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Cephaibols, New Peptaibol Antibiotics with Anthelmintic Properties from *Acremonium tubakii* DSM 12774

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Two groups of new peptaibol-type antibiotics termed cephaibols have been isolated from the fungus *Acremonium tubakii*, DSM 12774. These 16- or 17-unit straight-chain peptides, whose structures were characterized by amino acid analyses, 2-D NMR experiments, and by mass spectrometric sequencing, have a high content of the unusual amino acids aminoisobutyric acid and isovaline. The principal constituent of the novel peptaibol mixture is cephaibol A, which is formed in abundance in cultures of the wild strain. The striking biological property of cephaibol A is its pronounced anthelmintic action and activity against ectoparasites.

Peptaibols[1] are peptides containing α-aminoisobutyric acid (Aib) formed mainly by soil fungi. They are straight-chain compounds, usually comprising some 11~20 amino acid residues, in which the *N*-terminus is acylated and the *C*-terminus reduced to the alcohol, and are formed by peptide synthetases *via* a non-ribosomal biosynthesis pathway[2]. The synthesis products are often non-homogeneous, comprising so-called microheterogeneous mixtures of several closely related compounds in which Aib is frequently replaced by other α,α-dialkylated amino acids. The three-dimensional structures of various peptaibols have been determined by X-ray structure analyses performed by R. J. FOX and F. M. RICHARDS[3], I. L. KARLE *et al.*[4~6], and other authors[7]; a characteristic feature of this class of compound is the presence in the peptide chain of helical sections that can infiltrate lipid layers. The action of peptaibols derives from their interaction with biological phospholipid membranes, in which the formation of voltage-dependent ion channels results in increased ion permeability[8]. With this characterization of their action mechanism, theoretical interest in peptaibols has waned in recent years.

During target-oriented screening for pharmacologically active substances, we investigated cultures of the fungus *Acremonium tubakii*, DSM 12774. In the course of these investigations we came across a great many compounds with molecular weights of 1640~1690 daltons that were classified as peptaibols on the basis of their physicochemical data. However, renewed reports of peptaibols with surprising biological properties led us to examine these compounds we had found more closely, even though the basic screening had largely been completed. Ampullosporin[9] induces pigment formation in *Phoma destructiva* and produces neuroleptic effects in mice. Clonostachin[10] inhibits platelet aggregation. The antiviral peptaivirins[11] A and B have recently been described, and in another recent report constituents of bergofungin[12] were found to inhibit prolyl endopeptidase at submicromolar concentrations. These published biological effects would not have been predicted from the membrane action of peptaibols described above.

In this paper, we report the isolation, structural

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Table 1. Physico-chemical data of cephaibols.

	Appearance:	UV λ_{max} (MeOH) nm (log ε)	ESI-MS⁻ *m/z*, [M. W. found]	empirical formulae	M. W. [chem.]	HPLC Rt, minutes*	$[\alpha]_D^{21}$ c 1, CH_3OH
Cephaibol A	white powder	258, (2.6)	1669.9	$C_{82}H_{127}N_{17}O_{20}$	1671.03	12.1	+ 11.4°
Cephaibol A2	white powder	258, (2.6)	1684	$C_{83}H_{129}N_{17}O_{20}$	1685.06	16.9	n. t.
Cephaibol B	white powder	258, (2.6)	1684	$C_{83}H_{129}N_{17}O_{20}$	1685.06	16.0	+ 9.3°
Cephaibol C	white powder	258, (2.6)	1656	$C_{81}H_{125}N_{17}O_{20}$	1657.00	9.0	+ 4.9°
Cephaibol D	white powder	258, (2.6)	1641.8	$C_{80}H_{123}N_{17}O_{20}$	1642.97	7.1	n. t.
Cephaibol E	white powder	258, (2.6)	1656	$C_{81}H_{125}N_{17}O_{20}$	1657.00	9.3	n. t.
Cephaibol P	white powder	258, (2.6)	1872	$C_{89}H_{137}N_{19}O_{25}$	1873.20	17.9	n. t.
Cephaibol Q	white powder	258, (2.6)	1856	$C_{89}H_{137}N_{19}O_{24}$	1857.20	17.9	n. t.

* Column: Nucleosil 100-5 C18AB (4.0 x 250 mm); flow rate: 1 ml/minute; Solvent: 40% acetonitrile in 0.1% trifluoro acetic acid; detection at 210 nm.

characterization, and some biological properties of the new peptaibols, which have been termed cephaibols[13]. A. JAWORSKI and H. BRÜCKNER described antiamoebin III and VI in 2000 which are identical with two cephaibol components E and D[14].

Results and Discussion

The organism *Acremonium tubakii*, DSM 12774, is a fungal species with a mycelium that is beige-red on malt extract agar[15] and brown on Sabouraud agar. Only a faint aerial mycelium is formed in the center of the colony and exopigmentation is absent. The single phialides grow directly from the substrate mycelium and the chlamydospores are formed in chains[15]. On soya meal-based nutrient solutions *Acremonium tubakii*, DSM 12774, forms cephalosporin C. If the organism is fermented on sucrose-rich nutrient media containing yeast extract, $NaNO_3$ as the nitrogen source, and trace elements, it produces peptaibol antibiotics in abundance. The fermentation of *Acremonium tubakii*, DSM 12774, which is based on shake cultures, is described in the Experimental Section. Guided by HPLC monitoring, it was possible in later runs to harvest the fermentation after only 66 hours; the content of the cephaibol complex in such cases was more than 6 g in 60 liters, *i.e.* over 100 mg per liter of culture solution.

The cephaibols were isolated from the mycelium by methanol extraction, and the antibiotics were obtained from the culture filtrate by solid phase extraction. Both the cell extract and the material obtained from the aqueous phase were purified on an adsorption resin, gradient elution yielding a product of about 25% purity. Size exclusion chromatography increased the purity to about 75%. The individual constituents were separated by reversed-phase chromatography. Table 1 summarizes selected data for the isolated cephaibols.

The peptide nature of the new compounds was indicated by the presence of amide absorption bands at 1540 and 1654 cm^{-1} in the IR spectrum of the cephaibols. After hydrolysis in 6 N HCl, amino acid analysis of the investigated compounds cephaibol A, B, C, D and E revealed L-Hyp (hydroxyproline), L-Glu, L-Pro, Gly, L-Leu, L-Phe, Phe-ol (phenyl-alaninol), Iva (*iso*-valine) and Aib (amino-*iso*-butyric acid); the configurations of Iva and Phe-ol could not be determined (Table 2). The UV absorption maximum at 258 nm was consistent with the presence of phenylalanine. The molecular weights of the individual isolated compounds as determined by ESI-MS are given in Table 1.

In addition to the group comprising cephaibols A~E, which constituted the bulk of the mixture, a second group of peptaibol antibiotics were isolated from cultures of

Table 2. Amino acid analysis of cephaibol A, B, C, D and E.

	Cephaibol A	B	C	D	E
α-Amino-*iso*-butyric acid (Aib)	6.23 (6)	5.21 (5)	6.87 (7)	8.03 (8)	7.01 (7)
Iso-valine (Iva)	1.74 (2)	2.48 (3)	1.03 (1)	< 0.2 (0)	0.92 (1)
Glycine	0.93 (1)	0.90 (1)	1.04 (1)	0.92 (1)	1.02 (1)
L-Proline	1.10 (1)	1.28 (1)	1.24 (1)	1.10 (1)	1.11 (1)
L-Leucine	1.06 (1)	1.06 (1)	1.03 (1)	1.07 (1)	1.03 (1)
Phenylalaninol (Phe-ol)	0.77 (1)	0.75 (1)	0.78 (1)	0.80 (1)	0.80 (1)
L-Phenylalanine	0.92 (1)	0.99 (1)	0.90 (1)	0.96 (1)	0.91 (1)
L-Glutamic acid	1.08 (1)	1.16 (1)	1.02 (1)	1.06 (1)	1.03 (1)
L-Hydroxy-proline	2.17 (2)	2.19 (2)	2.09 (2)	2.06 (2)	2.18 (2)

The table shows the residues found in comparison with calculated numbers of amino acids per mole (in brackets).

Acremonium tubakii, DSM 12774, named cephaibol P and Q in recognition of their differing composition. The amounts isolated were 1.5 mg cephaibol P and 1.5 mg cephaibol Q.

In addition to the isolated compounds mentioned above, mass spectrometric fragmentation experiments revealed still more constituents, in particular a compound with a monoisotopic molecular mass of 1667 $[M-H]^-$, which has a sequence identical to that of the principal constituent, but with an aldehyde function instead of the alcohol group of pheol-16.

The structures of all the isolated peptaibols were determined by 2D-NMR experiments; to corroborate these results, the structure of cephaibol A was also independently determined by mass spectrometry.

Structural Elucidation by NMR

The structural elucidation of all compounds is based on analyses of several 2D-NMR experiments including DQF-COSY[16], TOCSY[17], NOESY[18], ROESY[19], HMQC[20], and HMBC[21] spectra. The proton resonances of amino acids other than Aib or Iva were assigned by DQF-COSY and TOCSY experiments following the standard procedure of WÜTHRICH[22]. Since the protons of the side chains of the Aib and Iva residues do not exhibit any scalar coupling to the corresponding amide proton, the assignment of these residues was achieved by correlations observed in the NOESY and HMBC spectra.

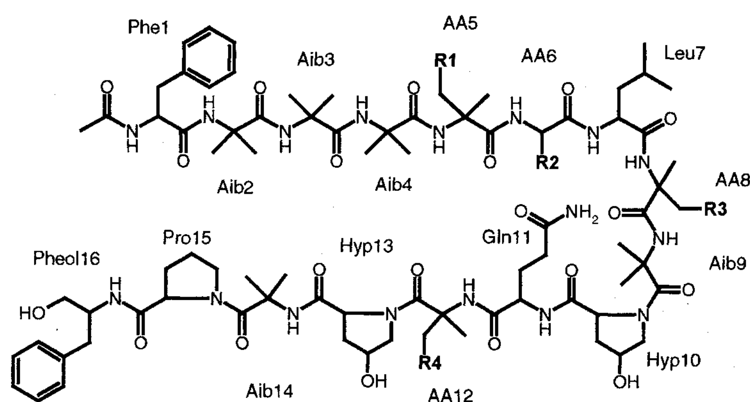
An initial sequence assignment was based on NOEs between amide protons of neighboring amino acids. In the case of peptaibols these NOEs are very intense owing to the helical conformation of these peptides. For the Pro and Hyp residues, connectivities were assigned on the basis of NOEs between the amide protons of the two adjacent amino acids and the δ-protons of the proline ring system. Unambiguous confirmation of the sequence assignment was ultimately obtained from correlations in the HMBC spectrum, in which the carbonyl carbon of each residue i experiences a scalar coupling to the amide proton resonance of the next residue in the sequence $i+1$ (Fig. 3).

Following this strategy, a complete assignment of the proton and carbon resonances for cephaibols A, A2, B, C, D, and E was achieved (see Tables 3a and 3b). In the case of cephaibols P and Q, the limited amount of sample available meant we were only able to assign the proton signals (see Table 4).

Mass Spectrometry of Cephaibol A

The ESI mass spectrum shows an intense $[MH]^+$ peak at *m/z* 1670.7 and a $[M+Na]^+$ peak at *m/z* 1692.7, giving a monoisotopic molecular weight of 1669.7, which is in close accordance with the theoretical mass of 1669.9. From the molecular mass, the presence of an odd number of nitrogens can be concluded.

Fig. 1. The chemical structures of cephaibol A, A2, B, C, D and E.



	R1	R2	R3	R4
Cephaibol A	H (AA5 = Aib)	H (AA6 = Gly)	CH_3 (AA8 = Iva)	CH_3 (AA12 = Iva)
Cephaibol A2	H (AA5 = Aib)	CH_3 (AA6 = Ala)	CH_3 (AA8 = Iva)	CH_3 (AA12 = Iva)
Cephaibol B	CH3 (AA5 = Iva)	H (AA6 = Gly)	CH_3 (AA8 = Iva)	CH_3 (AA12 = Iva)
Cephaibol C	H (AA5 = Aib)	H (AA6 = Gly)	CH_3 (AA8 = Iva)	H (AA12 = Aib)
Cephaibol D	H (AA5 = Aib)	H (AA6 = Gly)	H (AA8 = Aib)	H (AA12 = Aib)
Cephaibol E	H (AA5 = Aib)	H (AA6 = Gly)	H (AA8 = Aib)	CH_3 (AA12 = Iva)

AA = amino acid, Aib = α-amino *iso*-butyric acid, Iva = *iso*-valin

The MS/MS spectrum of the protonated molecule exhibits nearly exclusively the formation of B-type ions (nomenclature according to ROEPSTORFF[23]). At low collision energy the B_9, B_{12}, and B_{14} fragments predominate (spectrum not shown), demonstrating the lability of the proline and hydroxyproline amide bonds. This is a further example of the well-known proline effect[24~26].

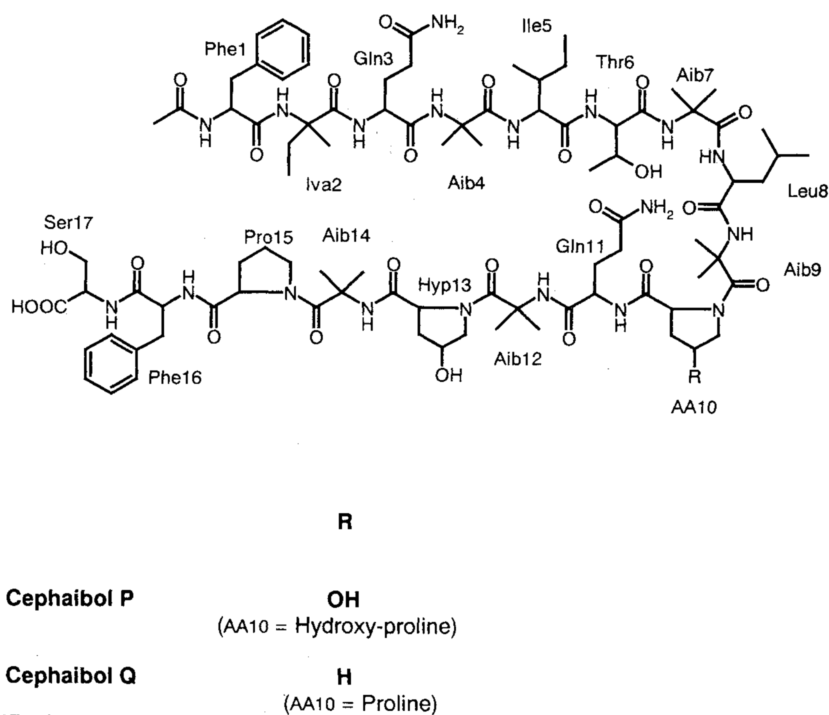
As the starting mass of the ion trap MS/MS scan is limited to about 30% of the parent mass, additional skimmer CID measurements were carried out in order to observe the smaller fragments. From this spectrum (Fig. 4), the entire series of B fragments except for B_{11} can be identified.

Interestingly, the MS/MS spectrum of the sodiated molecule (Fig. 5) shows the B and corresponding A fragments (formal loss of CO). In this spectrum the sodiated A_{11} and B_{11} fragments were also present.

Table 5 lists all the expected B and sodiated B and A fragments. The fragments present in the MS/MS spectra are shown in bold.

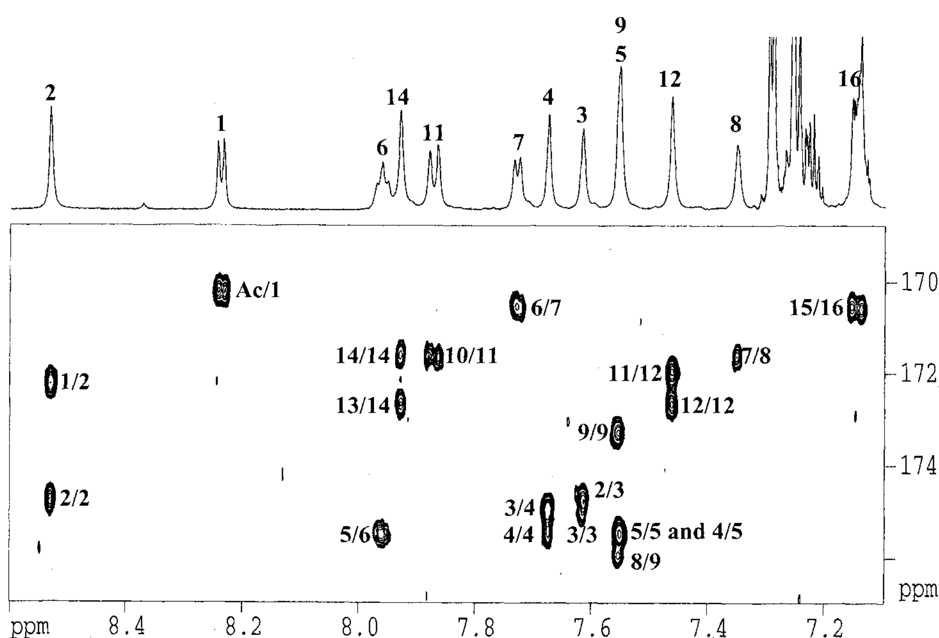
The fragmentation of cephaibol A reveals the complete sequence of the peptide, in agreement with the structure proposed from NMR measurements.

Fig. 2. The chemical structures of cephaibol P and Q.



AA = amino acid, Aib = α-amino *iso*-butyric acid, Iva = *iso*-valine

Fig. 3. Carbonyl carbon/amide proton region of the HMBC spectrum of cephaibol A.



Intraresidual three-bond C′(i)-HN(i) correlation peaks as well as two-bond interresidual C′(i)-HN(i+1) correlation peaks are indicated. The numbers correspond to residues 1 to 16.

Table 3a. Proton chemical shifts of cephaibols A, A2, B, C, D and E in DMSO at 300 K.

Proton	A	A2	B	C	D	E
Ac- Me	1.83	1.83	1.84	1.83	1.83	1.83
Phe1- NH	8.29	8.22	8.28	8.27	8.28	8.28
Hα	4.34	4.39	4.33	4.34	4.34	4.34
Hβ	2.97/2.83	3.04/2.83	2.97/2.83	2.97/2.83	2.97/2.83	2.98/2.84
Hδ	7.29	7.29	7.29	7.29	7.29	7.29
Hε	7.29	7.29	7.29	7.29	7.29	7.29
Hξ	7.22	7.21	7.22	7.22	7.22	7.23
Aib2- NH	8.59	8.50	8.58	8.58	8.59	8.58
HβMe1	1.28	1.31	1.28	1.28	1.28	1.28
HβMe2	1.27	1.30	1.27	1.27	1.27	1.27
Aib3- NH	7.64	7.73	7.62	7.63	7.62	7.62
HβMe1	1.32	1.32	1.32	1.32	1.32	1.32
HβMe2	1.29	1.29	1.29	1.29	1.29	1.29
Aib4- NH	7.69	7.80	7.73	7.69	7.69	7.69
HβMe1	1.39	1.35	1.38	1.38	1.38	1.38
HβMe2	1.38	1.34	1.38	1.38	1.37	1.38
AA5- NH	7.57	7.66	7.49	7.56	7.57	7.58
HβMe1	1.41	1.40	1.35	1.41	1.40	1.40
HβMe2/γMe	1.37	1.39	0.80	1.37	1.38	1.37
Hβ	-		1.99/1.70	-	-	-
AA6- NH	7.98	7.70	7.95	7.97	7.98	7.99
Hα	3.76/3.64	4.01	3.77/3.64	3.76/3.63	3.74/3.63	3.74/3.64
Hβ	-	1.38	-	-	-	-
Leu7- NH	7.74	7.67	7.74	7.72	7.66	7.68
Hα	4.02	3.96	4.02	4.03	4.05	4.04
Hβ	1.70/1.53	1.67/1.57	1.69/1.53	1.69/1.51	1.66/1.52	1.67/1.54
Hγ	1.69	1.72	1.69	1.69	1.66	1.67
Hδ	0.92	0.90	0.92	0.91	0.91	0.91
Hδ'	0.85	0.84	0.85	0.85	0.85	0.86
AA8- NH	7.39	7.18	7.41	7.43	7.78	7.72
HβMe1	1.27	1.28	1.27	1.29	1.45	1.45
HβMe2/γMe	0.73	0.70	0.74	0.74	1.35	1.34
Hβ	2.20/1.67	2.31/1.65	2.17/1.67	2.17/1.69	-	-
Aib9- NH	7.57	7.46	7.58	7.54	7.52	7.54
HβMe1	1.48	1.50	1.48	1.48	1.46	1.46
HβMe2	1.37	1.38	1.36	1.36	1.34	1.34
Hyp10- Hα	4.38	4.39	4.39	4.39	4.38	4.38
Hβ	2.16/1.77	2.17/1.78	2.16/1.78	2.16/1.77	2.15/1.78	2.16/1.79
Hγ	4.29	4.29	4.28	4.20	4.29	4.29
γ-OH	5.11	5.11	5.11	broad	broad	broad
Hδ	3.73/3.50	3.74/3.55	3.73/3.50	3.71/3.50	3.75/3.46	3.77/3.48
Gln11- NH	7.89	7.91	7.88	7.80	7.77	7.85
Hα	4.17	4.17	4.17	4.22	4.22	4.17
Hβ	2.16/1.86	2.15/1.86	2.15/1.87	2.18/1.83	2.18/1.84	2.16/1.89
Hγ	2.09	2.10	2.10	2.09	2.09	2.11
ε-NH_2	7.19/6.70	7.19/6.70	7.18/6.70	7.18/6.69	7.18/6.68	7.19/6.69
AA12- NH	7.47	7.47	7.46	7.75	7.76	7.47
HβMe1	1.41	1.42	1.42	1.50	1.50	1.41
HβMe2/γMe	0.74	0.74	0.74	1.38	1.38	0.75
Hβ	2.15/1.78	2.15/1.78	2.15/1.77	-		2.15/1.78
Hyp13- Hα	4.53	4.54	4.53	4.51	4.51	4.53
Hβ	2.17/1.67	2.17/1.68	2.18/1.68	2.16/1.69	2.16/1.70	2.17/1.68
Hγ	4.21	4.22	4.21	4.21	4.22	4.21
γ-OH	5.09	5.08	5.08	broad	broad	broad
Hδ	3.67/3.37	3.68/3.38	3.67/3.37	3.67/3.34	3.67/3.34	3.69/3.38
Aib14- NH	7.94	7.95	7.94	7.95	7.95	7.94
HβMe1	1.40	1.39	1.40	1.41	1.41	1.39
HβMe2	1.33	1.33	1.33	1.34	1.34	1.33
Pro15- Hα	4.13	4.13	4.13	4.13	4.13	4.13
Hβ	1.83/1.16	1.85/1.17	1.83/1.16	1.84/1.16	1.84/1.17	1.85/1.17
Hγ	1.58/1.48	1.59/1.48	1.58/1.47	1.58/1.49	1.58/1.49	1.59/1.48
Hδ	3.79/3.50	3.79/3.51	3.79/3.50	3.80/3.51	3.80/3.52	3.80/3.51
Phe16- NH	7.15	7.16	7.15	7.14	7.14	7.15
Hα	3.84	3.84	3.83	3.83	3.84	3.84
Hβ	2.99/2.57	2.99/2.58	2.99/2.57	2.98/2.57	2.98/2.58	2.98/2.58
Hδ	7.25	7.25	7.25	7.25	7.25	7.26
Hε	7.24	7.25	7.25	7.25	7.25	7.26
Hξ	7.13	7.14	7.14	7.13	7.13	7.14
CH_2-OH	3.39/3.24	3.39/3.25	3.39/3.24	3.38/3.24	3.39/3.24	3.39/3.25
CH_2-**OH**	4.57	4.56	4.57	broad	broad	broad

Table 3b. Carbon chemical shifts of cephaibols A, A2, B, C, D and E in DMSO at 300 K.

Carbon		A	A2	B	C	D	E
Ac-	Me	22.29	22.34	22.28	22.23	22.26	22.27
	C'	170.37	170.18	170.37	170.27	170.27	170.36
Phe1-	Cα	55.11	54.88	55.14	54.99	55.05	55.10
	Cβ	36.37	36.41	36.32	36.34	36.34	36.35
	Cγ	137.37	137.53	137.36	137.35	137.35	137.36
	Cδ	129.16	129.10	129.14	129.14	129.14	129.15
	Cε	128.09	128.04	128.05	128.05	128.05	128.06
	Cξ	126.39	126.32	126.37	126.38	126.38	126.39
	C'	172.41	172.09	172.40	172.39	172.40	172.42
Aib2-	Cα	55.77	55.73	55.79	55.75	55.76	55.76
	CβMe1	23.57	24.39	23.69	23.51	23.47	25.39
	CβMe2	25.37	24.60	25.24	25.34	25.45	23.51
	C'	174.92	174.55	174.81	174.81	174.89	174.90
Aib3-	Cα	55.86	55.73	55.84	55.87	55.87	55.87
	CβMe1	24.04	25.41	24.10	23.97	23.98	24.08
	CβMe2	24.62	23.12	24.60	24.55	24.84	24.63
	C'	175.11	175.38	175.10	175.08	175.04	175.06
Aib4-	Cα	55.86	55.82	55.96	55.87	55.87	55.87
	CβMe1	24.99	25.92	24.82	24.85	25.02	25.14
	CβMe2	24.55	23.12	24.82	24.55	24.48	24.94
	C'	175.62	175.60	175.45	175.62	175.59	175.60
AA5-	Cα	55.92	55.70	58.98	55.87	55.87	55.87
	CβMe1	24.99	26.24	21.23	24.85	24.84	24.94
	CβMe2/γMe	24.49	23.20	7.49	24.55	24.73	24.53
	Cβ	-	-	28.40	-	-	-
	C'	175.62	175.68	175.56	175.54	175.51	175.64
AA6-	Cα	43.43	50.69	43.34	43.35	43.28	43.35
	Cβ	-	16.47	-	-	-	-
	C'	170.69	174.55	170.67	170.63	170.35	170.43
Leu7-	Cα	53.18	53.49	53.06	53.04	52.56	52.73
	Cβ	39.40	39.29	39.40	39.41	39.25	39.24
	Cγ	24.13	24.18	24.10	24.11	24.07	24.08
	Cδ	22.62	22.58	22.63	22.60	22.67	22.67
	Cδ'	21.57	21.29	21.57	21.57	21.64	21.64
	C'	171.80	171.81	171.71	171.74	171.60	171.71
AA8-	Cα	59.34	59.29	59.35	59.34	56.15	56.18
	CβMe1	21.93	22.37	21.77	21.87	25.45	25.26
	CβMe2/γMe	7.27	7.15	7.32	7.31	25.02	25.66
	Cβ	27.60	26.62	27.89	27.87	-	-
	C'	176.07	176.12	176.08	176.04	175.80	175.84
Aib9-	Cα	56.22	56.16	56.21	56.21	56.22	56.22
	CβMe1	23.17	23.12	23.15	23.23	23.33	23.26
	CβMe2	25.72	25.65	25.75	25.49	25.45	25.66
	C'	173.44	173.39	173.44	173.59	173.64	173.48
Hyp10-	Cα	61.01	60.99	60.99	61.12	61.09	60.95
	Cβ	36.84	36.95	36.80	36.75	36.58	36.71
	Cγ	68.95	68.94	68.89	68.93	69.01	68.95
	Cδ	56.13	56.27	56.09	56.21	56.06	56.00
	C'	171.80	171.92	171.77	171.74	171.67	171.75
Gln11-	Cα	52.42	52.37	52.42	51.95	51.96	52.48
	Cβ	26.66	26.71	26.66	26.17	26.16	26.63
	Cγ	31.44	31.37	31.42	31.23	31.33	31.49
	Cδ	173.08	173.03	173.00	173.10	173.10	173.08
	C'	172.13	172.15	172.11	172.02	172.03	172.13
AA12-	Cα	58.48	58.47	58.47	55.87	55.87	58.47
	CβMe1	20.36	20.34	20.35	23.80	23.81	20.37
	CβMe2/γMe	6.97	6.94	6.97	25.73	25.76	7.01
	Cβ	28.06	28.04	28.06	-	-	28.09
	C'	172.83	172.82	172.81	172.26	172.27	172.83
Hyp13-	Cα	60.55	60.54	60.55	60.54	60.54	60.54
	Cβ	37.33	37.33	37.30	37.22	37.25	37.32
	Cγ	69.04	69.03	69.03	68.99	69.01	69.00
	Cδ	56.46	56.45	56.40	56.32	56.34	56.45
	C'	172.83	172.82	172.81	172.70	172.75	172.83

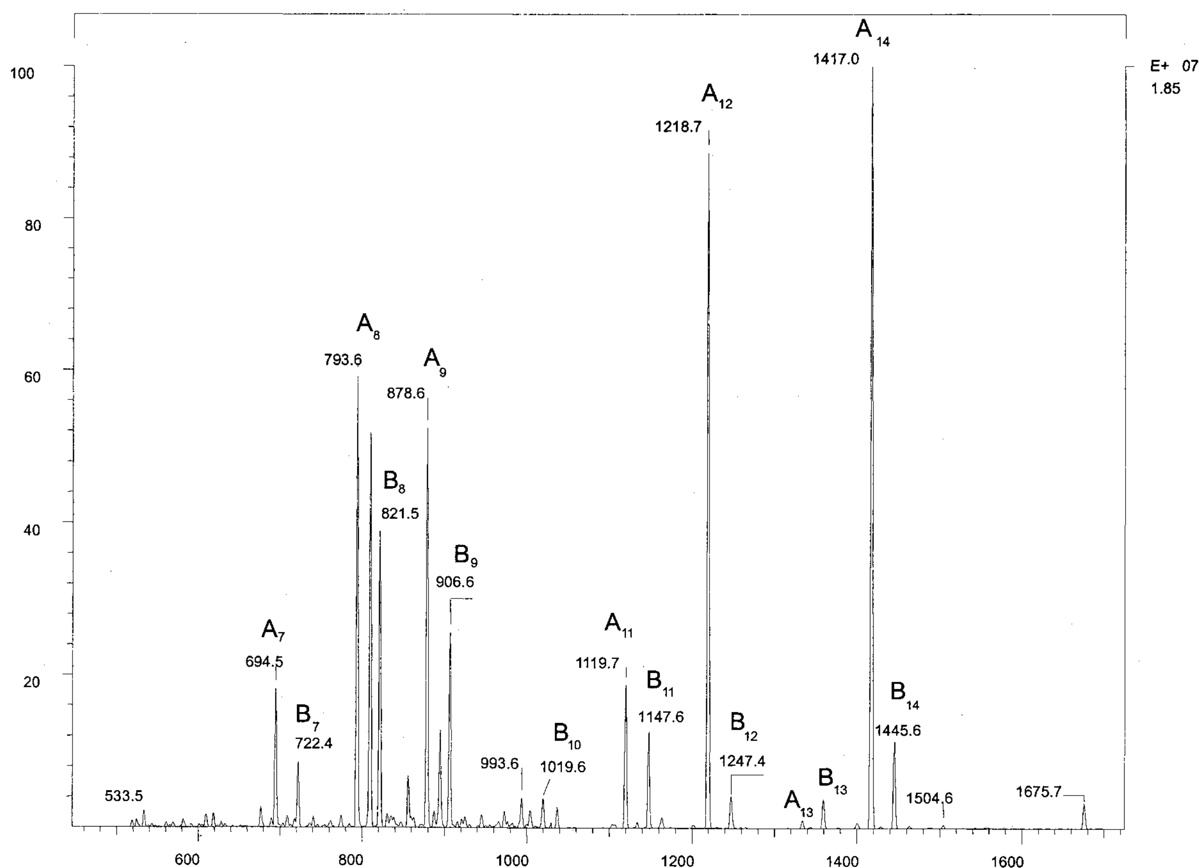
Table 3b. Continued.

Carbon		A	A2	B	C	D	E
Aib14-	Cα	55.65	55.63	55.63	55.65	55.66	55.64
	CβMe1	23.50	23.50	23.49	23.41	23.47	23.51
	CβMe2	25.66	25.65	25.65	25.58	25.60	25.66
	C'	171.73	171.71	171.71	171.70	171.73	171.71
Pro15-α		61.79	61.78	61.78	61.77	61.77	61.78
	Cβ	28.34	28.33	28.33	28.34	28.35	28.33
	Cγ	24.83	24.82	24.82	24.55	24.84	24.82
	Cδ	47.36	47.35	47.35	47.36	47.36	47.36
	C'	170.69	170.68	170.67	170.63	170.69	170.68
Phe16-	Cα	52.60	52.59	52.58	52.46	52.47	52.58
	Cβ	36.37	36.41	36.37	36.34	36.34	36.35
	Cγ	139.47	139.46	139.46	139.46	139.46	139.47
	Cδ	129.33	129.32	129.32	129.30	129.31	129.33
	Cε	127.93	127.92	127.91	127.90	127.90	127.92
	Cξ	125.65	125.64	125.63	125.63	125.63	125.64
	CH_2-OH	63.42	63.41	63.30	63.32	63.34	63.38

Table 4. Proton chemical shifts of cephaibols P and Q in DMSO at 300 K.

Proton		P	Q
Ac-	Me	1.83	1.83
Phe1-	NH	8.23	8.23
	Hα	4.48	4.48
	Hβ	3.05/2.84	3.05/2.84
	Hδ	7.27	7.27
	Hε	7.27	7.27
	Hξ	7.19	7.19
Iva2-	NH	8.28	8.28
	HβMe	1.27	1.27
	Hγ	0.74	0.74
	Hβ	1.86/1.66	1.86/1.66
Gln3-	NH	8.14	8.14
	Hα	3.97	3.97
	Hβ	1.88	1.88
	Hγ	2.18	2.18
	ε-NH_2	7.34/6.88	7.34/6.88
Aib4-	NH	7.99	7.99
	HβMe1	1.39	1.39
	HβMe2	1.35	1.35
Ile5-	NH	7.53	7.53
	Hα	3.88	3.88
	Hβ	1.88	1.88
	HγMe	0.84	0.84
	Hγ	1.45/1.18	1.45/1.18
	Hδ	0.78	0.78
Thr6-	NH	7.60	7.60
	Hα	3.82	3.83
	Hβ	4.09	4.09
	Hγ	1.12	1.11
	OH	4.96	4.96
Aib7-	NH	7.77	7.74
	HβMe1	1.36	1.36
	HβMe2	1.36	1.36
Leu8-	NH	7.26	7.28
	Hα	4.21	4.16
	Hβ	1.65/1.56	1.65/1.56
	Hγ	1.68	1.69
	Hδ	0.85	0.85
	Hδ'	0.78	0.78
Aib9-	NH	7.94	7.97
	Hβ	1.52	1.50
	Hβ'	1.42	1.42
Hyp/Pro10-Hα		4.39	4.30
	Hβ	2.16/1.77	2.22/1.69
	Hγ	4.26	1.84
	γ-OH	5.12	-
	Hδ	3.76/3.44	3.75/3.53
Gln11-	NH	7.85	7.79
	Hα	4.22	4.24
	Hβ	1.89	1.88
	Hγ	2.14/2.12	2.14/2.12
	ε-NH_2	7.20/6.70	7.19/6.71
Aib12-	NH	7.79	7.78
	HβMe1	1.51	1.50
	HβMe2	1.39	1.39
Hyp13-	Hα	4.53	4.53
	Hβ	2.18/1.71	2.18/1.71
	Hγ	4.22	4.22
	γ-OH	5.09	5.09
	Hδ	3.67/3.38	3.67/3.38
Aib14-	NH	8.07	8.07
	HβMe1	1.47	1.47
	HβMe2	1.38	1.38
Pro15-	Hα	4.17	4.17
	Hβ	1.90/1.04	1.90/1.04
	Hγ	1.63/1.51	1.63/1.51
	Hδ	3.87/3.58	3.87/3.58
Phe16-	NH	7.67	7.67
	Hα	4.35	4.35
	Hβ	3.26/2.80	3.26/2.80
	Hδ	7.32	7.32
	Hε	7.26	7.26
	Hξ	7.19	7.19
Ser17-	NH	7.39	7.39
	Hα	4.24	4.24
a.	*Hβ*	3.71	3.71
	β-OH	4.77(broad)	4.77(broad)

Fig. 4. Skimmer CID mass spectrum of cephaibol A.



Biological Activity

Cephaibols have antibacterial properties; some minimum inhibitory concentration values are listed in Table 6. The investigated cephaibols were found to have an inhibitory action against Gram-positive bacteria, but were inactive against Gram-negative bacteria. What is most remarkable is the significant differences in the inhibitory effects of the investigated constituents. Up to now, the microheterogeneity of peptaibols has been assumed to be a consequence of feed-related changes in the biosynthetic pathway[2]. The demonstrated differences in the inhibitory concentrations of the different constituents mean we must now appraise this series of homologues in a new light.

A further point of note is that cephaibols exhibit no diminution in their activity on addition of serum.

In addition to the inhibition values listed in Table 6, cephaibol A was shown to be active against *Mycoplasma gallisepticum* and *Mycoplasma mycoides* at concentrations of 64 μg/ml.

Cephaibols show anthelmintic activity and an insecticidal action against some ectoparasites. In larval development tests with *Ascaria galli*, cephaibol A showed 100% lethality within 24 hours at concentrations of 200, 100, 50, and 25 μg/ml, and at 12.5 μg/ml was still 60% lethal with severe impairment of mobility in the surviving larvae.

In addition to its anthelmintic activity, cephaibol A also has an ectoparasiticidal action. Concentrations of 1000, 500, and 250 μg/ml resulted in 100% lethality within 72 hours after membrane feeding in *Cimex lectularius*; even the lower concentration of 62.5 μg/ml achieved the significant result of 80% mortality. On the other hand, in feeding toxicity tests with *Lucilia cuprina*, cephaibol A was found to be less active at comparable active substance concentrations, with mortality of only 57% at the highest concentration (500 μg/ml).

Coccidia of the species *Eimeria tenella* (Houghton) showed little or no inhibition by cephaibol A; in the invasion inhibition test 10 and 100 μg/ml cephaibol A reduced protozoal infection by 24 and 31% respectively.

Fig. 5. MS/MS spectrum of the sodiated molecule (*m/z* 1692.7).

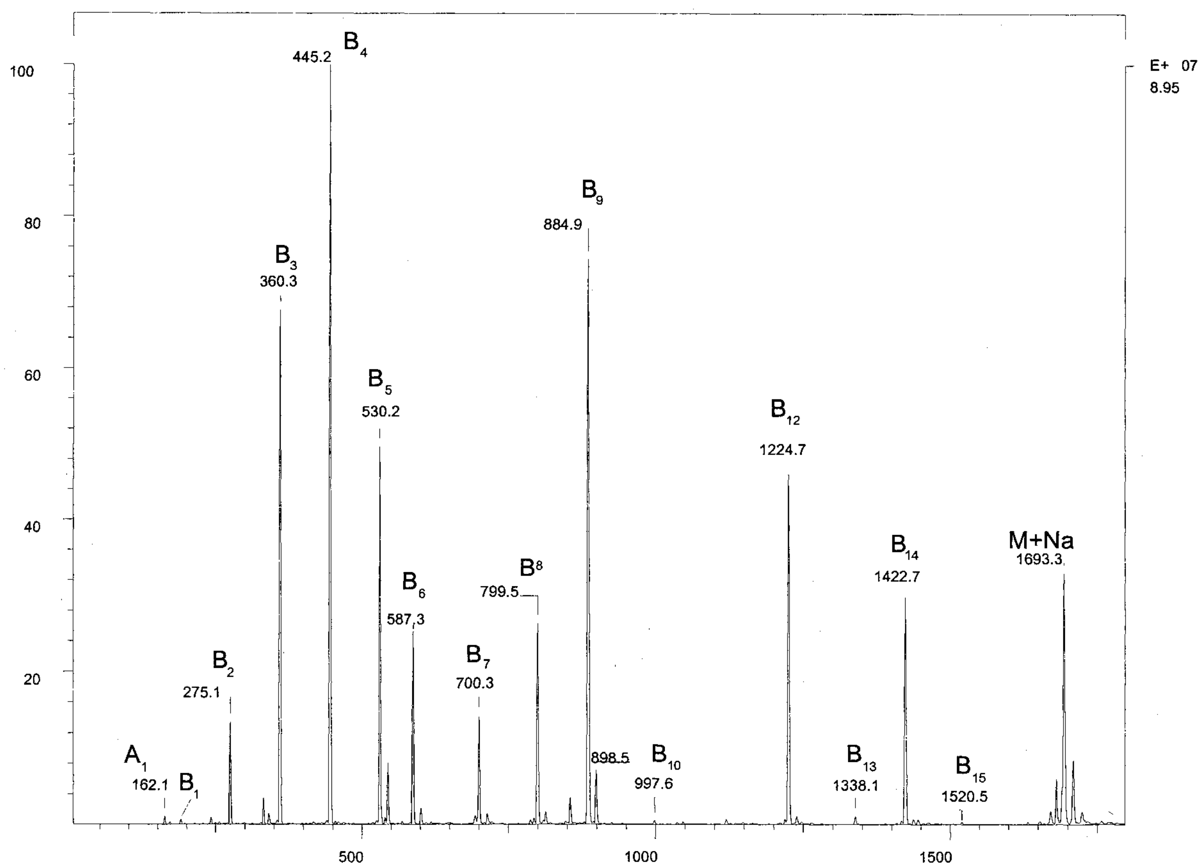


Table 5. Normal B-fragments in the first row, sodiated B- and A-fragments in the second and third row; observed ions are printed in bold.

B_1	**190,1**	B_1	*212,1*	A_1	*184,1*
B_2	**275,1**	B_2	*297,1*	A_2	*269,1*
B_3	**360,2**	B_3	*382,2*	A_3	*354,2*
B_4	**445,2**	B_4	*467,2*	A_4	*439,2*
B_5	**530,3**	B_5	*552,3*	A_5	*524,3*
B_6	**587,3**	B_6	*609,3*	A_6	*581,3*
B_7	**700,4**	B_7	**722,4**	A_7	**694,4**
B_8	**799,5**	B_8	**821,5**	A_8	**793,5**
B_9	**884,5**	B_9	**906,5**	A_9	**878,5**
B_{10}	**997,6**	B_{10}	**1019,6**	A_{10}	*991,6*
B_{11}	*1125,6*	B_{11}	**1147,6**	A_{11}	**1119,6**
B_{12}	**1224,7**	B_{12}	**1246,7**	A_{12}	**1218,7**
B_{13}	**1337,7**	B_{13}	**1359,7**	A_{13}	**1331,7**
B_{14}	**1422,8**	B_{14}	**1444,8**	A_{14}	**1416,8**
B_{15}	**1519,9**	B_{15}	*1541,9*	A_{15}	*1513,9*

Schizont maturation was not affected.

The efficiency-enhancing activity of aibellin on rumen fermentation has been described in an earlier paper[27].

There is, however, little mention in the literature of the anthelmintic properties of peptaibols[28,29]. Further studies are necessary to establish the entire spectrum of activity of cephaibols.

Likewise still outstanding is a description of the three-dimensional structure of cephaibols, which would be necessary for a comparison with known peptaibols.

Experimental

Analytical Chromatography

Cephaibol concentrations were determined by reversed-phase HPLC using the following equipment: autosampler (TSP, model SP 8880), low-pressure HPLC pump (LKB, type 2150), photometric detector (Jasco UV-975, 210 nm detection wavelength), and a TSP Chromjet integrator. The

Table 6. Comparative antibacterial potency (MIC, μg/ml) of cephaibol A, B and E.

	Cephaibol A	B	E
Staphyloc. aureus SG511	10	5	80
Staphyloc. aureus SG511 + 10% serum	5	2.5	40
Staphyloc. aureus Exp54146	10	5	80
Streptoc. pyogenes A561	10	5	160
Enteroc. faecium M78L	20	10	>160
Escherichia coli	>160	>160	>160

separations were performed using a steel column (4.0× 250 mm) packed with Nucleosil $100C_{18}AB$ 250/4 (Macherey-Nagel, Düren, Germany) as the stationary phase. The mobile phase was 0.1% trifluoroacetic acid in 40% acetonitrile. The analyses were carried out with a flow rate of 1.0 ml/minute and an injection volume of 20 μl. Detection: UV absorbance at 210 nm. The amino acid analyses were carried out by C.A.T. GmbH, D-72070 Tübingen.

Fermentation Conditions

The *Acremonium tubakii*, DSM 12774, stock culture was a glycerol culture prepared by inoculation of 100 ml of nutrient solution [malt extract 2.0%, yeast extract 0.2%, glucose 1.0%, $(NH_4)_2HPO_4$ 0.05%, pH 6.0] in a sterile 300 ml conical flask with *Acremonium tubakii*, DSM 12774, followed by incubation for 7 days at 25°C on a rotary shaking machine at 140 rpm. 1.5 ml of this culture was then diluted with 2.5 ml of 80% glycerol and stored at −20°C.

Cultures/precultures of *Acremonium tubakii*, DSM 12774, were prepared in 300 ml conical flasks containing 100 ml of the following nutrient solution: 30 g/liter sucrose, 5 g/liter yeast extract, 1 g/liter K_2HPO_4, 3 g/liter $NaNO_3$, 0.5 g/liter $MgSO_4 \cdot 7H_2O$, 0.01 g/liter $FeSO_4 \cdot 7H_2O$, 0.5 g/liter KCl, +1.0 ml of trace element solution [2 g/liter $ZnSO_4 \cdot 7H_2O$ and 0.7 g/liter $CuSO_4 \cdot 5H_2O$], which were incubated on a rotary shaking machine at 25°C and 140 rpm. Maximum cephaibol production in the shake cultures was achieved after about 120 hours.

Cephaibol production was carried out in 30 liter fermenters under the following conditions. Nutrient medium: 30 g/liter sucrose, 5 g/liter yeast extract, 3 g/liter $NaNO_3$, 0.5 g/liter KCl, 0.5 g/liter $MgSO_4$, 0.1 g/liter K_2HPO_4, 10 μM $FeCl_3 \cdot 6H_2O$, +1.0 ml of trace element solution; pH 6.5 (prior to sterilization). The trace element solution consisted of 2 g/liter $ZnSO_4 \cdot 7H_2O$ and 0.7 g/liter $CuSO_4 \cdot 5H_2O$. Incubation was for 96 hours at a temperature of 25°C, a stirrer speed of 300 rpm, and aeration of 15 liters/minute.

Isolation of cephaibols

At the end of the fermentation of *Acremonium tubakii*, DSM 12774, the culture broth from three fermenters (70 liter) was filtered with the addition of approx. 2% Celite (filtration aid) and the cell mass (6 liter) was extracted with 20 liter methanol. The methanolic solution containing the peptide active substances was filtered from the mycelium and concentrated under vacuum. This concentrate was loaded together with the culture filtrate (63 liter) onto a prepared MCI gel, CHP20P column (capacity 4 liter), which was eluted with a gradient of 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 2-propanol. The eluate was collected in 2.5 liter fractions at a flow rate of 12 liters/hour, and the fractions (21~24) containing the peptide active substances were pooled. Concentration under vacuum followed by freeze-drying gave 4 g of a light brown powder.

4 g of the product was loaded onto a column (capacity 3.9 liter; dimensions 10×50 cm) packed with Fractogel® TSK MW-40s. The methanol eluent was pumped through the column at a flow rate of 50 ml/minute and the eluate was

collected in 65 ml fractions. The cephaibols were for the most part eluted in fractions 40~80. These fractions were pooled and solvent was stripped under reduced pressure, yielding 1.3 g of the peptide active substance mixture.

The peptide active substance mixture (1.3 g) was loaded onto a preparative HPLC column (capacity 500 ml; dimensions 4.0 (i.d.) ×25 cm) packed with Nucleosil® 100-7 C18 HD, which was eluted with 40% acetonitrile in 0.1% aqueous trifluoroacetic acid at a flow rate of 50 ml/minute, collecting the eluate in 125 ml fractions. Cephaibol D was eluted in fraction 46, cephaibol C in fractions 49 and 50, cephaibol E in fraction 51, cephaibol A in fractions 60~64, and cephaibols B and A1 in fractions 66 and 67. Fraction 68 contained a mixture of cephaibols. After concentration under vacuum and freeze-drying, the following quantities were obtained:

Cephaibol A: 520 mg, ESI-MS: 1671 Da $[M+H]^+$,
Cephaibol A2: 4 mg, ESI-MS: 1685 Da $[M+H]^+$,
Cephaibol B: 38 mg, ESI-MS: 1685 Da $[M+H]^+$,
Cephaibol C: 195 mg, ESI-MS: 1657 Da $[M+H]^+$,
Cephaibol D: 16 mg, ESI-MS: 1643 Da $[M+H]^+$,
Cephaibol E: 76 mg, ESI-MS: 1657 Da $[M+H]^+$.

After freeze-drying, fraction 68 (4.1 mg) was dissolved in 20% aqueous acetonitrile and loaded onto a 250/10 Nucleosil C18 300-7® column, which was eluted with 0.005% ammonium acetate buffer in 40% acetonitrile. This gave after drying 1.5 mg cephaibol P and 1.5 mg cephaibol Q, plus a small amount of cephaibol B.

Cephaibol P: ESI-MS: 1873 Da $[M+H]^+$,
Cephaibol Q: ESI-MS: 1857 Da $[M+H]^+$.

Under the above conditions the cephaibols had the following retention times: cephaibol A: 12.1 minutes, cephaibol A2: 16.9 minutes, cephaibol B: 16.0 minutes, cephaibol C: 9.0 minutes, cephaibol D: 7.1 minutes, cephaibol E: 9.3 minutes, cephaibol P: 17.9 minutes, cephaibol Q: 17.9 minutes.

NMR Spectroscopy

All NMR spectra were recorded on Bruker DRX 600 spectrometers operating at 600 MHz (^{1}H) and 150 MHz (^{13}C). Data were processed on an indigo2 station (Silicon Graphics) using Bruker XWINNMR software. Homonuclear COSY, TOCSY, NOESY, and ROESY experiments were performed with a spectral width of 9 ppm. In all experiments, spectra were recorded with 1024 increments in t_1 and 4096 complex data points in t_2. For the NOESY and ROESY experiments 32 transients were averaged for each t_1 value, and for COSY and TOCSY, 8 transients. A mixing time of 150 msec was used for the NOESY and ROESY spectra.

For the HMQC spectra, 512 increments (16 scans) with 4096 complex data points in t_2 were collected using a sweep width of 9 ppm in the proton and 160 ppm in the carbon dimension. The HMBC spectra were acquired with a sweep width of 9 ppm in the proton and 200 ppm in the carbon dimension. A total of 96 transients were averaged for each of 512 increments in t_1, and 2048 complex points in t_2 were recorded. A delay of 70 msec was used for the development of long range correlations.

Mass Spectrometry

Mass spectra were recorded on a Finnigan MAT LCQ ion trap mass spectrometer equipped with an electrospray ionization source. The sample solution was introduced using a syringe pump. The mass spectrometer was operated in the positive mode, with electrospray ionization (ESI). The heated capillary was kept at 220°C, and an 11 V potential was applied. The conversion dynode was set at 15 kV, the electron multiplier at 0.9 kV, and the spray voltage at 5.2 kV.

The sheath gas was nitrogen at a pressure of 70 psi. No auxiliary gas was used.

For the MS/MS analysis, a 3 u wide window was used for isolation of the precursor.

A relative excitation energy of 20% was applied to dissociate the precursor ions (MS/MS). Skimmer CID fragmentation was performed with an excitation energy of 25%. The daughter ions were unit resolved across the scan range. Three microscans were acquired over the mass range of 265~2000, with a target threshold of 1×10^6 for automatic gain control (AGC). The mobile phase was 50 : 50 acetonitrile:water (0.01 M NH_4OAc, pH 4.5) with a flow rate of 3 μl/minute. The peptide was dissolved in the mobile phase to a concentration of 0.5 mg/ml.

The software package Navigator (Finnigan), version 1.1, was used for instrument control and data processing.

In Vitro Demonstration of Anthelmintic Activity

Embryonated eggs of *Ascaridia galli* were surface-sterilized with 6% sodium hypochlorite solution. After washing several times to remove traces of chlorine, the embryonic membranes were mechanically opened using slowly rotating glass beads (diameter 5 mm). Viable L2 larvae were separated from the cell debris at 41°C over a 6 hours period by means of a larval accumulation process. The highly motile larvae were then suspended in KW2 medium (NCTC 135+0.13% yeast extract+0.14% peptone+0.14% dextrose, pH 6.8), diluted, and transferred to a microtiter plate at a density of 20 larvae per well. Before addition of the larvae, the microtiter plate was

treated with medicated medium in which cephaibol A was tested at end concentrations of 200~0.1 μg/ml. In addition to the medicated samples, a solvent control (1% DMSO) and a non-medicated control were also carried out. After incubation for 5 days at 41°C and 10% CO_2, neutral red was added to an end concentration per well of 0.16%. The larval culture was then incubated at 41°C for a further 24 hours (=end of test). Larval morphology and mortality were monitored daily under a microscope; vitality was quantified according to oral ingestion of neutral red and its accumulation in the gut of the larvae and was investigated at the end of the experiment.

Test of Ectoparasiticidal Activity

a. Membrane feeding experiment with *Cimex lectularis*

Defibrinated sheep blood (5 ml aliquots) was mixed with cephaibol A stock solution to give a range of active substance concentrations (1000~2 μg/ml). 20 adult bedbugs were used per product concentration and were fed warm (37°C) medicated blood in a special membrane feeding system. The duration of bloodsucking was about 10 minutes.

The fully engorged bedbugs were then transferred to Petri dishes and kept in a climatic chamber at 28°C. Product activity was assessed according to mortality, egg-laying, and larval development.

b. Feeding toxicity test with *Lucilia cuprina*

Adult flies were fed with medicated sugar solution. Product concentrations of 500~31.25 μg/ml were prepared from the cephaibol A stock solution, with 300 flies used per concentration. A sugar solution containing 3% dimethyl formamide served as a solvent control and a non-medicated control was also carried out. Mortality was determined 24 hours after feeding.

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