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BIOSYNTHETIC STUDIES ON THE CHAETOGLOBOSINS K AND L. ORIGIN OF THE EXTRA METHYL GROUPS AT C(10) AND C(11)

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Dedicated to Professor Sir Derek Barton on the occasion of his 70th birthday.

Abstract - Incorporation experiments on chaetoglobosin K (1) established that the 10'- as well as the 11'-methyl group are derived as C_1-units from methionine. The methylation was shown to occur with retention of all three H-atoms in the cases of the 11'-, 12- and 18'-methyl groups, agreeing with the "CD_3"-mechanism. The two diastereoisomeric racemates of 3-methyltryptophan with a ^{14}C-label at the 2'-position were synthesized and administered to chaetoglobosin K (1) producing cultures, both leading to high incorporation rates. The deuterium atom of (2S,3R)-3-methyl-[2-^{2}H]tryptophan, obtained by selective hydrolysis of the methyl ester with α-chymotrypsin was partially incorporated into the metabolite, establishing the (2S,3R)-enantiomer of the amino acid as a direct precursor.

INTRODUCTION

Chaetoglobosin K (1)[1] and chaetoglobosin L (2)[2], two minor metabolites of Diplodia macrospora, belonging to the class of cytochalasans, differ from all other members of this group of microbial metabolites by two extra methyl groups at C(10) and C(11). Whereas a general scheme for the biogenetic building blocks of the cytochalasans was derived by a series of studies on the biosynthesis of cytochalasin B[3] and D[4] as well as 19-O-acetylchaetoglobosin A[5], the origin of the two extra methyl groups of the 10,11-bis-homochaetoglobosins has not been clarified. A polyketide-derived chain with a various number of methyl groups of methionine, combined

with phenylalanine, tryptophan or leucine, forms the carbon skeleton of the cytochalasans. In analogy to this scheme, the 10'-methyl group at the side chain of the amino acid part in the chaetoglobosins K (1) and L (2) suggests 3-methyltryptophan (3) to be a precursor of these metabolites (Fig. 1). Incorporation of this amino acid was demonstrated in the cases of indolmycin[6], indolmycenic acid[7], indolisopropionic acid[8] and streptonigrin[9]. The Peptide antibiotic telomycin contains 3-methyltryptophan besides 10 other amino acids[10]. The 11'-methyl group is located at the C-atom derived from the methyl group of the starter acetate unit of the polyketide chain, according to the general biogenetic scheme of the cytochalasans. Methylation of the potential chain initiating unit of a polyketide occurs in the biosynthesis of aurovertin B[11], asteltoxin[12], barnol[13] and a metabolite of *Aspergillus ustus*[14] and was supposed to occur in the biosynthesis of stellatin[15]. However, in all these cases, either further investigations favoured an alternative proposed pathway, consisting in methylation of an originally longer polyketide chain and subsequent loss of the starter unit, or no attempt to identify the real starter unit was carried out. An alternative possible origin of C(5), C(11) and C(11') in the chaetoglobosins K (1) and L (2), besides methylation of acetate, is propionate as a starter unit of the polyketide. In order to determine the origin of the 10'- and 11'-methyl groups, incorporation of L-[methyl-^{13}C]methionine, L-[methyl-$^{13}C^{2}H_3$]methionine and [1-^{13}C]propionate into chaetoglobosin K (1) was tested. Further investigations on the amino acid precursor were carried out by incorporation experiments with the two diastereoisomeric racemates of [2'-^{14}C]3-methyltryptophan and (2S,3R)-3-methyl-[2-^{2}H]tryptophan.

INCORPORATION EXPERIMENTS

The strain of *Diplodia macrospora* was grown on a solid wheat medium. The production curve revealed that the formation of chaetoglobosins begins after 13-16 days, reaching a maximum after 27 days. The production of diplosporin (4), the main metabolite, reached a maximum after less than 13 days. The precursors were administered after 10 days. The cultures were incubated for 27 days. Isolation of the metabolites yielded 150 mg of 1, 50 mg of 2 and 1.5 g of 4 per litre of culture filtrate. In view of ^{13}C-incorporation experiments, the ^{13}C-nmr signals of 1 and 2 (Table 1)

have been reassigned by a HC-correlated nmr spectrum of 1. In some cases they differed from former assignments of 1[1].

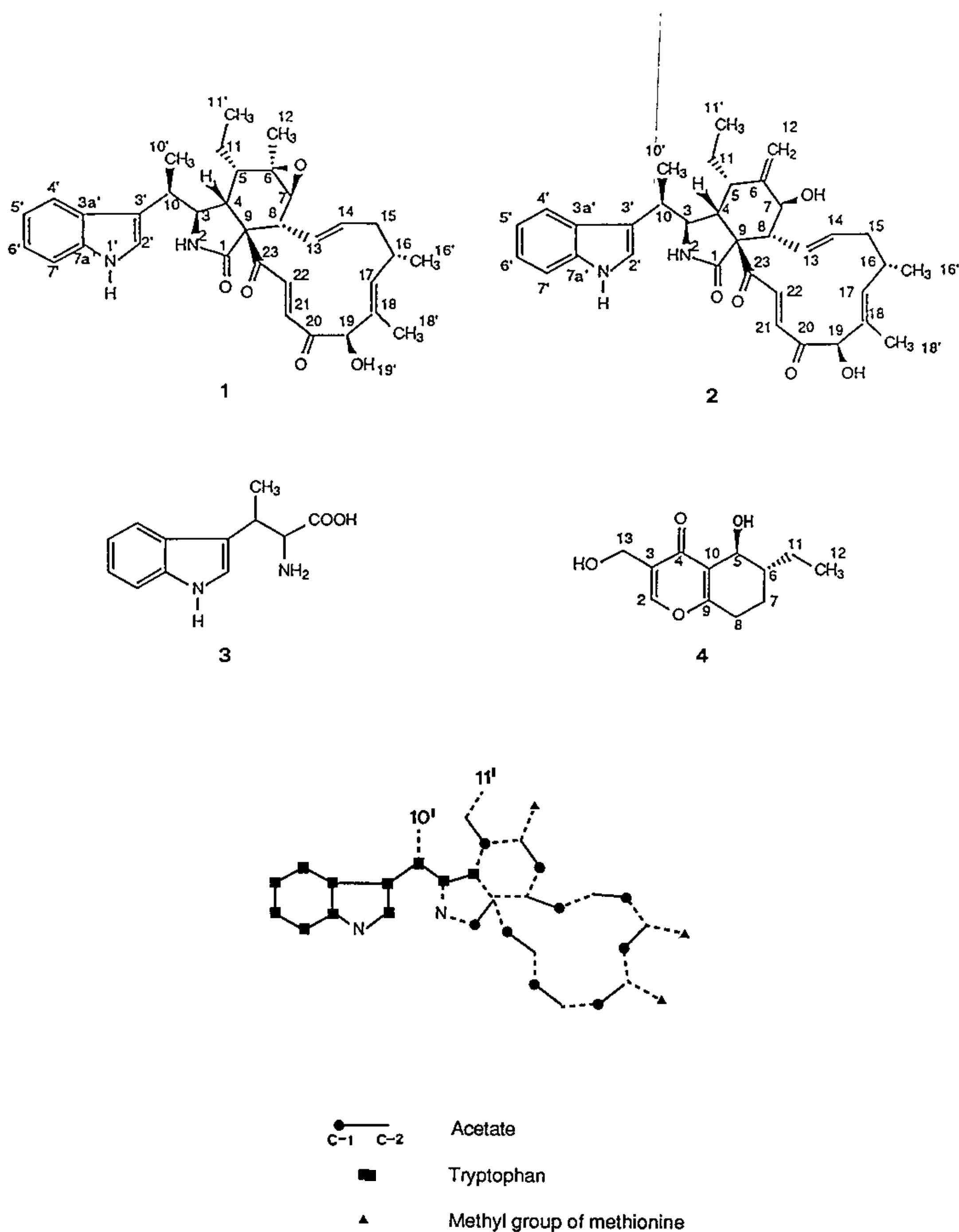


Figure 1 Biogenetic building blocks of the chaetoglobosins K and L, according to the general biogenetic scheme of the cytochalasans

Table 1. Assignments of the ^{13}C-nmr signals of chaetoglobosin K (1) (90.5 MHz) and chaetoglobosin L (2) (100.6 MHz) in $CDCl_3$

Position	(C) of 1 (ppm)	(C) of 2 (ppm)
1	173.5	172.6
3	56.8	56.1
4	44.1	39.8[a)]
5	44.1	41.0[a)]
6	57.4	146.8
7	61.7	71.7
8	49.3	50.6
9	63.9	62.2
10	36.3	35.0
11	21.8	21.4
12	19.7	114.3
13	128.4	128.5
14	133.5	136.0
15	42.0	42.0
16	32.0	32.1
17	140.4	140.3
18	132.4	132.7
19	81.7	81.7
20	201.8	201.6
21	131.2	131.5
22	136.6	137.0
23	197.9	197.7
2'	121.9	121.4
3'	116.7	117.7
4'	118.6	118.7
5'	112.5	122.5
6'	120.0	119.8
7'	111.7	111.6
10'	13.8	12.9[b)]

11'	12.7	12.1[b)]
16'	21.0	21.1
18'	10.6	10.7
3a'	126.1	126.0
7a'	136.6	136.6

a),b): exchangeable

3 to 8-Fold enhancements of the ^{13}C-nmr signals of all 5 methyl groups of 1 after addition of L-[methyl-^{13}C]methionine to growing cultures of Diplodia macrospora established their origin from the methyl group of methionine. No significant enhancements were observed after addition of [1-^{13}C]propionate. In order to elucidate the mechanisms of the methylation processes, L-[methyl-$^{13}C^2H_3$]methionine was fed to growing cultures of Diplodia macrospora. The ^{13}C-nmr spectrum of the resulting chaetoglobosin K (1) (Fig. 2) showed, in addition to the $^{13}CH_3$-signals, septets for C(11'), C(12) and C(18') with an upfield shift of the resonance frequencies (0.75-0.9 ppm), corresponding to $^{13}C^2H_3$-groups. These results are consistent with the reaction at an enolic double bond and agree, concerning C(12) and C(18'), with the findings in biosynthetical studies on 19-O-acetylchaetoglobosin A[5]. The mechanism of methylation could not be determined in the cases of C(10') and C(16'), because septets were not unequivocally detectable. The triplets in the cases of all methyl groups, belonging to $^{13}C^2H^1H_2$-groups according multiplicity and upfield shift (0.25-0.3 ppm), are difficult to explain. They are not consistent with any known methylation mechanism. Precedent metabolic exchange of the protons of the methyl group of methionine is, however, not obvious from primary metabolism.

An attempt to identify the starter unit of the polyketide chain by feeding [2-$^{13}C^2H_3$]acetate and observing the deuterium retention at C(11) of chaetoglobosin K (1) failed. Due to unfavourable conditions of the solid medium, promoting dilution of precursors particularly of unspecific ones like acetate, neither ^{13}C- nor ^{2}H-enrichments were observed in the ^{13}C-nmr spectrum of 1. This effect was not prevented with the pulse feeding method, keeping a low actual concentration of the precursor.

In view of the investigations about the amino acid precursor of 1 and 2, the two diastereoisomeric racemates of 3-methyltryptophan (3) were synthesized according to the method of Snyder and Matteson[16] (Scheme 1). A ^{14}C-label at C(2') was introduced by starting from [2-^{14}C]indole. In the reaction with ethylideneisopropylamine, 3-(isopropylaminoethylidene)indole was obtained, which was converted into dibenzyl (3-indolylethylidene)acetamidomalonate with dibenzyl acetamidomalonate and sodium methoxide. The free dicarboxylic acid was formed by hydrogenation with palladium catalyst and was subsequently decarboxylated in pyridine to give N-acetyl-3-methyltryptophan. The diastereoisomers of the product were separated by fractional crystallisation and converted into the free amino acids with sulphuric acid. (2RS,3SR)-3-methyl[2'-^{14}C]tryptophan and (2RS,3RS)-3-methyl-[2'-^{14}C]tryptophan were administered to growing cultures of Diplodia macrospora. The incorporation rates into chaetoglobosin K (1) are presented in Table 2. The high relative incorporation rates allow the unambiguous conclusion that the carbon skeleton of both diastereoisomers is the precursor of the metabolite. The absolute incorporation rates do not have a high validity, because the precursor was diluted with inactive material. The (2S,3R)-enantiomer of (2RS,3SR)-3-methyltryptophan could be incorporated directly or via a deaminated or dehydrated stage. The significant incorporation of (2RS,3RS)-3-methyltryptophan implies transformation of configuration of at least one enantiomer either at C(2) or C(3) and is most probably caused by transamination of the (2R,3R)-enantiomer to 3-indolylpyruvate.

Table 2. Incorporation rates of [2'-^{14}C]3-methyltryptophan into chaetoglobosin K (1)

Precursor	Incorporation rates	
	relative	absolute
(2RS,3SR)-3-methyl-[2'-^{14}C]tryptophan	27 %	5.9 %
(2RS,3RS)-3-methyl-[2'-^{14}C]tryptophan	36 %	3.3 %

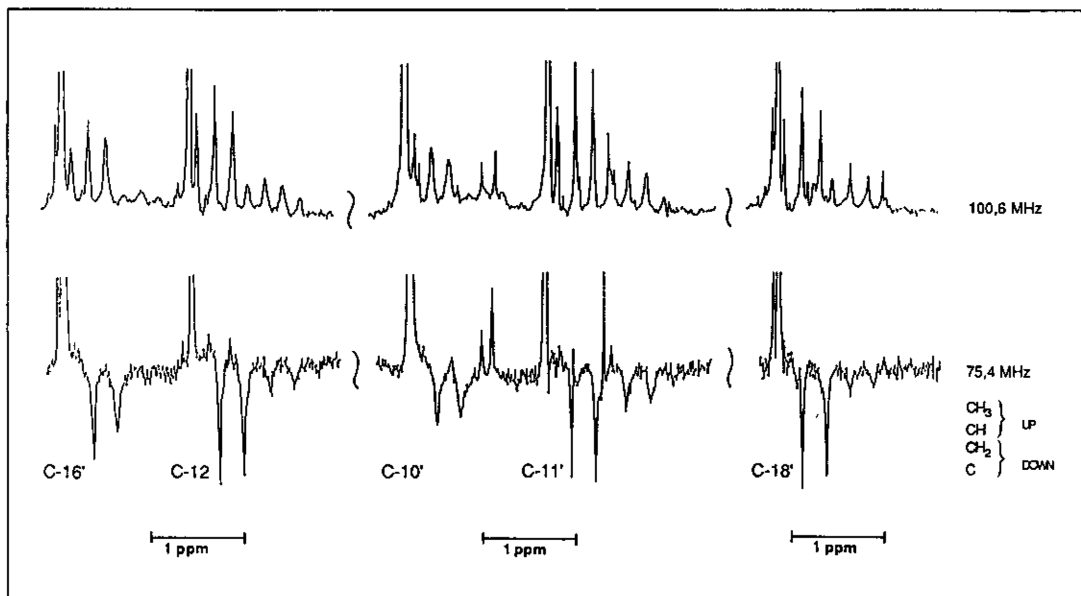
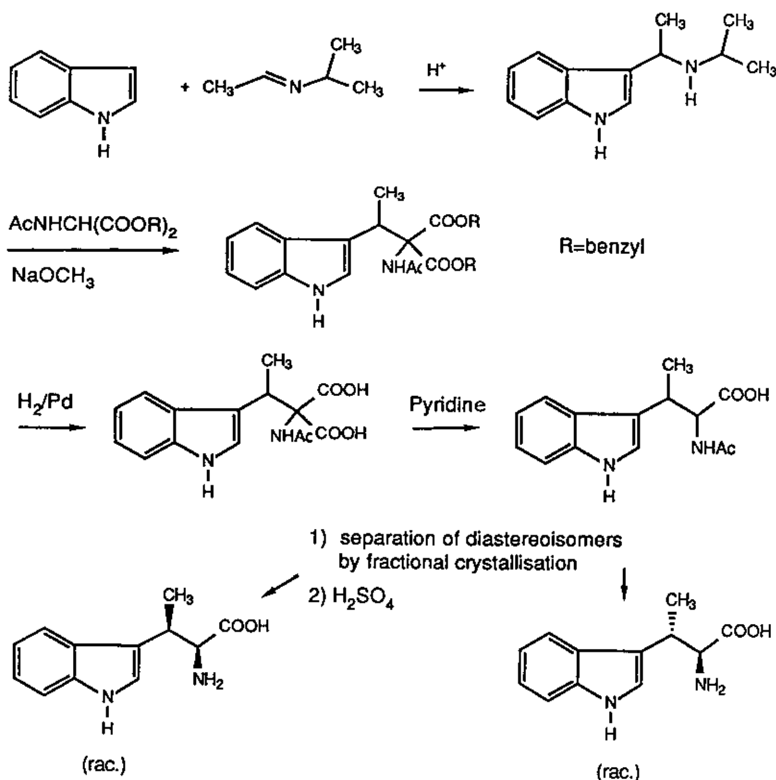


Figure 2 ^{1}H-Decoupled ^{13}C-nmr spectrum of chaetoglobosin K after incorporation of [methyl-$^{13}C^{2}H_3$]methionine ($CDCl_3$)

Scheme 1 Synthesis of 3-methyl tryptophan (3)



In order to distinguish the direct incorporation of (2S,3R)-3-methyltryptophan from a pathway via a deaminated or dehydrated stage of the amino acid, a ^{2}H-atom at the α-position of the precursor was introduced and the racemate of (2RS,3SR)-3-methyltryptophan was separated into the enantiomers. After the synthetic steps according to the method of Snyder and Matteson[16], the ^{2}H-atom was introduced at N-acetyl-3-methyltryptophan by treatment with acetanhydride and subsequent hydrolysis with NaOD. Attempts to separate the enantiomers of (2RS,3SR)-3-methyltryptophan by selective removal of the N-acetyl group of (2S,3R)-N-acetyl-3-methyltryptophan with 14 different proteases (Table 3) failed. The separation was finally achieved by selective hydrolysis of (2S,3R)-N-acetyl-3-methyltryptophan methyl ester, yielding (2S,3R)-N-acetyl-3-methyltryptophan, by catalysis of α-chymotrypsin, followed by acid catalyzed removal of the N-acetyl group. The absolute configuration of the product was assigned according to the selectivity of the enzyme for L-amino

Table 3. *Enzymes, tested for catalysis of the removal of the N-acetyl group of* N-acetyl-3-methyltryptophan

Enzyme
Acylase I from Hog Kidney (Sigma)
Carboxypeptidase I (Boehringer)
Acylase I from Aspergillus species (Sigma)
Protease Lot 7729 (Gist. brocades)[1)]
Protease type XIV (Sigma)[1)]
Protease maxacal (high alkaline)[1)]
Neutral protease dispase[1)]
Protease XVIII Rhizopus[1)]
α-Chymotrypsin (Sigma)
Penicillin G-amidase[1)]
Papain[1)]
Ficin[1)]
Pepsin[1)]
Bromelain[1)]

[1)] These enzymes were a gift from CIBA-GEIGY AG, Basel which is gratefully acknowledged.

acids. Enantiomeric purity of the product was determined gas chromatographically on Chirasil-VAL-L where the corresponding racemate was shown to be separated into enantiomers. (2S,3R)-3-Methyl-[2-^{2}H]tryptophan was administered to growing cultures of Diplodia macrospora. The deuterium enrichment in the resulting chaetoglobosin K (1) was calculated by comparing the intensities in the mass spectrum with those in the spectrum of the unlabelled metabolite. A relative incorporation rate of 4% established (2S,3R)-3-methyltryptophan as a direct precursor. Due to lack of an internal standard[1)], the exact percentage of the loss of the α-deuterium atom could not be determined. However, according to the relatively low incorporation rate compared with that of (2RS,3SR)-3-methyl-[2-^{14}C]tryptophan, the precursor seems to be subjected to a high exchange of the α-^{2}H atom, probably caused by the transamination equilibrium.

[1)] Originally intended incorporation experiments included separate administration of all four isomers of the α-deuterated amino acid precursor with a 5'-deuterium atom as an internal standard. Introduction of the 5'-label was carried out by subjecting 5-bromoindole to lithium halide exchange, according to a method of H. Rapoport et al.[17], followed by hydrolysis with D_2O. Separation of the enantiomers of (2RS,3RS)-N-acetyl-3-methyltryptophan by selective removal of the N-acetyl group was tried with the 14 proteases listed in Table 3 and succeeded, contrary to the other diastereoisomer, with acylase I from Aspergillus species as well as by selective hydrolysis of (2S,3S)-N-acetyl-3-methyltryptophan methyl ester to (2S,3S)-N-acetyl-3-methyltryptophan with α-chymotrypsin. Acid catalyzed formation of the free amino acids from N-acetyl-3-methyltryptophan and N-acetyl-3-methyltryptophan methyl ester caused, however, a complete loss of the 5'-deuterium atom. Therefore, the only double labelled isomer was (2S,3S)-3-methyl-[2-^{2}H,5'-^{2}H]tryptophan. Administration of this precursor to growing cultures of Diplodia macrospora did not lead to significant deuterium enrichments in the resulting chaetoglobosin K (1). Administration of (2R,3S)- and (2R,3R)-3-methyl-[2-^{2}H]tryptophan was not carried out, because they would have lost the deuterium atom in case of incorporation.

CONCLUSIONS

The biogenetic scheme of the cytochalasans has been extended for the 10',11'-bis-homo-chaetoglobosins. Both extra methyl groups, C(10') and C(11'), are derived from methionine. Tryptophan is methylated at C(3) and the resulting (2S,3R)-methyl-tryptophan is incorporated directly with intact chirality. This results agree with those found in biosynthetic studies on 19-O-acetylchaetoglobosin A[5] where L-tryptophan was determined as a direct precursor. Incorporation of the 11'-methyl group occurs with retention of three H-atoms and is consistent with the reaction at an enolic double bond of the polyketide chain. This finding agrees with a pathway proposed for the biosynthesis of aurovertin B[11] consisting of methylation of the methylene group of an originally longer polyketide chain and subsequent loss of the starter acetate unit, rather than with methylation of the starter unit, a reaction difficult to explain in mechanism.

EXPERIMENTAL

General methods. Labelled compounds were purchased from Medipro, Teufen AR, Switzerland, Amersham International, England and Aldrich Chemical Company, Milwaukee, USA.
Production of chaetoglobosin K (1) and L (2). The strain of *Diplodia macrospora* (ATCC 36 896) was grown in standing cultures on a solid medium containing 400 g of shredded wheat, 150 g of sucrose, 50 g of mycological broth (Difco) and 20 g of yeast extract (Difco) per 1000 ml of water. After incubation for 27 days at 27°, the cultures were refluxed with chloroform. The extract was filtered, dried with Na_2SO_4 and evaporated *in vacuo*. The residue was washed with petroleum ether and purified on a silica gel column, using increasing amounts of ethyl acetate in methylene chloride, yielding 1 as yellow prisms, 2 as a yellow gum, and diplosporin (4) as coulourless crystals. The metabolites were identified by the ^{1}H-nmr, ^{13}C-nmr and ms dates.
Production curve. The production curve was measured by examining the crude extracts in HPLC, using a 100Å-Styragel column (Waters).
Administration of precursors. Aqueous solutions of the precursors were filtered through 0,22 µm membrane filters (Sartorius) and injected into the medium through the hydrophobic surface of the cultures. L-[Methyl-^{13}C]methionine (isotopic enrich-

ment: 90%), L-[methyl-$^{13}C^{2}H_3$]methionine (isotopic enrichment: ^{13}C 90%, ^{2}H 98%) and [1-^{13}C]propionate (isotopic enrichment: 90%) were fed in two portions after 10-11 and 15 days in a quantity of 0.8-1 g per litre. Na [2-$^{13}C^{2}H$]acetate (isotopic enrichment: ^{13}C 95%, ^{2}H 97%) was fed in 4 portions after 10-16 days in quantity of 1.6 g per litre. Both diastereoisomeric racemates of 3-methyl-[2'-^{14}C]tryptophan (7.0 µCi/mmol) and (2S,3R)-3-methyl-[2-^{2}H]tryptophan (isotopic enrichment: 90%) were administered in 4 portions after 10-16 days in a quantity of 0.6-0.7 g per litre.

3-Methyl-[2'-^{14}C]tryptophan: The two diastereoisomeric racemates of 3-methyl-[2'-^{14}C]tryptophan were prepared according to the method of Snyder and Matteson[16] starting from [2-^{14}C]indole.

(2RS,3SR)-3-Methyl-[2'-^{14}C]tryptophan:

mp 220-228° (d)

^{1}H-Nmr (90 MHz, D_2O, chem. shifts refer to CH_3CN=2.0 ppm): 1.3 (d, J=7Hz, 3H); 3.9 (dxq, J=7Hz/4Hz, 1H); 4.1 (d, J=4Hz, 1H); 7.1-7.8 (m, 5H)

(2RS,3RS)-3-Methyl-[2'-^{14}C]tryptophan:

mp 230-237° (d)

^{1}H-Nmr (90 MHz, D_2O, chem. shifts refer to CH_3CN=2.0 ppm): 1.5 (d, J=7Hz, 3H); 3.6 (dxq, J=6-7Hz, 1H); 3.9 (d, J=6Hz, 1H); 6.9-7.7 (m, 5H)

N-Acetyl-3-methyl-[2-^{2}H]tryptophan. 5.37 g (20.6 mmol) of N-acetyl-3-methyltryptophan (diastereoisomeric mixture) were dissolved in 1 M NaOD to give a neutral solution. After addition of 10 ml of acetanhydride the mixture was stirred for 30 min at 50° and for further 60 min at 40°. The solution was then adjusted to pH 2 with conc. HCl and stored at 4° for 4 h. The crystals were collected to give 2.58 g (48%) of the (2RS,3SR)-diastereoisomer:

mp 216-217°

^{1}H-Nmr (90 MHz, NaOD in D_2O, chem. shifts refer to dioxane=3.71): 1.3 (d, J=7Hz, 3H); 1.4 (s, 1H); 1.8 (s, 3H); 3.7 (q, J=7Hz, 1H); 7.1-7.8 (m, 5H);

mass spectrum (EI): m/e 261, 144 (base peak)

(2RS-3SR)-N-Acetyl-3-methyl-[2-^{2}H]tryptophan methyl ester. To 1.32 g (5.04 mmol) of (2RS,3SR)-N-acetyl-3-methyl-[2-^{2}H]tryptophan 30 ml of abs methanol and 0.6 ml thionyl chloride were added. After stirring for 24 h at room temperature the solution was neutralized with 10%-$NaHCO_3$ and evaporated in vacuo. The residue was extracted with methylene chloride. The extract was evaporated *in vacuo* yielding

1.38 g (quant.) of a colourless oil containing 5% sodium acetate according to the nmr spectrum.

^{1}H-Nmr (90 MHz, $CDCl_3$): 1.4 (d, J=7Hz, 3H); 1.9 (s, 3H); 3.5 (m, 4H); 6.0 (s, 1H); 6.9-7.6 (m, 5H); 8.3 (s, 1H)

(2S,3R)-3-Methyl[2-^{2}H]tryptophan. To 1.38 g (5.02 mmol) of (2RS,3SR)-N-acetyl-3-methyl-[2-^{2}H]tryptophan methyl ester 1000 ml of phosphate buffer (pH 7.0) and 100 mg of α-chymotrypsin were added. The mixture was stirred at room temperature for 45 h and then extracted with methylene chloride. The extract was evaporated *in vacuo* and the residue (1.11 g) was subjected to hydrolysis for further 64 h under the same conditions as described above. Subsequent extraction yielded a residue of 0.73 g which was hydrolyzed for further 24 h in the same way, because the reaction was obviously stopped by product inhibition. The combined methylene chloride extracts were evaporated yielding 0.64 g of a residue which was identified by TLC as the starting material of the reaction. The combined aqueous layers were adjusted to pH 2.0 and extracted with the ethyl acetate. Evaporation of the extract yielded 0.60 g of a residue identified by TLC as N-acetyl-3-methyltryptophan. The product was refluxed in 15 ml of 4 N sulphuric acid for 5 h. The solution was then adjusted to pH 8, neutralized with dry ice, filtered through cellite, purified with charcoal and evaporated *in vacuo*. The residue (0.46 g) was recrystallized from water, yielding 130 mg (26%) of (2S,3R)-3-methyltryptophan which contained according to the ^{1}H-nmr spectrum, 6% barium acetate. 100% ee of the product was determined by gas chromatography of the N-pentafluoropropionyl-isopropyl ester derivative on a Chirasil-VAL-L-column (Chrompack) at 170° and a pressure of 135 kPa, yielding a single peak, whereas the corresponding racemate yielded two peaks under the same conditions.

mp 222-225° (d)

^{1}H-Nmr (400 MHz, D_2O, chem. shifts refer to $\underline{CH_3}CN$=2.0 ppm): 1.36 (d, J=7Hz, 3H); 3.89 (q, J=7Hz, 1H); 4.11 (d, 0.10 H); 7.16 (t, J=6-8Hz, 1H); 7.20-7.30 (m, 2H); 7.49 (d, J=8Hz, 1H); 7.73 (d, J=8Hz, 1H)

^{13}C-Nmr (100.6 MHz, D_2O, chem. shifts refer to $\underline{CH_3}CN$=1.3 ppm): 13.0; 31.5; 112.5; 114.3; 119.0; 119.9; 122.8; 124.2; 125.9; 137.1; 174.2

mass spectrum (EI): m/z 219, 174, 159, 144 (base peak), 130, 115

$[\alpha]_D^{22}$: -23.9° (water, c=0.92).

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