine (91% ^{13}C, 97.3% ^{15}N) was analyzed using ^{1}H-, ^{13}C- and ^{15}N NMR spectroscopy. Graphic integration of the signals shown in Fig. 5 permitted the calculation of the portion of each of the four possible isotopomers contained in the sample. These contributions were used to calculate the specific incorporation rates and the ^{15}N-retention (Table II).

The analysis of the data of Table II reveals that the four isotopomers of cytochalasin D are distributed according to a pattern expected for independent incorporation of ^{13}C- and ^{15}N-atoms. Thus *e.g.*, the portion of $^{13}C^{15}N$-isotopomers (1.53%) corresponds within experimental error to the probability to find a ^{13}C- and a ^{15}N-atom in the same molecule (1.51%),

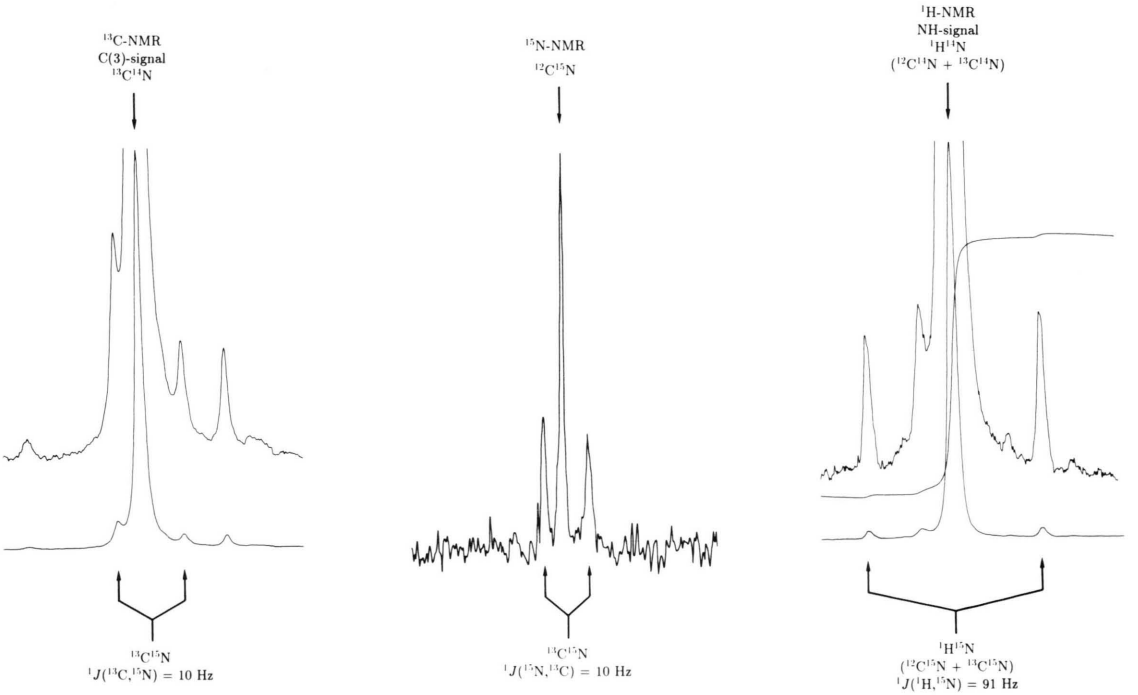


Fig. 5. NMR spectroscopy of cytochalasin D obtained after incorporation of L-phenyl-[2-^{13}C,^{15}N]alanine.

Table II. Incorporation of L-phenyl-[2-^{13}C,^{15}N]alanine (91% ^{13}C, 97.3% ^{15}N, 0.4 g/l) into cytochalasin D. No DL-glutamic acid was added to the minimal medium. Data in parentheses denote estimated standard deviations.

C(3)N-isotopomers of cytochalasin D	
$^{12}C^{14}N$ [%]	55.5 (2.2)
$^{12}C^{15}N$ [%]	2.02(0.03)
$^{13}C^{14}N$ [%]	40.9 (1.2)
$^{13}C^{15}N$ [%]	1.53(0.03)
^{13}C-content [%]	42.5 (1.2)
$I_{spec.}(^{13}C)$ [%]	46.7 (1.4)
^{15}N-content [%]	3.55(0.05)
$I_{spec.}(^{15}N)$ [%]	3.65(0.05)
^{15}N-retention [%]	7.82(0.26)

i.e. to the product of the probabilities to find a ^{13}C-atom (42.5%) and a ^{15}N-atom (3.55%), respectively.

Discussion

Considerable effort has been made to examine the incorporation of L-phenylalanine into cytochalasin D. Since both the α-H- and the α-N-atoms are lost, a proof for the intact incorporation of L-phenylalanine is still lacking. The assumption of a N-free precursor and/or a N-free intermediate in the course cytochalasin D biosynthesis is possible. However, we suggest that the hypothesis of an intact incorporation be maintained for several reasons. (1) A similar loss of ^{15}N from L-phenyl-[^{15}N]alanine occurs at the stage of L-phenylalanine before its (intact) incorporation into the cell proteins. This is a specific proof for the existence of exchange processes taking place while L-phenylalanine is part of the free amino acid pool. (2) Considerable loss of ^{15}N from phenylalanine precursors has also been observed in the biosyntheses of other secondary metabolites derived from intact L-phenylalanine, *e.g.* pseurotin A [9]. (3) Since analogous building blocks have been found for the biosyntheses of cytochalasin B, cytochalasin D and chaetoglobosin A [4, 10], it is reasonable to assume a general pathway for the biosynthesis of the cytochalasans. The key steps in the formation of the cytochalasan skeleton may easily be formulated starting with an intact L-amino acid and a hypothetical polyketide [4]. In the case of 19-O-acetylchaetoglobosin A, a cytochalasan produced by *Chaetomium globosum* and carrying a tryptophan moiety in place of the phenylalanine substructure, the participation of intact L-tryptophan has been proven [10].

The variety of possible enzymatic transformations involving the exchange of α-N-atoms, of L-phenylalanine is remarkable. The existence of aminotransferases [11, 12] and amino acid oxidases has already been mentioned and may also account for a considerable loss of H-atoms at the C(3)-position [13]. In addition, the participation of amino acid racemases [14] or of a stereospecifically operating phenylpyruvate tautomerase [15, 16] cannot be ruled out. Apart from normal amino acid uptake systems, a unique transport mechanism for phenylalanine in *Neurospora crassa* has been described [17], involving an extracellular deaminase activity and an α-oxo acid transport system.

With respect to the markedly medium- and concentration-dependent incorporation of exogeneous D-phenylalanine into cytochalasin D, it is reasonable to attribute these effects to the medium-dependent activities of various uptake systems for amino acids. Amino acid, peptide and protein transport systems of microorganisms have been extensively reviewed in 1980 [18]. A more recent survey of amino acid transport systems of some eucaryotic microorganisms has been published by Horák [19]. Apart from the fact that the transport of a given amino acid is generally mediated by more than one system, three features characteristic for eucaryotic microorganisms are noteworthy. (1) They generally possess two types of transport systems: Systems that are specific for only one amino acid or a family of structurally related amino acids; and general systems, shared by a large number of amino acids. (2) The substrate fluxes are practically unidirectional, *i.e.* from the medium into the cells. (3) The systems are partly regulated by feedback mechanisms known as trans-inhibition, *i.e.* the ability of intracellular amino acids to inhibit the influx of the same and of other amino acids from the medium, the degree of inhibition increasing with increasing intracellular amino acid concentration.

Based on this concept, a simple model for the use of exogeneous phenylalanine enantiomers by *Z. masonii* may be outlined as shown in Fig. 6. Different transport systems are proposed for the uptake of D- and L-phenylalanine. Inside the cell, the carbon skeleton of the D-enantiomer enters the normal primary metabolism by oxidative deamination to phenylpyruvate. Using this model, the behaviour of *Z. masonii* in response to the composition of the

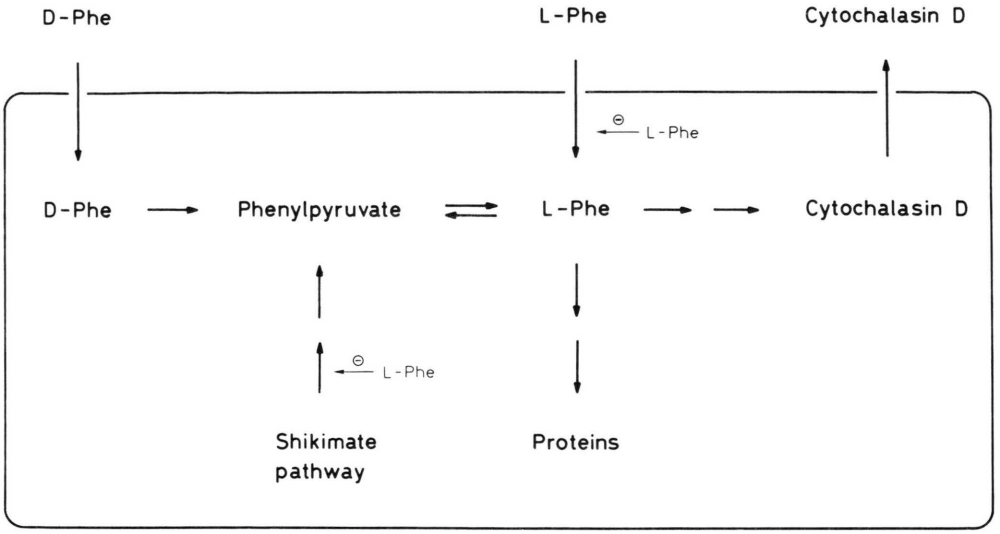


Fig. 6. Proposed model for the utilization of both enantiomers of phenylalanine by *Z. masonii*.

nutrient medium (*cf.* Fig. 3) may be explained without difficulty.

Most evidently, the systems responsible for the transport of both enantiomers of phenylalanine must be constitutive. This conclusion is based on the fact that in the minimal medium both substrates are efficiently utilized even when supplemented in minute amounts, *i.e.* at concentrations lower than 10^{-7} M. If now, starting from this situation, the concentration of exogeneous D-phenylalanine is increased, leaving L-phenylalanine highly diluted (empty symbols in Fig. 3), the material flow from exogeneous D-phenylalanine to cytochalasin D remains practically unaffected, whereas $I_{abs.}$(L-Phe) decreases. This suggests that intracellular L-phenylalanine, which is generated from the D-enantiomer *via* phenylpyruvate, is able to inhibit both its own *de novo* biosynthesis and the transport of the exogeneous natural enantiomer into the cell. No trans-inhibition of the D-phenylalanine transport system by intracellular L-phenylalanine seems to occur. These conclusions are confirmed by the effects observed at similarly increased concentrations of exogeneous L-phenylalanine (filled symbols in Fig. 3). Both $I_{abs.}$(L-Phe) and the material flow from the shikimate pathway are further reduced by inhibition effects, whereas the incorporation rates of D-phenylalanine remain unaffected. To explain the poor incorporation of D-phenylalanine in the complex medium, several mechanisms for transport inhibition are conceivable. Since many transport system prefer L-amino acids over the D-enantiomers [19], the presence of a wide variety of exogeneous L-amino acids and small peptides may lead to a saturation of the system responsible for the uptake of D-phenylalanine. On the other hand, transinhibition effects due to high intracellular concentrations of many amino acids may prevent D-phenylalanine from being taken up.

Experimental

1. General

L-[4-^{3}H]Phenylalanine (30 Ci/mmol, DL-phenyl-[3-^{14}C]alanine (50 mCi/mmol), DL-[ring-U-^{14}C]phenylalanine (195 mCi/mmol), DL-[ring-U-^{14}C]phenylalanine (188 mCi/mmol), L-phenyl-[^{15}N]alanine (95% ^{15}N), L-[U-^{14}C]phenylalanine and DL-[^{15}N]glutamic acid (96% ^{15}N) were purchased from Radium Chemie (Medipro AG), Teufen. L-[4-^{3}H]Phenylalanine (26 Ci/mmol, D-phenylalanine content < 4%) and L-[2,6-^{3}H$_2$]phenylalanine (57 Ci/mmol, D-phenylalanine content < 1%) were obtained from Amersham International plc, England. L-Phenyl-[2-^{13}C,^{15}N]alanine (91% ^{13}C, 97.3% ^{15}N) and

L-[2-^{2}H]phenyl-[2-^{2}H]alanine (84% ^{2}H-ar, 96% α-^{2}H) were prepared according to Hädener and Tamm [7]. Column chromatography: Silica gel (40–63 μm, 63–200 μm, Merck); starch (Lehner, Muttenz). The organic extracts were dried over Na_2SO_4 and evaporated below 50 °C. Unless otherwise stated, solvent proportions are given in v/v. Prior to analytical procedures, samples were dried *in vacuo* (0.02 Torr) for at least 5 h. M.p.: Kofler block; corrected. Elemental analyses were performed by E. Thommen. IR: Perkin Elmer model 177 grating spectrometer. Optical rotation: Perkin Elmer model 141 polarimeter. Radioactivity: Nuclear Chicago scintillation counter (Dr. H. G. Seiler); Liquimat 220 (Picker, Connecticut) (Dr. H. Mäcke). HPLC: pump: Altex model 100A/101A (Pye Unicam); injection valve: Rheodyne model 7120; UV-detection (254 nm): Absorbance Monitor model UA-5 with optical unit Type 6 (Instrumentation Specialties); integration: HP 3380A (Hewlett Packard). MS: AEI-MS 30 instrument (R. Fink, Dr. M. Wachtl, Dr. J.-P. Stadelmann, Dr. A. Wehrli); VG-70-250 instrument (Dr. H. Nadig). NMR: Bruker-WH-90 spectrometer (^{1}H, 90 MHz; ^{13}C, 22.63 MHz) (K. Aegerter); Bruker spectrometer (^{1}H, 400 MHz; ^{13}C, 100.6 MHz; ^{15}N, 40.55 MHz) (Prof. H. Fritz, Dr. D. Zbinden). We are indebted to Prof. Dr. A. Amberger and Dr. K. Vilsmeier (Technische Universität München, Freising-Weihenstephan, F.R.G) for some ^{15}N-determination on a JASCO model NIA-1 instrument.

2. *Microbiological Materials and Methods*

2.1 General

Nutrient media were composed as follows. Oatmeal agar: $MgSO_4$ 1.0 g, $NaNO_3$ 1.0 g, KH_2PO_4 1.5 g, agar 15 g, a freshly prepared, hot aqueous extract of 30 g oatmeal (Knorritsch), H_2O ad 1000 ml. Liquid minimal medium: D-Glucose 30 g, NH_4NO_3 9.0 g, NaCl 5.0 g, Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (Difco Laboratories) 1.7 g, H_2O 1000 ml. Liquid complex medium: D-Glucose 30 g, Bacto-peptone (Difco Laboratories) 20 g, NaCl 5.0 g, H_2O 1000 ml. Unless otherwise stated, labelled compounds and other admixtures were added to the media prior to sterilization. The addition of DL-glutamic acid, if required, was accompanied by the addition of equimolar amounts of KOH (*i.e.* 15 ml 1.0 M KOH per 2.2 g of glutamic acid).

2.2 Maintenance and Cultivation of *Zygosporium masonii*

Stock cultures of *Z. masonii* were maintained on oatmeal agar slants, which were stored at 4 °C after incubation. Spore suspensions (*ca.* 10^7 spores ml^{-1}) were prepared from fresh cultures on the same medium in Erlenmeyer flasks by suspending the mycelial pads in a 0.1 M phosphate buffer (pH 7) containing 0.1% Tween 80 (Merck) and filtering the suspension through glass wool. Liquid nutrient media were inoculated with a minimum of 10^8 spores/l. Incubation was always carried out at 26–28 °C in the dark. Liquid cultures (10–500 ml) in Erlenmeyer flasks (50–1000 ml) were shaken on a rotary shaker (150–300 rpm).

For a series of experiments shown in Fig. 3, fermentation of *Z. masonii* was carried out in 150 ml-portions (500 ml Erlenmeyer flasks) of the liquid complex and the liquid minimal medium, both media being appropriately supplemented with inactive DL- or D-phenylalanine. ^{3}H-Labelled L-phenylalanine and ^{14}C-labelled DL-phenylalanine were mixed in a proportion between 7:1 and 10:1 with respect to radioactivity. Aliquots (filtered, Sartorius SM 16534 K, 0.2 μm) of these mixtures were added to each Erlenmeyer flask after sterilization, or subjected to a determination of ^{14}C- and ^{3}H-activities. For each concentration of DL- or D-phenylalanine 3 equivalent 150 ml-cultures were independently fermented and combined before work-up. In cases where no inactive DL- or D-phenylalanine had been added, this procedure was carried out twice (minimal medium), or three times (complex medium), using different pairs of ^{3}H- and ^{14}C-labelled phenylalanines each time. The yields of cytochalasin D were 62–75 mg (complex medium), and 64–118 mg (minimal medium) per 450 ml of culture broth, showing no dependence on the concentration of phenylalanine enantiomers in the media.

3. *Analytical Methods*

3.1 General

TLC: Silicagel 60 F_{254} (Merck); the following solvents and methods of detection were used; for crude CH_2Cl_2-extracts of culture broths of *Z. masonii* and preparations of cytochalasin D: CH_2Cl_2/MeOH (9:1), CH_2Cl_2/MeOH/HCOOH (90:5:5), $CHCl_3$/acetone (3:1), Et_2O/MeOH (95:5), and CH_2Cl_2/isopropanol (9:1), detection with UV, I_2, H_2SO_4; for amino acids:

phenol/H_2O (3:1, w/w) and BuOH/CH_3COOH/H_2O (4:1:1), detection with ninhydrin soln.; for N-[(2-nitrophenyl)thio]-amino acid ethyl esters: CH_2Cl_2, benzene, and Et_2O/hexane (2:1), detection with UV. HPLC: μStyragel 100 Å and 500 Å (10 μm, 7.8 × 300 mm, Waters), using $CHCl_3$ (Merck, Nr. 2444) as the mobile phase (1.0 ml/min). The calculations for the determination of the retention of the aliphatic ^{3}H-atoms of phenylalanine were carried out according to Hädener [20]. Standard methods for data reduction and error analysis were employed [21]. Multiple determinations of a quantity were pooled by weight.

The data shown in Fig. 4 were obtained as follows. At different times of incubation, 100 ml-cultures were sampled by adding 240 ml EtOH, cooling overnight and filtering. The dry weight of the mycelium was taken as a measure of the cell mass. The filtrate was concentrated and diluted to 100 ml with H_2O. D-Glucose was determined in this soln. using Merckotest Nr. 14335 (Merck, Glucose dehydrogenase method). The soln. was then extracted once with 25 ml $CHCl_3$. An equivalent of this extract was subjected to HPLC analysis on μStyragel. Quantification was achieved by internal standardization of the work-up procedure with known amounts of cytochalasin D.

3.2 Radioactivity measurements

The simultaneous determination of ^{14}C- and ^{3}H-activities was carried out using the channels ratio technique and standards from ICN Radiochemicals, U.S.A. ([^{3}H]toluene, 2.34×10^6 dpm/ml; [^{14}C]toluene, 4.40×10^5 dpm/ml), or from New England Nuclear, U.S.A. ([^{3}H]-toluene, 2.22×10^6 dpm/ml; [carboxyl-^{14}C]benzoic acid, 5400 dpm/mg) [22]. With the ^{14}C- and ^{3}H-activities obtained, the data shown in Fig. 3 were calculated, making use of the following equations (*c*, *h*, specific ^{14}C- and ^{3}H-activities of cytochalasin D [dpm/mmol]; *y*, yield of cytocholasin D [mmol]; C, H, total ^{14}C- and ^{3}H-activities of the precursors added at the beginning [dpm]; L, D, total amounts of exogeneous L- and D-phenylalanines at the beginning [mmol]).

$$I_{abs.}(\text{L-Phe}) = \frac{hy}{H}; \; I_{spec.}(\text{L-Phe}) = \frac{hL}{H};$$

$$\frac{I_{abs.}(\text{D-Phe})}{I_{abs.}(\text{L-Phe})} = \frac{2cH}{hC} - 1; \; I_{spec.}(\text{D-Phe}) = D\left(\frac{2c}{C} - \frac{h}{H}\right).$$

The sole determination of ^{14}C-activity (*cf.* Table I) was carried out without standardization. The relative radioactivities of the precursor (L-[U-^{14}C]-phenylalanine) and the isolated products (cytochalasin D and the N-[(2-nitrophenyl)thio]-amino acid ethyl esters of phenylalanine, leucine and isoleucine) were determined by measurement at the same time and under identical conditions. Quenching corrections had to be applied for the amino acid derivatives.

3.3 Determination of ^{15}N-contents

a) Using MS. Mass spectra of labelled and unlabelled compounds were recorded under identical conditions. The ratios [M + 1]/[M] for appropriate peak groups were determined, [M] being the intensity of the main signal at *m/z* (479 for cytochalasin D, 346 and 273 for the phenylalanine derivative, 312 and 239 for the leucine/isoleucine derivatives) and [M + 1] being the intensity of the signal at *m/z* + 1. These ratios were used to calculate the ^{15}N-contents according to the equation.

$$^{15}\text{N-content} = \frac{\text{number of } ^{15}\text{N-atoms}}{\text{number of } ^{15}\text{N-atoms} + \text{number of } ^{14}\text{N-atoms}} = \frac{y}{1 + y}$$

where

$$y = \left(\frac{[M + 1]}{[M]}\right)_{labelled} - \left(\frac{[M + 1]}{[M]}\right)_{unlabelled} + 0.003^7$$

b) Using ^{1}H NMR. NMR spectra (^{1}H, 400 MHz, Py-d_5) of the labelled compounds were recorded and enlarged plots of the NH-signals (similar to the corresponding plot of Fig. 5) were graphically integrated with respect to the areas of the H−^{14}N-signal and the H−^{15}N-satellites. This method allowed the separate determination of the ^{15}N-contents of the leucine and isoleucine derivatives in mixtures of these compounds.

3.4 Determination of Isotopomer Proportions in ^{13}C, ^{15}N-Doubly Labelled Samples of Cytochalasin D

High resolution NMR spectra (^{1}H, 400 MHz; ^{13}C, 100.6 MHz; ^{15}N, 40.55 MHz; Py-d_5) of the doubly labelled compound were recorded and enlarged plots of the signals corresponding to H−N, ^{13}C(3)−N, and ^{15}N−C(3) (Fig. 5) were graphically integrated. The following proportions are obtained from the spectra, [^{x}C^{y}N] being the portion of the corresponding isotopomer contained in the sample:

$r_1 = \frac{[^{12}C^{15}N] + [^{13}C^{15}N]}{[^{12}C^{14}N] + [^{13}C^{14}N]}$, from the ^1H NMR spectrum;

$r_2 = \frac{[^{13}C^{15}N]}{[^{12}C^{15}N]}$, from the ^{15}N NMR spectrum;

$r_3 = \frac{[^{13}C^{15}N]}{[^{13}C^{14}N]}$, from the ^{13}C NMR spectrum.

Using these proportions and the additional requirement

$$[^{12}C^{14}N] + [^{12}C^{15}N] + [^{13}C^{14}N] + [^{13}C^{15}N] = 1$$

the desired isotopomer portions $[^xC^yN]$ are calculated.

4. *Isolation of Cytochalasin D and Protein Amino Acids*

4.1 Cytochalasin D

Cytochalasin D was isolated from *ca.* 8 day old culture broths as previously reported [5]. M.p. 234–236 °C. The material was identical with an authentic specimen from Lebet and Tamm [5] with respect to m.p., m.p. of mixtures, and TLC. The spectral properties and $[\alpha]_D$ were the same as reported in the literature [4, 5]. Anal. calc. for $C_{30}H_{37}NO_6$ (507.60): C 70.98, H 7.35, N 2.76; found C 70.86, H 7.56, N 2.72. ^{15}N NMR (40.55 MHz, Py-d_5): 129.5 (ref. CH_3NO_2, 380.23). $^1J(^{13}C,^{15}N)$ and $^1J(^1H,^{15}N)$, see Fig. 5.

4.2 Amino Acids (modified according to [23–25])

4.2.1 Preparation of a starch column. 500 g starch with known H_2O-content (typically 10%) was suspended in 1 l BuOH containing enough H_2O to obtain a total H_2O-content of 30%. The starch was left to soak for 3 days. Then, the suspension was poured into a column of 73 mm i.d., and after the starch had settled, the excess of BuOH was withdrawn and replaced by 1.6 l of a mobile phase (benzyl alcohol/BuOH/H_2O [4:4:1]). The mobile phase was allowed to run overnight. After the addition of another 500 ml of the mobile phase the starch was vigorously stirred up for 3 h, again allowed to settle to a height of *ca.* 20 cm and protected with a filter paper. The layer of mobile phase was replaced by a soln. of 1.0 g 8-hydroxychinolin in 80 ml of mobile phase. This compound was allowed to pass the column using the same mobile phase, the eluate being discarded.

4.2.2 Accumulation of phenylalanine, leucine, and isoleucine. Dry mycelium (2–3 g), which had been obtained in the course of the isolation of cytochalasin D from culture broth (300–500 ml), was crushed and added to distilled 6 N HCl (830 ml). The mixture was boiled for 48 h and filtered (Whatman GF/F, 0.7 μm). The filtrate was concentrated and repeatedly evaporated from H_2O (200 ml), yielding a resinous residue (1.8–2.7 g) which was suspended in benzyl alcohol/BuOH (1:1) (25 ml). The suspension was treated in an ultrasonic bath, filtered, and the filtrate was loaded onto the starch column. Isocratic elution with the mobile phase mentioned above was started, with fractions (100–180 ml) being collected and tested for radioactivity and reactivity with ninhydrin soln. [26]. Fractions containing phenylalanine were combined and extracted with H_2O. The extract was washed with Et_2O and CH_2Cl_2 and evaporated, yielding a yellow residue (*ca.* 0.3 g) containing mainly leucine/isoleucine and phenylalanine in minor amounts (TLC).

4.2.3 Conversion into derivatives and purification. While refluxing a soln. of the residue in a mixture of 100 ml abs. EtOH, 75 ml benzene and 15 ml conc. HCl, a total of 190 ml was slowly (1:20) distilled off, the corresponding volume being continuously replaced by benzene/abs. EtOH (18:5). The mixture was concentrated and repeatedly evaporated from $CHCl_3$ (100 ml). The resulting oil was mixed with 5 ml $CHCl_3$, a 0.88-fold amount (w/w) of triethylamine and a 0.83-fold amount (w/w) of (2-nitrophenyl)sulfenyl chloride. The soln. was stirred overnight at r.t. and then washed with H_2O, 0.2 M AcOH, and 0.2 M bicarbonate soln. After evaporation, 0.1–0.3 g of a yellow oil was obtained, which was chromatographed twice on silicagel (40–63 μm) using first CH_2Cl_2 and then benzene as the mobile phases. Thus, 2–16 mg of N-[(2-nitrophenyl)thio]-phenylalanine ethyl ester was obtained, m.p. 92.5–95 °C. Anal. calc. for $C_{17}H_{18}N_2O_4S$ (346.40): C 58.94, H 5.24, N 8.09; found: C 58.63, H 5.49, N 7.91. For comparison, a sample of the same derivative was synthesized in 79% yield from L-phenylalanine ethyl ester hydrochloride by a general procedure [25]. M.p. 98–98.5 °C. $[\alpha]_D^{23} = -28° \pm 1°$ ($c = 3$ in DMF). IR (KBr): 3320 (N–H), 2980, 1735 (C=O), 1590, 1565, 1510 (NO_2, asym.), 1445, 1335 (NO_2, sym.), 1300, 1270, 1210, 1180, 1100, 740, 705. ^1H NMR (90 MHz, $CDCl_3$): 8.2 (dd, 1 H, $J = 8$, $J' = 2$); 7.6–7.0 (m, 8 H); 4.23 (q, $J = 7$, ethyl); 3.9–3.5 (6 signals, C(2); after addition of D_2O: 4 signals); 3.4–2.7 (10 signals, 3 H, C(3) and NH; after addi-

tion of D_2O: 8 signals, 2 H); 1.27 (t, J = 7, ethyl). ^{13}C NMR ($CDCl_3$): 173.2 (s, C(1)); 144.8 (s); 142.4 (s); 136.9 (s); 133.7 (d); 129.5 (d); 128.6 (d); 127.0 (d); 125.4 (d); 124.6 (d); 124.5 (d); 65.8 (d, C(2)); 61.4 (t, CH_2 (ester)); 39.7 (t C(3)); 14.1 (q, CH_3 (ester)). MS: 346 (M^+), 273 ($M^+-COOEt$), 255 ($M^+-C_7H_7$), 169 ($NO_2C_6H_4SNH^+$), 154 (base peak, $NO_2C_6H_4S^+$), 91 ($C_7H_7^+$). Anal. calc. for $C_{17}H_{18}N_2O_4S$ (346.40): C 58.94, H 5.24, N 8.09, S 9.26; found: C 59.15, H 5.29, N 8.26, S 9.30. Both compounds were identical with respect to TLC, HPLC (μStyragel 100 Å), ^1H NMR and MS.

In other fractions, 11–44 mg of a mixture of the leucine and isoleucine derivatives were obtained as an oil. Anal. calc. for $C_{14}H_{20}N_2O_4S$ (312.38): C 53.83, H 6.45, N 8.97, S 10.27; found: C 53.63, H 6.50, N 8.86, S 10.09. MS: 312 (M^+), 239 ($M^+-COOEt$), 169 ($NO_2C_6H_4SNH^+$), 154 (base peak, $NO_2C_6H_4S^+$), 77, 106, 105, 98. ^1H NMR (400 MHz, Py-d_5): signals attributed to the leucine derivative: 0.93 (dd, $J = J'$ = 6.6, 2 CH_3); 3.84 (m, H–C(2)); 5.99 (d, J = 8.8 H–^{14}N); 5.99 (dd, J = 8.8, J' = 83, H–^{15}N); signals attributed to the isoleucine derivative: 0.89 (t, J = 7.5, CH_3); 1.09 (d, J = 7.2, CH_3); 3.65 (m, H–C(2)); 5.93 (d, J = 8.8, H–^{14}N); 5.93 (dd, J = 8.8, J' = 83, H–^{15}N); signals attributed to both derivatives: 1.15 (t, J = 7.2, CH_3 (ester)); 1.81 (m); 2.06 (m); 4.22 (m, CH_2 (ester)); 7.20 (m), 7.67 (m), 8.26 (m), 8.42 (m), aromatic; leucine/isoleucine proportions ranged between 72:28 and 85:15, as estimated by the area proportions of the NH-signals.

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