Liebigs Ann. Chem. **1985**, 2285–2300

Structure Elucidation of the Peptide Antibiotics Herbicolin A and B

Mitat Aydin[a], *Norbert Lucht*[a], *Wilfried A. König**[a], *Rudolf Lupp*[b], *Günther Jung**[b], and *Günther Winkelmann*[c]

Institut für Organische Chemie der Universität Hamburg[a], Martin-Luther-King-Platz 6, D-2000 Hamburg 13

Institut für Organische Chemie der Universität Tübingen[b], Auf der Morgenstelle 18, D-7400 Tübingen

Institut für Biologie I der Universität Tübingen[c], Auf der Morgenstelle 1, D-7400 Tübingen

Received April 1, 1985

The structures of the amphiphilic peptide antibiotics herbicolin A and B were determined by application of physical methods, chemical degradation, and partial syntheses. Herbicolin B is a lipodepsinonapeptide with the sequence DH-Abu-L-Thr-D-aThr-D-Leu-Gly-D-Gln-Gly-*N*-Me-L-aThr-L-Arg (DH-Abu = 2,3-dehydro-α-aminobutyric acid). The C-terminal Arg residue forms a lactone ring with the hydroxy group of L-Thr, while the N-terminus is acylated by an (*R*)-3-hydroxytetradecanoic acid residue. The main component herbicolin A differs from herbicolin B in an additional D-glucose moiety linked in an 1-α-glycosidic bond to the 3-hydroxytetradecanoic acid residue. Thus herbicolin A constitutes the first glycolipodepsinonapeptide antibiotic known so far.

Strukturaufklärung der Peptidantibiotika Herbicolin A und B

Die Struktur der amphiphilen Peptidantibiotika Herbicolin A und B konnte mit physikalischen Methoden sowie durch chemischen Abbau und Partialsynthesen identifiziert werden. Herbicolin B ist ein Lipodepsipeptid der Sequenz DH-Abu-L-Thr-D-aThr-D-Leu-Gly-D-Gln-Gly-*N*-Me-L-aThr-L-Arg (DH-Abu = 2,3-Dehydro-α-aminobuttersäure), dessen C-terminaler Argininrest mit der Hydroxygruppe von L-Thr lactonisiert und dessen N-Terminus durch (*R*)-3-Hydroxytetradecansäure acyliert ist. In der Hauptkomponente Herbicolin A ist im Unterschied zu Herbicolin B zusätzlich ein Molekül D-Glucose 1-α-glycosidisch mit der 3-Hydroxytetradecansäure verknüpft. Somit ist Herbicolin A das erste bekannte Glycolipodepsinonapeptid-Antibiotikum.

Herbicolins A and B were isolated from a bacterial strain A 111, identical with *Erwinia herbicola*, in a screening program for antifungal agents. Both antibiotics are highly active against yeasts and filamentous fungi but not active against bacteria[1,2].

The herbicolins show minimal inhibition concentrations (MIC) of 0.2–0.6 μg/ml in serial dilution tests against *Trichophyton rubrum, Epidermophyton floccosum,* and *Microsporum canis*[3]. Herbicolin A inhibits the growth of the sterol-requiring *Mycoplasma, Ureaplasma,*

© VCH Verlagsgesellschaft mbH, D-6940 Weinheim, 1985
0170–2041/85/1111–2285 $ 02.50/0

and *Spiroplasma species* with MIC of 1.5–100 µg/ml. Except for *Acholeplasma modicum*, all non-sterol-requiring species of *Acholeplasmataceae* were resistant on serum-containing medium, but were inhibited somewhat on media lacking serum, sterols, or fatty acids[4]. Ultrastructural changes in mollicutes were induced by herbicolin A, whereby filamentous structures in sterol-containing mycoplasmas were observed[5]. These filaments show twisted ribbon-like structures of 80–100 Å in diameter and also long rigid rod-like shapes.

Herbicolin A activates to a very small extent membrane-bound Ca^{2+}/calmodulin-dependent guanylate cyclase of *Paramecium*[6]. These observations indicate that lipid-membrane alterations are part of the mode of action of the herbicolins possibly by replacing sterol. The antibiotics have no ionophoric properties, and they show almost no effects on the permeability of protoplasts and chloroplasts. Herbicolins induce no mitogenic activity in B-lymphocytes. At micromolar concentrations ($c_{50} = 6 \times 10^{-6}$ mol/l) the highly amphiphilic herbicolins cause hemolysis on erythrocytes[1,2].

Fermentation, assay, isolation, and purification of herbicolin have been described[1,2]. Herbicolin A is labile in acidic and basic medium and loses activity even on silica gel RP chromatography. However, it can be purified to homogeneity (HPLC and TLC) by counter-current distribution in a neutral system.

Identification of constituents of herbicolin A and B

In previous communications the presence of two Gly, one L-Thr, one D-aThr, one D-Glu or D-Gln, one D-Leu, one L-Arg, and of 3-hydroxytetradecanoic acid as constituents of herbicolin A was shown by GC-MS investigations and amino acid analysis[1,2]. ^{13}C NMR indicated the presence of a double bond and a sugar moiety. The more detailed investigations described in the present communication show the following additional constituents of herbicolin A: 2,3-dehydro-α-aminobutyric acid (DH-Abu), L-*N*-methyl-allothreonine, D-glucose, and D-glutamine.

The *R* configuration of 3-hydroxytetradecanoic acid was determined by GC on the chiral stationary phase XE-60-L-valine (*S*)-α-phenylethylamide[7] after acid hydrolysis of herbicolin A and formation of *N*-isopropyl-3-(isopropylcarbamoyloxy)tetradecanamide with isopropyl isocyanate[8].

The L-*N*-Me-aThr moiety was identified by GC-MS of herbicolin hydrolyzates as volatile trimethylsilyl derivative and the *N,O*-bis(trifluoroacetyl) methyl ester derivative by comparison with a synthetic reference compound. It was also proved by comparison of gas chromatographic and mass spectrometric properties that the herbicolin constituent is not identical with *N*-Me-Thr, a constituent of the peptide antibiotic stendomycin[9] or with homothreonine. The configuration of *N*-Me-aThr from herbicolin could be determined after conversion to the diastereomeric *N,O*-trimethylsilylated (+)-1,2-dimethylpropyl ester[10]. Knowing the order of elution of the stereoisomers of the amino acid derivatives, it is possible to determine an unknown configuration by coinjection with the derivatives obtained from the racemic alcohol as demonstrated in Figure 1. In this case it is not necessary to have both enantiomers of the unusual amino acid as a reference for comparison.

The DH-Abu residue in the herbicolins could not be determined directly because of decomposition during acid hydrolysis. DH-Abu was identified as α-amino-*n*-

butyric acid (Abu) by GC-MS investigation of a sample of herbicolin A after catalytic hydrogenation followed by hydrolysis and by comparison with the reference compound. The Abu residue was also found in some peptide fragments of herbicolin A and B by GC-MS of partial hydrolyzates after *N*-acetylation, diborane reduction, and trimethylsilylation (see below). DH-Abu was also identified as a constituent of stenothricin[11].

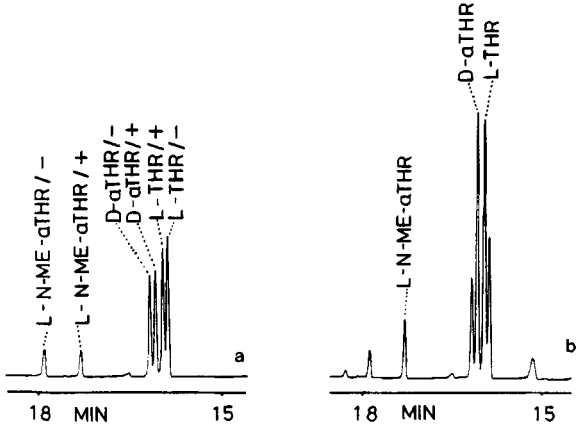

Fig. 1. Partial gas chromatogram of the total hydrolyzate of herbicolin A; a) after esterification with racemic 3-methyl-2-butanol and trimethylsilylation and b) coinjection of this sample with a sample obtained after esterification with (+)-3-methyl-2-butanol and trimethylsilylation. The peaks of L-Thr, D-aThr and *N*-Me-L-aThr are enhanced. 25-m fused silica capillary with SE 54 CB, 80 °C; temperature program: 3 °C/min

For the identification of glucose as trimethylsilyl derivative by GC-MS it was necessary to use a hydrolysis time of only 10 min (6 N HCl at 100 °C). After prolonged hydrolysis, *e.g.* 18 h, glucose was no longer detectable in the hydrolyzate because of decomposition in the presence of free amino acids.

Determination of the amino acid sequence

Attempts to cleave the peptide bonds enzymatically were unsuccessfull with trypsin, pepsin, and an Arg specific protease because of the high D-amino acid content. Attempts for the selective chemical cleavage and subsequent Edman degradation of partial sequences isolated in preparative amounts were not satisfying. Therefore sequence investigations of herbicolin A and B were performed after partial HCl hydrolysis by GC-MS of trimethylsilyl derivatives, trifluoroacetylated peptide esters[12,13], and trimethylsilylated polyamino alcohols which were obtained after *N*-acetylation and diborane reduction[14,15]. In order to obtain a maximum of structure and molecular mass information, electron-impact (EI) and chemical-ionization (CI) mass spectra using isobutane or ammonia as reactant gases were taken. As shown in Table 1 for herbicolin A, a complete set of overlapping fragments was identified. This enabled us to derive an unambiguous sequence except

for the position of L-Thr and D-aThr. This problem could, however, be solved by capillary GC in comparing by coinjection of synthetic D-aThr-D-Leu and L-Thr-D-Leu with a partial hydrolyzate of herbicolin A after formation of trifluoroacetylated methyl ester derivatives. The D-aThr-D-Leu derivative was coeluted with the corresponding peak of the partial hydrolyzate, while the diastereoisomeric L-Thr-D-Leu derivative was eluted at a position where there is no peak in the chromatogram of the partial hydrolyzate.

Table 1. List of overlapping fragments as obtained by partial HCl hydrolysis of herbicolin A and identified by GC-MS of volatile derivatives (see footnotes); the same fragmentation pattern was found for herbicolin B

(*R*)-3-OH-C_{14}–DH-Abu[a]										
(*R*)-3-OH-C_{14}–DL-Abu[b]										
(*R*)-3-OH-C_{14}–DL-Abu–L-Thr[b]										
	DL-Abu–L-Thr[b]									
		L-Thr–D-aThr[c]								
		L-Thr–D-aThr–D-Leu[d]								
		L-Thr–D-aThr–D-Leu–Gly[b]								
			D-aThr–D-Leu[e]							
			D-aThr–D-Leu–Gly[c]							
			D-aThr–D-Leu–Gly–D-Glx[a]							
				Gly–D-Glx[f]						
					D-Glx–Gly[d]					
					D-Glx–Gly–L-Me-aThr[b]					
						Gly–L-Me-aThr[a]				
							L-Me-aThr–L-Arg[c]			
(*R*)-3-OH-C_{14}–DH-Abu–L-Thr–D-aThr–D-Leu–Gly–D-Glx–Gly–L-Me-aThr–L-Arg										

[a] TMS. – [b] B_2H_6. – [c] TMS, B_2H_6. – [d] TMS, Tfa, B_2H_6. – [e] Tfa, TMS, B_2H_6. – [f] TFA, TMS.

Direct proof for the presence of DH-Abu linked to (*R*)-3-hydroxytetradecanoic acid was possible by GC-MS of the trimethylsilyl derivatives of a partial hydrolyzate. The EI mass spectrum shows a molecular ion signal at $m/z = 471$ and an $M - CH_3$ ion at $m/z = 456$. In addition, α-cleavage products at $m/z = 257$, 299, 316, and 354 and a fragment of the C-terminal residue at $m/z = 173$ confirm the proposed structure.

The presence of the C-terminal L-Arg residue is proved by the mass spectrum of a derivative of *N*-Me-L-aThr-L-Arg formed by *N*-acetylation, diborane treatment, and silylation. An identical derivative was obtained when a synthetic reference compound was treated the same way. The Arg residue is converted into an *N*-methylornithine residue by reductive cleavage with diborane[16].

It is not possible to distinguish between glutamic acid and glutamine after partial hydrolysis, however, the presence of Gln and not of Glu is indicated by molecular mass determination, by fast atom bombardment (FAB) mass spectrometry, and by the fact that even extensive treatment with diazomethane does not effect a modification in the molecular mass or in the thin-layer chromatographic behaviour of herbicolin A. Concerning the sequence investigations, completely identical

results were obtained for herbicolin A and B indicating the same sequence of constituents for both compounds.

Linkage of the glucose moiety in herbicolin A

As mentioned above, glucose as a constituent of herbicolin A was identified as trimethylsilyl derivative or as pertrifluoroacetylated methyl glycoside by GC-MS after very short acidic hydrolysis. The latter derivative was also used for the determination of the D configuration[7,17]. In an analogous investigation of herbicolin B no glucose could be detected. In order to determine the type of linkage of the glucose molecule to the peptide chain, herbicolin A was permethylated by treatment with NaH/dimethyl sulfoxide and CD_3I and subsequently hydrolyzed. After trimethylsilylation of the hyrolyzate a monosilylated derivative (molecular mass: 320) was obtained. An identical derivative was found when saccharose was treated in the same manner and compared by GC-MS, giving proof for the glycosidic linkage of glucose in herbicolin A.

In addition, the D-aThr residue was identified as *N,O*-perdeuteriomethylated derivative indicating that glucose is not linked to D-aThr, otherwise the hydroxy group of D-aThr would not have been methylated. In contrast to this, the L-Thr residue and the 3-hydroxytetradecanoic acid residue were found with free hydroxy groups after permethylation and hydrolysis. This means that either one of these residues could be linked to glucose.

A subsequent investigation of herbicolin A and B after chromic acid oxidation, however, indicated that the hydroxy group of L-Thr forms a lactone bond with the C-terminal L-Arg residue. Therefore the glucose residue is most probably linked to the hydroxy group of 3-hydroxytetradecanoic acid.

The configuration of the glycosidic bond in herbicolin A was investigated by ^{13}C NMR. In comparing the spectra of α- and β-methyl glucopyranosides with herbicolin A it can be concluded from the chemical shifts that the glucose residue is α-glycosidically linked (Table 2).

Table 2. ^{13}C NMR signals of herbicolin A and derivatives in [^{12}C,$^{2}H_4$]methanol in comparison to some reference compounds (carbonyl parts omitted, tentative assignments, differentiation between L-Thr, D-aThr and *N*-Me-L-aThr signals is arbitrary; n.a. = not assigned)

Carbon atom		Reference compounds[a-e]	Herbicolin A	Dihydroherbicolin A	Dihydroherbicolin A-OMe	Herbicolin A acid
3-OH-C_{14}	C-2	43.2[a]	44.8	44.4/44.6	44.1/44.3	44.7
	C-3	69.3	69.7	69.8	69.6	69.9
	C-4	38.1	38.4	38.3/38.4	38.1/38.2	38.4
	C-5	33.0	33.0	32.9	32.8	33.0
	C-(6–10)	30.7 (5×)	30.7	30.6	30.5	30.7
	C-11	30.4	30.3	30.3	30.2	30.4
	C-12	26.6	26.6	26.5	26.4	26.6
	C-13	23.6	23.6	23.6	23.4	23.7
	C-14	14.4	14.4	14.4	14.4	14.4

Table 2 *(Continued)*

Carbon atom		Reference compounds[a–e]	Herbicolin A	Dihydroherbicolin A	Dihydroherbicolin A-OMe	Herbicolin A acid
DH-Abu	C_α	130.2[b]	132.3			132.1
	C_β	121.6	122.9			123.7
	C_γ	14.4	14.3			13.7
Abu	C_α	58.6[c]		56.4/57.1	56.2/56.9	
	C_β	27.3		26.0/26.1	25.7/25.8	
	C_γ	11.2		10.9/11.2	10.9/11.2	
L-Thr and D-aThr	C_α	58.0[d]	57.6	57.6/57.7	57.3/57.4	60.6
			61.7	60.9/61.6	60.6/61.4	61.8
	C_β	66.6	68.8	68.6/68.9	68.4/68.8	68.2
			71.3	70.4/70.7	70.0/70.3	68.2
	C_γ	19.7	16.2	15.9	15.7/15.8	15.8
			17.5	16.8/17.2	16.8/17.1	20.3
Leu	C_α	50.5[d]	n. a.			
	C_β	41.0	42.2	42.0/42.1	41.8/41.9	42.2
	C_γ	24.0	26.8	25.8	25.5/25.6	25.8
	$C_{\delta a}$	21.6	21.9	21.8	21.7	21.7
	$C_{\delta b}$	23.0	23.6	23.6	23.5	23.6
Gly	CH_2	42.1[d]	42.6	42.6	42.3/42.3	42.4
			43.9	43.8/44.1	43.5/43.9	43.9
Gln	C_α	51.7[d]	n. a.			
	C_β	27.9	29.9	29.0/29.3	29.0/29.2	28.7
	C_γ	31.1	32.5	32.5/32.6	32.2/32.4	32.5
Me-aThr	C_α	67.7[c]	62.4	63.0	62.4	60.6
	C_β	71.1	72.1	72.3/72.9	71.9/72.6	70.4
	C_γ	19.6	21.7	20.9/21.3	20.8/21.3	20.6
	N-CH_3	35.4	32.1	32.6/32.8	32.8	32.3
Glc	C-1	101.2[e]	97.6	96.3/96.9	96.0/96.6	96.4
	C-2	73.5	73.9	74.0/74.2	73.7/73.8	74.0
	C-3	75.1	74.9	75.0	74.7	74.8
	C-4	71.8	71.6	71.7	71.4	
	C-5	73.5	73.1	73.1/73.2	72.9/73.7	73.5
	C-6	62.7	62.6	62.6	62.4	
Arg	C_α	51.7[d]	n. a.			
	C_β	29.5	30.7	29.6/30.1	29.5/29.9	30.7
	C_γ	24.7	26.6	26.4/26.6	26.1/26.3	26.3
	C_δ	40.3	40.6	40.5/40.7	40.2/40.4	41.0
	C_g	159.3	158.5	158.5	158.1	158.5
O-Me (methyl ester)					49.8	

[a] 3-Hydroxytetradecanoic acid methyl ester (3-OH-C_{14}-OMe) in [$^2H_4$]methanol. – [b] Compare ref.[27,28], published assignments deviate. – [c] D_2O, pD = 7.0. – [d] Tfa-Gly-Gly-L-X-L-Ala-OMe in [$^2H_6$]DMSO; Arg as Arg(NO_2)[21]. – [e] Methyl α-D-glucopyranoside in [$^2H_4$]methanol, assignment see ref.[25].

Lactone structure of herbicolin A and B

From the molecular mass of herbicolin A (1299.6) as determined by FAB mass spectrometry (see below) it was concluded that the peptide is cyclized in a lactone

structure. Experimental proof was obtained in analogy to the investigation of stenothricin[11] by reduction of herbicolin A with $NaBH_4$ (in methanol-water solution, 4 days at 20°C). In the amino acid analysis of a total hydrolyzate of the reduction product Arg had disappeared completely, indicating that the carboxylic group of Arg is involved in the lactone bond.

As concluded from the permethylation experiment the hydroxy group of either L-Thr or of 3-hydroxytetradecanoic acid could be connected to the carboxy group of L-Arg. By oxidation of herbicolin A and B with chromic acid in pyridine and acetic acid[5,18,19] all residues with free hydroxy groups are destroyed. Amino acid analysis and GC-MS investigations clearly showed that of the hydroxy amino acids only L-Thr was still present but not D-aThr nor L-*N*-Me-aThr. From these results it can be concluded that in herbicolin A the carboxy group of L-Arg and the hydroxy group of L-Thr form a lactone structure. If the glucose residue were linked to L-Thr and the lactone were formed by the carboxy group of L-Arg and the hydroxy group of (*R*)-3-hydroxytetradecanoic acid, L-Thr would have been destroyed by chromic acid in herbicolin B in which the glucose residue is not present.

The *N*-Me-aThr→Arg bond seems to be unusually labile against alkaline media. After treatment of herbicolin A with concentrated ammonia for 12 h and countercurrent separation of the cleavage mixture a ninhydrin positive lipopeptide was isolated which contained N-terminally free arginine as found *via* Edman degradation. Isolation of the degradation products by gel chromatography on Sephadex LH-20 showed the remaining lipopeptide to be ninhydrin-negative and revealed PTH-Arg. Thus, only one Edman degradation cycle was possible cleaving the Arg→O^3-Thr ester bond. End group determination with dansyl chloride showed also only Dns-Arg.

Spectra

Fast atom bombardment and field-desorption mass spectra

Positive ion FAB mass spectra as well as FD mass spectra of herbicolin A gave intense protonated molecular ions at m/z = 1300. Upon catalytic hydrogenation a shift in the protonated molecular ion by two mass units to m/z = 1302 was observed, giving additional proof for the DH-Abu residue. By mild alkaline treatment (5-% $NaHCO_3$/ethanol) the lactone is hydrolyzed and the mass of the protonated molecular ion is shifted to m/z = 1318. In the positive ion FAB mass spectrum of herbicolin B a protonated molecular ion at m/z = 1138 is observed. This result is in agreement with the depsipeptide devoid of the glucose residue.

^{13}C NMR spectra of herbicolin A and derivatives

Routine ^{13}C NMR spectra (100.6 MHz) of herbicolin A and its derivatives were recorded from methanolic solutions. Most signal assignments had to be made by comparison with known data[20–25] and by *J*-modulated spin-echo spectra. Nevertheless, the assignments of the signals summarized in Table 2 are only partially arbitrary, since the spectrum of the native compound (Fig. 2) could be compared with those of three derivatives (Table 2).

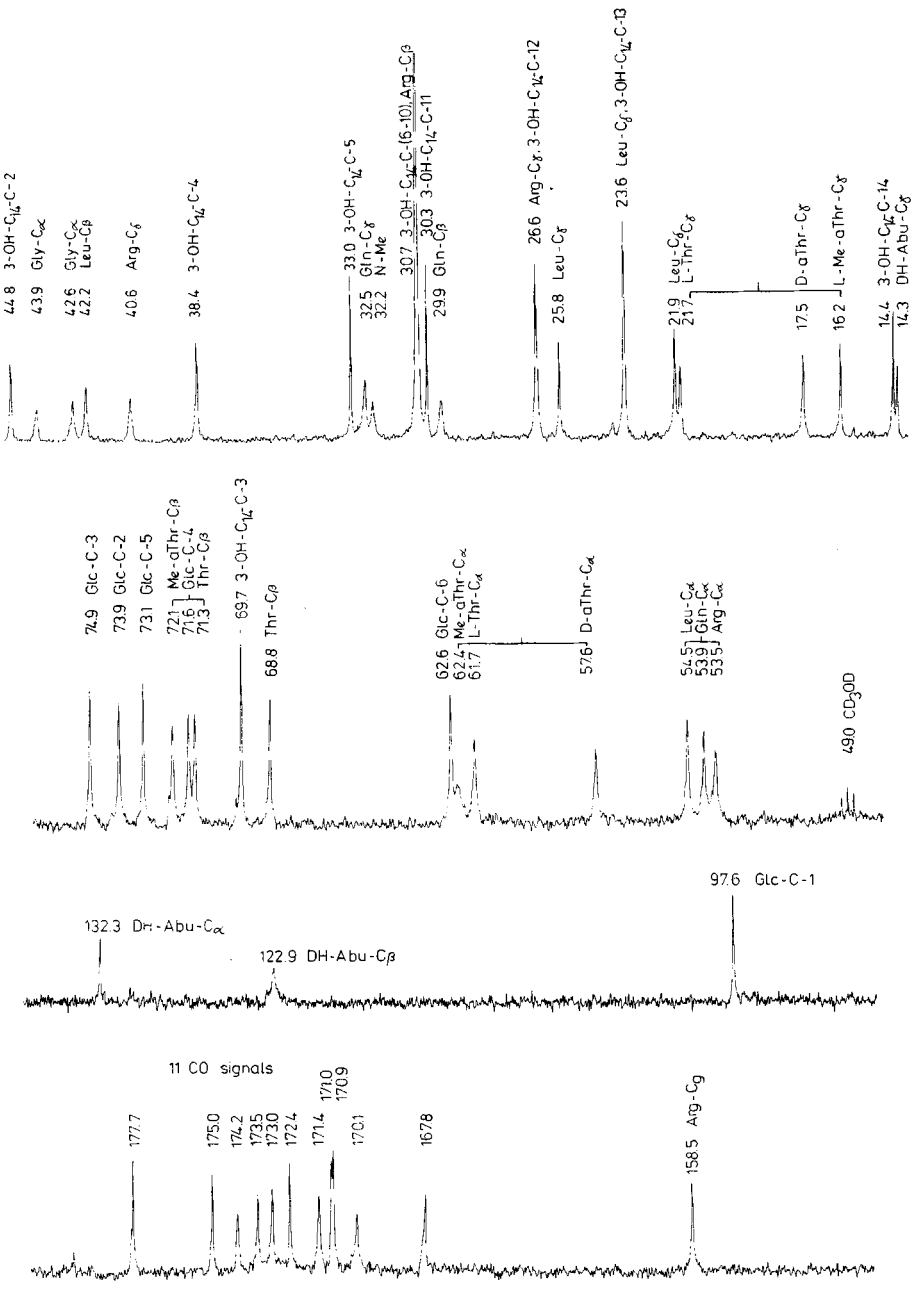

Fig. 2. ^{13}C NMR spectrum of herbicolin A (100.6 MHz; [^{12}C,$^{2}H_4$]methanol; regions of δ = 13–45, δ = 50–80, δ = 91–139, and δ = 154–183)

The spectra demonstrate clearly the presence of the non-peptidic constituents β-hydroxytetradecanoic acid (intense peak at δ = 30.7)[23,24] and glucose (δ = 97.6 for the anomeric carbon atom, and signals at δ = 67–75)[25]. The higher mobility of these two constituents relative to the peptide lactone ring is reflected in the sharper signals of the carbon atoms of the fatty acid and the sugar moiety. Eleven carbonyl signals and the signal of the guanidino group of Arg are consistent with the peptide backbone of herbicolin A.

The occurrence of an *N*-methylated residue is reflected by the methyl signal at δ = 32.2 which could be distinguished from the Gln-C_γ signal at δ = 32.5. As already found for the pairs Val/Me-Val and Ile/Me-Ile, *N*-methylation shifts the C_α signal about 9 ppm to lower field also for aThr/Me-aThr. A strong indication for the presence of an α,β-dehydroamino acid[26] are the signals at δ = 122.9 (C_β) and 132.3 (C_α). The chemical shift values for DH-Abu found in micrococcin P_1[27] and the thiocillins I–III[28] deviate considerably from those in herbicolin A. This can be explained by the particular *N*-acylation of DH-Abu with 3-hydroxytetradecanoic acid in comparison to the unusual thiazol derivatives in the case of micrococcin and thiocillin.

In the ^{13}C NMR spectrum of dihydroherbicolin A these two olefinic signals are not present, and one methyl signal is shifted from δ = 14.3 to 10.9 respectively 11.2. Corresponding strong changes are found downfield for one carbonyl signal and in the C_α and C_β region. The conversion of DH-Abu to DL-Abu implies the creation of diastereomeric herbicolins, and consequently most signals were doubled. Furthermore, the conformational restriction of native herbicolin A around the DH-Abu residue ought to be diminished for dihydroherbicolin A.

The spectrum of dihydroherbicolin A methyl ester obtained by methanolysis of the lactone confirms the previous assignments. Except for the additional methyl ester signal at δ = 49.8 no further signals or significant signal shifts were detectable, and the signal doublings found for dihydroherbicolin A were still present.

Finally we recorded the ^{13}C NMR spectrum of herbicolin A acid (Na^+ salt), which was obtained by mild alkaline hydrolysis of the lactone group. This ring opening is reflected in strong shifts up to about 3 ppm of most of the threonine signals (L-Thr, D-aThr, L-*N*-Me-aThr). The signals of D-glucose, glycines, Gln-C_β and -C_γ, *N*-Me-aThr-C_α, and *N*-Me as well

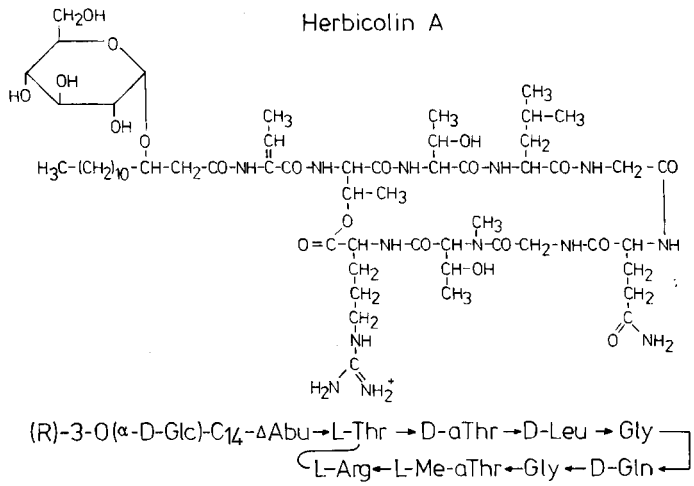

Fig. 3. Structural formula and three letter symbolism of herbicolin A

as 4 – 5 carbonyl signals are considerably broadened in this open-chain derivative compared to the cyclic antibiotic.

All results of the chemical and spectroscopic investigations on the new antifungal peptide antibiotic are consistent with the structural formula shown in Fig. 3.

This monocyclic depsipeptide antibiotic is a heterodetic-heteromeric glycolipononapeptide. The natural analogue herbicolin B is lacking the D-glucose moiety (3-OH-C_{14} = 3-hydroxytetradecanoic acid, Glc = glucose, ΔAbu = 2,3-dehydro-α-aminobutyric acid (DH-Abu), aThr = allothreonine, *N*-Me-aThr = *N*-methyl-allothreonine).

This work has been supported by the *Deutsche Forschungsgemeinschaft* (SFB 76, project B2 and projects Ko 608/5 – 4 and 608/7 – 1) and the *Fonds der Chemischen Industrie.* We thank Mrs. *U. Becker-Sanzenbacher,* Prof. Dr. *W. Bessler,* Prof. Dr. *G. Boheim,* Dr. *C. Langebartels,* and Mrs. *G. Lupp* for testing the herbicolins in biological and biophysical systems.

Experimental

Abbreviations: 3-OH-C_{14} = 3-hydroxytetradecanoic acid, *N*-Me-L-aThr = L-*N*-methyl-allothreonine; DH-Abu = 2,3-dehydro-α-aminobutyric acid; FAB = fast atom bombardment; FD = field desorption; Glc = D-glucose; TDM = *N,N,N′,N′*-tetramethyl-4,4′-methylenebis(benzeneamine); TMS = trimethylsilyl; Tfa = trifluoroacetyl. Amino acids and peptides are abbreviated according to IUPAC-IUB recommendations: Eur. J. Biochem. **138**, 9 – 37 (1984).

Chemicals: HPLC solvents were LiChrosolv (Merck) and Chromosolv (Riedel de Häen). All other solvents were purified and distilled before use. Sephadex LH-20 was from Pharmacia, Servachrom XAD-2 from Serva. DL-Allothreonine was purchased from Ferak (Berlin), D-allothreonine from Aldrich, L-allothreonine from Calbiochem, and methyl (*R*)-3-hydroxytetradecanoate from Applied Science. 2-Oxobutyric acid and other chemicals were from Merck.

Instruments and measuring methods: Mass spectra were recorded with a Varian-MAT 311 A instrument and SS 200 data system under electron-impact (70 eV) and chemical ionization conditions with isobutane as reactant gas. FAB mass spectra were taken with the same instrument with glycerol as solvent and a xenon ionizing beam produced with a saddlefield primary atom gun (Ion Tech, England). FD mass spectra were recorded with a Varian-MAT 711 instrument and SS 200 data system at an ion-source temperature of 64 °C. – NMR investigations were performed with a Bruker WH-400 instrument at 400.13 MHz (^{1}H-NMR) and 100.6 MHz (^{13}C-NMR); solvent: CD_3OD, *c* = 50 – 200 mg/ml; 25 °C. – Gas chromatography was performed with capillary columns coated with Chirasil-Val[33] and XE-60-L-valine (*S*)-α-phenylethylamide[7] for configurational analysis and OV-1, CpSil-5, and SE 54 CB for GC-MS investigations of partial hydrolyzates and total hydrolyzates of herbicolin A and B. – CD spectra[1,2] were obtained from a Jasco-J-20 A and a Roussel Jouan CD 185 dichrograph.

Thin-layer chromatography of herbicolins: Silica gel plates Kieselgel 60 F_{244} (5 × 20 cm; Merck No. 5714; saturated glass chambers; samples 2 µl of 1% solutions; detection by spraying with water (a), chlorine/TDM (b), and orcine/sulfuric acid (c); (a) to (c) have to be applied one after the other. Benzidine/periodate may be used also for detection of herbicolin A due to its D-glucose residue. Solvent systems and R_F values for components A and B:

I = chloroform/methanol/acetic acid/water (40:35:3:4), R_F = 0.37 (A) and 0.91 (B); II = chloroform/methanol/acetic acid/water (65:25:3:4), R_F = 0.12 (A) and 0.46 (B); III = chloroform/methanol/acetic acid/water (35:40:3:4), R_F = 0.28 (A) and 0.72 (B); IV = chloroform/methanol/water (65:25:4), R_F = 0.02 (A) and 0.10 (B); V = chloroform/methanol/17-% ammonia (14:7:2), R_F = 0.07 (A) and 0.31 (B); VI = 1-butanol/acetic acid/water (4:1:1); R_F = 0.16 (A) and 0.27 (B); VII = 1-propanol/water (7:3), R_F = 0.09 (A) and 0.38 (B); VIII = methyl ethyl ketone/pyridine/acetic acid/water (70:15:2:15), R_F = 0.37 (A) and 0.50 (B).

HPLC separations: HPLC system of Waters (pump 6000 A; gradient programmer 660); column LiChrosorb-NH_2 (5 μm, 250 × 4 mm; Knauer); solvent system acetonitrile/0.01 M KH_2PO_4 (4:1), 1 ml/min; detection (Spectroflow monitor 770 of Schöffel) at λ = 210 nm, R_t = 8 min (herbicolin A) and 14 min (herbicolin B).

With minor changes and a gradient the same system can be used for the separation of hydrolyzates of herbicolin A and the detection of the D-glucose moiety (λ = 190 nm). Preparative separations were carried out on a larger column (250 × 8 mm).

Amino acid analyses: Total hydrolyzates (6 N HCl, 110°C, 18 h) were analyzed with a LC 6000 E (Biotronik). DH-Abu can be detected due to its hydrolytic conversion to 2-oxobutyric acid, the characteristically smelling component of soup flavour. Alkaline hydrolysis [4% $Ba(OH)_2$, 105°C, 8 h] led to loss of Thr, conversion of Arg to Orn and Glu to Abu. Abu has the same R_t value as Cys on the amino acid analyzer (standard program), it is also formed upon catalytic hydrogenation of herbicolin.

The amino acid composition was also determined by two-dimensional TLC of a dansylated total hydrolyzate in comparison with a known mixture of dansylated amino acids[29] [1st dimension: formic acid/water (1:24), 2nd dimension: acetic acid/toluene (1:5)].

Isolation of herbicolins: The isolation procedure described by us earlier[1,2] was optimized as follows: After 3 d of cultivation the fermentation broth (20 l) was directly applied (without removal of cells by centrifugation) to a column (2.5 × 100 cm) filled with Servachrom XAD-2 (0.3–1 mm). After washing with water (1 l) the antibiotic was eluted quantitatively (TLC control) with methanol (about 600 ml). The column was regenerated with acetone (1 l) and water (2 l). The combined herbicolin-positive fractions were evaporated *in vacuo* to yield about 2 g of a residue. The yields thus obtained of 10 fermentations were collected, dissolved in methanol (75–100 ml), filtered, and chromatographed on Sephadex LH-20 in methanol; it is recommendable to use a precolumn (2.5 × 100 cm) before a larger column (5 × 100 cm). Fractions containing herbicolins (detection by TLC and antifungal activity against *Neurospora crassa*) were evaporated and chromatographed again on a fresh column. Finally the crude herbicolin was lyophilized; yield 4.5 g.

Purification by counter-current distribution: Crude herbicolin was purified on a Craig apparatus TM 402 with 440 tubes à 10 ml (Labortec, Bubendorf) using 2-butanol/ethyl acetate/water (2:4:3). Because of the low solubility of herbicolin in this system (about 15 mg/ml) 20 tubes were filled with herbicolin dissolved in the lower phase. In order to avoid formation of emulsions the following parameters were adjusted: number of steps 1000–1200, movements/step 50, relative intensity of shaking 30 (later 50), separation time 5 min. Pure herbicolin A was found in tubes 50–130. These fractions were evaporated and lyophilized from *tert*-butyl alcohol/water to yield colorless antibiotic which crystallizes from methanol in thin needles; overall yield from 200 l fermentation broth: 2 g. The herbicolin A thus obtained is pure according to TLC (R_F values see above) and HPLC. NMR (^{13}C, ^1H), MS (FD, FAB), and amino acid analysis revealed no impurities. – FD-MS: (m/z = 1300 (M + 1).

– ^{13}C-NMR: See Table 2. – Due to the Arg residue herbicolins contain varying amounts of anions depending on the isolation procedure:

$C_{58}H_{101}N_{13}O_{20} \times HCl \times 5\ H_2O$ (1427.1)	Calc.	C 48.82	H 7.92	N 12.75
$C_{58}H_{101}N_{13}O_{20} \times H_2SO_4 \times 2\ H_2O$ (1418.6)	Calc.	C 49.11	H 7.60	N 12.84
	Found	C 49.34	H 8.26	N 12.28

Herbicolin B: The more lipophilic component B, which lacks the sugar moiety, is produced only in small amounts. It was isolated from the faster migrating lipophilic fractions of the counter-current distribution. The purification was performed by preparative TLC and HPLC (R_F values and conditions see above).

Lytic activities: A dose response curve of the hemolytic action of herbicolin on human erythrocytes was determined according to published procedures[30]. The following c_{50} values (mol/l) compared to other hemolytically active polypeptides[30] were obtained: melittin 2.8×10^{-7}, herbicolin A 6×10^{-6}, trichotoxin A 40 3.8×10^{-5}, alamethicin 1.6×10^{-5}. Similar cytotoxic effects were found on lymphocytes (*W. Bessler*, unpublished results). Lytic activities were also found on protoplasts isolated from callus cells of *Daucus carota* L. (*C. Langebartels*, unpublished results).

Effects on chloroplasts and lipid bilayers: In contrast to the decouplers alamethicin and trichotoxin, herbicolin A show no effects on the oxygen development by chloroplasts (*G. Lupp* and *K. Fischer*, unpublished results). This is consistent with the findings that herbicolin A has no ionophoric properties in lipid bilayers (*G. Boheim*, unpublished results).

Dihydroherbicolin A: Herbicolin A (200 mg) was hydrogenated in methanol (10 ml) or, in order to avoid esterification, in acetone/water (2:1) in the presence of Pd-charcoal. The progress of hydrogenation is followed by TLC [R_F(II) = 0.15]. After about 3 h the catalyst is filtered off, the solvent removed *in vacuo*, and the residue lyophilized from water/*tert*-butyl alcohol (1:1); yield quantitative. – ^{13}C NMR: See Table 2. – FD-MS: m/z = 1302 (M + 1). – Antibiotic activity: fully active against *Neurospora crassa.*

Herbicolin A acid: Herbicolin A (150 mg) in ethanol (0.5 ml) is added to 5-% $NaHCO_3$ solution (0.5 ml). After 3 h at 60°C the solution is neutralized at ca. 20°C with 1 N acetic acid. After evaporation to dryness the peptide acid is chromatographed on Sephadex LH-20 (column 1 × 50 cm); yield 138 mg (ca. 90%) of antibiotically inactive peptide. – ^{13}C NMR: See Table 2. – FD-MS: m/z = 1318 (M + 1), 1348 (M + Na). – Amino acid analysis identical to that of herbicolin A.

Herbicolin A and dihydroherbicolin A methyl esters: Herbicolin A (or dihydroherbicolin A) (150 mg) is dissolved in methanol (1 ml). After about 2 weeks the antibiotic is converted almost quantitatively into its inactive methyl ester. The amino acid analyses were identical to those of herbicolin A and dihydroherbicolin A, respectively. – ^{13}C NMR: See Table 2. – R_F(II) = 0.16 (for both esters).

Cleavage of herbicolin with ammonia: Herbicolin A (10 mg) was reacted in conc. ammonia (5 ml) for 12 h. The solution was evaporated to dryness and the residual mixture was separated by counter-current distribution [280 tubes à 2 ml; 2-butanol/ethyl acetate/water (1:2:1); 135 steps]. The tubes 6–15 contained a ninhydrin-positive peptide (about 3 mg) which was pure according to TLC. Attempts for Edman degradation[31] yielded the water soluble PTH-Arg[32] which was separated from the remaining peptide by gel chromatography on Sephadex LH-20 in methanol. This ninhydrin-negative peptide could not be subjected to further degradation steps. End group determinations were performed *via* dansyl derivatives

on polyamide sheets (Macherey-Nagel & Co.)[29,31] confirming the result obtained from Edman degradation.

Preparation of derivatives: Partial hydrolysis of herbicolin A and B and preparation of trifluoroacetylated peptide esters were performed as described in ref.[13]. Trimethylsilylated polyamino alcohols of peptide fragments were prepared according to ref.[15]. Trimethylsilyl derivatives were obtained by heating partial hydrolyzates of herbicolins in trifluoro-*N*-methyl-*N*-(trimethylsilyl)acetamide (MSTFA, Macherey-Nagel & Co.). The formation of (+)-*N,O*-bis(trimethylsilyl)threonine 1,2-dimethylpropyl esters proceeded according to ref.[10]. Derivatization of 3-hydroxytetradecanoic acid for enantiomer separation was carried out as described in ref.[8]. Catalytic hydrogenation, chromic acid oxidation, and $NaBH_4$ reduction of herbicolin proceeded as described for stenothricin[11]. For permethylation herbicolin was treated with methylsulfinyl carbanion and with CD_3I according to *Vilkas* and *Lederer*[33].

Synthesis of N-methyl-D-allothreonine. – 1) *H-D-aThr-OMe · HCl:* To 25 ml of dry methanol 6.5 ml of $SOCl_2$ was added dropwise, and the solution was cooled to −18 °C. To this solution 3.7 g (21 mmol) of D-aThr · HCl was added, and the mixture was stirred at room temp. for about 12 h. The reaction mixture was evaporated to dryness, and the residue was dissolved in a small quantity of methanol. After addition of ether a colorless solid precipitated which was washed with ether and dried *in vacuo;* yield 3.96 g (98%), m.p. 101 – 103 °C, $[\alpha]_D^{20} = -26.1$ ($c = 1$ in CH_3OH).

2) *Z-D-aThr-OMe:* To 3.38 g (20 mmol) of H-D-aThr-OMe · HCl in 25 ml of dry chloroform 4.04 g (5.56 ml, 40 mmol) of triethylamine and 3.59 g (21 mmol) of benzyl chloroformate were added dropwise after cooling to 0 °C. The reaction mixture was stirred for about 12 h. Then the solvent was removed *in vacuo,* the solid residue taken up in water and acidified with 5-% $KHSO_4$ solution. The aqueous solution was extracted three times with ethyl acetate, the combined extracts were washed with 5-% $NaHCO_3$ solution and with water and dried with $MgSO_4$. After removal of the solvent a colorless solid remained; yield 5.25 g (98%), m.p. 64 °C, $[\alpha]_D^{20} = -17.2$ ($c = 1$ in methanol). – ^1H NMR ($CDCl_3$): $\delta = 1.19$ (d, $J = 8.4$ Hz; 3H, $CHCH_3$), 2.85 (d, $J = 7.4$ Hz; 1H, OH), 3.77 (s; 3H, OCH_3), 4.15 (m; 1H, $CHCH_3$), 4.44 (m; 1H, $CHNH$), 5.13 (s; 2H, $C_6H_5CH_2$), 5.84 (broad; 1H, NH), 7.35 (m; 5H, C_6H_5).

3) *Z-D-aThr(Bu^t)-OMe:* To 5.2 g (19 mmol) of Z-D-aThr-OMe, dissolved in 50 ml of CH_2Cl_2 in a pressure bottle, 50 ml (33.3 g) of isobutene was added by condensation at −50 °C. After addition of 0.15 ml of conc. H_2SO_4 the reaction mixture was kept at 20 °C for 5 days. The solution was cooled to −15 °C, and the excess of isobutene and the solvent were removed in a stream of N_2. The residue was taken up in CH_2Cl_2 and washed with a 5-% solution of $NaHCO_3$ at 0 °C and with water until neutral pH, and dried with $MgSO_4$. After evaporation of the solvent the product remained as an oil; yield 4.32 g (68%), $[\alpha]_D^{20} = -17.1$ ($c = 2.25$ in CH_3OH). – ^1H NMR ($CDCl_3$): $\delta = 1.15$ [s; 9H, $C(CH_3)_3$].

4) *Z-D-aThr(Bu^t)-OH:* 4.28 g (13.2 mmol) of Z-D-aThr(Bu^t)-OMe was dissolved in 50 ml of dioxane/water (1:1), and 14.5 ml of 1 N NaOH was added, and the solution was stirred for 4 h at 20 °C. The solvent was removed *in vacuo,* the product was taken up in a 5-% solution of $KHSO_4$ and extracted several times with ethyl acetate. The combined extracts were shaken three times with 30 ml of a 5-% $NaHCO_3$ solution. To the aqueous solution a 5-% $KHSO_4$ solution was added until a pH of 2 – 3 was reached, and the solution was extracted with ethyl acetate. The extract was dried with $MgSO_4$, and the solvent was removed *in vacuo;* yield 3.4 g (85%), m.p. 79 – 81 °C, $[\alpha]_D^{20} = -28.4$ ($c = 1$ in $CHCl_3$).

5) *Z-N-Me-D-aThr(Bu^t)-OH:* 3.37 g (10.9 mmol) of Z-D-aThr(Bu^t)-OH was dissolved in 30 ml of dry THF, and 5.5 ml of CH_3I was added. After cooling to 0°C 1.41 g (33 mmol) of NaH (from a dispersion in paraffin oil) was added in small portions under stirring. After addition of another 50 ml of THF stirring of the reaction mixture was continued for 24 h at 20°C. The reaction was interrupted by dropwise addition of water under ice cooling. After removal of the solvents *in vacuo* the residue was taken up in 100 ml of water and 50 ml of ether. The ether phase was extracted three times with 30 ml of a 5-% solution of $NaHCO_3$. The combined aqueous extracts were acidified to pH = 2–3 with a 5-% $KHSO_4$ solution and several times extracted with ethyl acetate. The ethyl acetate extracts were washed twice with 50 ml of water, twice with 50 ml of a 5-% solution of $Na_2S_2O_3$ and again with 50 ml of water and dried with $MgSO_4$. Removal of the solvent yielded 3.0 g (87%) of an oil; $[\alpha]_D^{20} = -15.5$ (c = 1.8 in $CHCl_3$). – ^{1}H NMR ($CDCl_3$): δ = 3.0 (s; 3H, NCH_3).

In an analogous procedure Z-*N*-Me-DL-Thr(Bu^t)-OH and Z-*N*-Me-DL-aThr(Bu^t)-OH were prepared.

6) *H-N-Me-D-aThr-OH:* For gas chromatographic and mass spectrometric comparison small samples of synthetic Z-*N*-Me-D-aThr(Bu^t)-OH were treated for 1 h with 6 N HCl at 100°C to remove the protecting groups before derivatives were formed. – MS of TMS derivative (70 eV): m/z = 349 (1.8%, M^+), 334 (6.4%, M – CH_3), 306 (4%, M – CH_3CO), 232 (100%, M – COOTMS), 117 (11%, $CH_3CHOTMS$), 73 (52%, TMS). High resolution of fragment $[M - CH_3]^+$ ($C_{13}H_{32}NO_3Si_3$): Calc. 334.1690, found 334.1679. – ^{13}C NMR of Me-aThr: See Table 2 (for assignment of *N*-methylated amino acids compare ref.[34]).

Synthesis of L-threonyl-D-leucine. – 1) *H-D-Leu-OMe · HCl:* This was prepared from 1.05 (7.6 mmol) of D-Leu, 5 ml of CH_3OH, and 2.2 ml of $SOCl_2$ according to the procedure described above; yield 1.3 g (94%), m.p. 151°C, $[\alpha]_D^{20} = -19.5$ (c = 1.0 in CH_3OH).

2) *Boc-L-Thr(Bzl)-D-Leu-OMe:* To 250 mg (0.81 mmol) of Boc-L-Thr(Bzl)-OH (Fluka) in 10 ml of dry THF 216 mg (1.6 mmol) of 1-hydroxybenzotriazol, 159 mg (0.88 mmol) of H-D-Leu-OMe · HCl, and 104 mg (0.8 mmol) of *N*-ethylmorpholine were added. 206 mg (1 mmol) of dicyclohexylcarbodiimide in 5 ml of THF was added after cooling to 0°C and the solution stirred for 1 h at 0°C and for about 12 h at 20°C. The precipitate of dicyclohexylurea was filtered off, the filtrate was evaporated almost to dryness, and taken up in ethyl acetate. The solution was washed three times each with 5-% $NaHCO_3$, 5-% citric acid, 5-% $NaHCO_3$ solutions, and water and dried with $MgSO_4$. Removing of the solvent yielded 290 mg (82%) of an oil. – ^{1}H NMR ($CDCl_3$): δ = 0.90 (dd; 6H, $CHCH_3$), 1.2 (d; 3H, $CHCH_3$), 1.5 [s; 9H, $C(CH_3)_3$], 1.6 (m; 2H, $CHCH_2CH$), 1.7 [m; 1H, $CH_2CH(CH_3)_2$], 1.9 (m; 1H, $CHCH_3$), 3.75 (s; 3H, OCH_3), 4.15 (m; 1H, NHC*H*), 4.3 (m; 1H, NHC*H*), 4.65 (s; 2H, $C_6H_5CH_2$), 5.45 (broad; 1H, NH), 6.8 (d; 1H, NH), 7.30 (s; 5H, C_6H_5).

In an analogous way Boc-D-aThr(Bzl)-D-Leu-OMe was prepared.

3) Of both, Boc-L-Thr(Bzl)-D-Leu-OH and Boc-D-aThr(Bzl)-D-Leu-OMe, small samples were hydrogenated (H_2, Pd-C), treated with trifluoroacetic acid to split off the Boc group and subsequently with trifluoroacetic anhydride to yield the *N,O*-Tfa derivatives. Both derivatives were compared by capillary GC and GC-MS with the partial hydrolyzate of herbicolin A and B to identify the partial sequence D-aThr-D-Leu in the herbicolins. – High-resolution *MS* of TMS-L-Thr(TMS)-D-Leu-OMe, fragment $[M - CO_2CH_3]^+$ = ($C_{13}H_{17}N_2O_4F_6$): Calc. 379.1092, found 379.1078.

Synthesis of N-methyl-D-allothreonyl-L-arginine. – 1) *H-L-Arg(NO$_2$)-OMe · HCl:* To 5 ml of CH_3OH, cooled at –18°C, dropwise 2.2 ml of $SOCl_2$ and subsequently 1.0 g (4.5 mmol)

of L-Arg(NO_2)-OH were added, and the suspension was stirred at 20 °C for about 12 h. After evaporation of the solvent 1.16 g (94%) of a yellow oil remained.

2) *Z-N-Me-D-aThr(Bu^t)-L-Arg(NO_2)-OMe:* To a solution of 618 mg (2 mmol) of Z-*N*-Me-D-aThr(Bu^t)-OH in 5 ml of dry THF 540 mg (4 mmol) of 1-hydroxybenzotriazol, 538 mg (2 mmol) of H-L-Arg(NO_2)-OMe · HCl, and 230 mg (2 mmol) *N*-ethylmorpholine were added. After cooling the solution to 0 °C 412 mg (2 mmol) of dicyclohexylcarbodiimide in 1 ml of cooled THF was added and kept at 0 °C for 1 h and at 20 °C for 12 h. The precipitated dicyclohexylurea was filtered off, the filtrate concentrated to almost dryness, and taken up in ethyl acetate. This solution was washed three times each with 5-% $NaHCO_3$, 5-% $KHSO_4$, 5% $NaHCO_3$ solutions, and water and dried with $MgSO_4$. After evaporation of the solvent the solid residue was chromatographed over silica gel; yield 770 mg (74%), m.p. 86 °C (decomp.). – ^{1}H NMR ($CDCl_3$): δ = 1.21 (d, *J* = 8.8 Hz; 3H, $CHCH_3$), 1.24 [s; 9H, $C(CH_3)_3$], 1.71 (m; 4H, $CHCH_2CH_2$), 1.92 (m; 2H, CH_2NH), 3.00 (s; 3H, NCH_3), 3.33 (m; 1H, NH), 3.59 (m; 1H, NH), 3.77 (s; 3H, OCH_3), 4.12 (m; 1H, $CHCH_3$), 4.21 (m; 1H, $CHCH_2CH_2$), 4.36 (t, *J* = 6.6 Hz; 1H, $CHCH_2$), 4.64 (m; 1H, NH), 5.16 (s; 2H, $CH_2C_6H_5$), 7.36 (s; 5H, C_6H_5), 7.83 (broad; 1H, CO – NH).

3) *N-Me-D-aThr-L-Arg-OH:* 267 mg (0.5 mmol) of Z-*N*-Me-D-aThr(Bu^t)-L-Arg(NO_2)-OMe in 0.4 ml of dioxane and 1.2 ml of CH_3OH was treated with 0.65 ml (0.65 mmol) 1 N NaOH for 2 h in an ultrasonic bath. Subsequently the reaction mixture was stirred for 12 h at 20 °C. The mixture was concentrated *in vacuo*, 3 ml of water added, and four times extracted with ether. The aqueous phase was then acidified with a 5-% solution of $KHSO_4$ under ice cooling, extracted three times with ethyl acetate, the organic phase evaporated, and the residue dried over P_2O_5 without removal of inorganic salts.

To 10 ml of a solution of BBr_3 in CH_2Cl_2 (10 mmol) 0.77 ml of trifluoroacetic acid was added dropwise at 0 °C. The mixture was kept at 20 °C for 10 min, and the solvents were removed in a stream of dry N_2. The residue was taken up in 0.5 ml of trifluoroacetic acid and added dropwise to a solution of the dry peptide derivative (see above) in trifluoroacetic acid according to *Pless* and *Bauer*[35]. The solution was stirred at 20 °C for 12 h, refluxed for 1 h, and evaporated to dryness. In order to remove volatile boronic acid methyl ester, to the residue 1 ml of CH_3OH was added repeatedly and evaporated in a stream of N_2. The residue was dissolved in 2 ml of water, and the solution was extracted three times with ether. The aqueous phase was lyophylized and not further purified; yield 161 mg of salt-containing product. – High-resolution FAB-MS of $[M + H]^+$ ion ($C_{11}H_{24}N_5O_4$): Calc. 290.1828, found 290.1824 (peak matching against $[M + H]^+$ ion of Leu-Tyr ($C_{15}H_{23}N_2O_4$): 295.1657). – ^{1}H NMR (CD_3OD): δ = 1.27 (d; 3H, $CHCH_3$), 1.77 (m; 2H, $CHCH_2CH_2$), 1.88 (m; 2H, $CHCH_2CH_2$), 2.02 (m; 2H, CH_2NH), 2.73 (s; 3H, NCH_3), 3.94 (d; 1H, $CHCHCH_3$), 4.23 (m; 1H, CHOH), 4.49 (m; 1H, $CHCH_2$).

4) A small quantity of *N*-Me-D-aThr-L-Arg-OH was used for comparison with the partial hydrolyzate of herbicolin A and B after formation of a volatile derivative by *N*-acetylation, diborane reduction and trimethylsilylation. – MS (70 eV): *m/z* = 362 (0.5%, M – CH_3), 274 (21%, M – CH_2OTMS), 217 [26%, M – $CH_3NHCHCH(CH_3)OTMS$], 160 [61%, $CH_3NHCHCH(CH_3)OTMS$], 84 (97%, $C_5H_{10}N$), 73 (100%, TMS).

[1] *G. Winkelmann, R. Lupp,* and *G. Jung,* J. Antibiot. **33**, 353 (1980).
[2] *G. Winkelmann, C. Glück, R. Lupp,* and *G. Jung* in Structure and Activity of Natural Peptides, Selected Topics (edit. *W. Voelter* and *G. Weitzel*), p. 237, de Gruyter, Berlin 1981.
[3] *G. Winkelmann* and *W. Adam,* Mykosen **23**, 290 (1980).
[4] *E. A. Freundt* and *G. Winkelmann,* Antimicrob. Agents Chemother. **26**, 112 (1984).
[5] *S. Birkelund-Anderson, E. A. Freundt,* and *G. Christiansen,* Antimicrob. Agents Chemother., in print.
[6] *S. Klumpp, G. Jung,* and *J. E. Schultz,* Biochim. Biophys. Acta **800**, 145 (1984).
[7] *W. A. König, I. Benecke,* and *S. Sievers,* J. Chromatogr. **217**, 71 (1981).
[8] *W. A. König, I. Benecke, N. Lucht, E. Schmidt, J. Schulze,* and *S. Sievers,* J. Chromatogr. **279**, 555 (1983).
[9] *M. Bodanszky, J. Izdebski,* and *I. Muramatsu,* J. Am. Chem. Soc. **91**, 2351 (1969).
[10] *W. A. König, I. Benecke,* and *J. Schulze,* J. Chromatogr. **238**, 237 (1982).
[11] *M. Rinken, W. D. Lehmann,* and *W. A. König,* Liebigs Ann. Chem. **1984**, 1672.
[12] *F. Weygand, A. Prox, H. H. Fessel,* and *K. KunSun,* Z. Naturforsch., Teil B, **20**, 1169 (1960).
[13] *W. A. König, C. Engelfried, H. Hagenmaier,* and *H. Kneifel,* Liebigs Ann. Chem. **1976**, 2011.
[14] *J. A. Kelley, H. Nau, H.-J. Förster,* and *K. Biemann,* Biomed. Mass Spectrom. **2**, 213 (1975).
[15] *M. Aydin, D. H. Bloß, W. A. König, H. Brückner,* and *G. Jung,* Biomed. Mass Spectrom. **9**, 39 (1982).
[16] *H. Nau, H.-J. Förster, J. A. Kelley,* and *K. Biemann,* Biomed. Mass Spectrom. **2**, 326 (1975).
[17] *W. A. König, I. Benecke,* and *H. Bretting,* Angew. Chem. **93**, 688 (1981); Angew. Chem., Int. Ed. Engl. **20**, 693 (1981).
[18] *J. C. Sheehan, S. Nakamura, J. A. Stock,* and *K. Maeda,* J. Am. Chem. Soc. **90**, 462 (1968).
[19] *H. Laatsch,* Liebigs Ann. Chem. **1982**, 28.
[20] *W. Voelter, G. Jung, E. Breitmaier,* and *E. Bayer,* Z. Naturforsch., Teil B, **26**, 213 (1971).
[21] *K. Wüthrich,* NMR in Biological Research: Peptides and Proteins, North-Holland, Amsterdam, 1976.
[22] *D. Leibfritz, E. Haupt, N. Dubischar, H. Lachmann, R. Oekonomopulos,* and *G. Jung,* Tetrahedron **38**, 2165 (1982).
[23] *G. Jung, C. Carrera, H. Brückner,* and *W. G. Bessler,* Liebigs Ann. Chem. **1983**, 1608.
[24] *K.-H. Wiesmüller, W. G. Bessler,* and *G. Jung,* Hoppe-Seyler's Z. Physiol. Chem. **364**, 593 (1983).
[25] *E. Breitmaier, W. Voelter, G. Jung,* and *C. Tänzer,* Chem. Ber. **104**, 1147 (1971).
[26] *K. Noda, Y. Shimohigashi,* and *N. Izumiya* in The Peptides, Analysis, Synthesis, Biology (edit. *E. Gross* and *J. Meienhofer*), Vol. 5, p. 285, Academic Press, New York – London 1983.
[27] *B. W. Bycroft* and *M. S. Gowland,* J. Chem. Soc., Chem. Commun. **1**, 256 (1978).
[28] *J. Shoji, T. Kato, Y. Yoshimura,* and *K. Tori,* J. Antibiot. **34**, 1126 (1981).
[29] *H. Laatsch,* J. Chromatogr. **173**, 398 (1979).
[30] *G. Irmscher* and *G. Jung,* Eur. J. Biochem. **80**, 165 (1977).
[31] *G. Bovermann, H. Rautenstrauch, G. Seybold,* and *G. Jung,* Hoppe-Seyler's Z. Physiol. Chem. **363**, 1187 (1982), and references cited therein.
[32] *G. Pataki,* Dünnschichtchromatographie in der Aminosäure- und Peptidchemie, de Gruyter, Berlin, 1966.
[33] *E. Vilkas* and *E. Lederer,* Tetrahedron Lett. **1968**, 3089.
[34] *A. Kern, U. Kabatek, G. Jung, R. G. Werner, M. Poetsch,* and *H. Zähner,* Liebigs Ann. Chem. **1985**, 877.
[35] *J. Pless* and *W. Bauer,* Angew. Chem. **85**, 142 (1973); Angew. Chem., Int. Ed. Engl. **12**, 147 (1973).

[64/85]