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Memnopeptide A, a Novel Terpene Peptide from *Memnoniella* with an Activating Effect on SERCA2

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The terpene peptide memnopeptide A (**1**), $C_{76}H_{108}N_{16}O_{18}S$, MW 1564, was isolated from a culture of the fungus *Memnoniella echinata* FH 2272 on casein peptone. The structure of the novel compound was elucidated with the aid of 2D NMR experiments and from amino acid analysis and mass spectrometric sequencing of the peptide. The compound consists of a known phenylspirodrimane subunit linked to the decapeptide Met-His-Gln-Pro-His-Gln-Pro-Leu-Pro-Pro. This proline-rich peptide is a subsequence of β-casein. From the observed absence in the literature of any other highly significant sequence homologues, memnopeptide A can be assumed to arise from metabolic products of the fungus with direct incorporation of constituents of the nutrient medium. The formation of memnopeptide A suggests this may be a mechanism for storage of amines by the fungus. Memnopeptide A has weak antibacterial activity against Gram-positive bacteria and effects half-maximal activation of sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA2) at a concentration of 12.5 μM.

The isolation of the inositol phosphatase inhibitor L-671,776 from cultures of the strain *Memnoniella echinata* ATCC 20928 was reported in 1992 by Y. K. T. Lam, *et al.* In this initial report a phenylspirodrimane structure[1] was postulated for the compound, which was subsequently revised on the basis of a synthesis described by J. R. Falck, *et al.*[2] The initial paper from Merck Sharp and Dohme[1] prompted a number of research groups to launch investigations into the metabolic products of the fungus *Memnoniella echinata*[3], leading to the discovery of further sesquiterpenyl phenols, the memnobotrins[4] and memnoconols[4].

In addition to *Memnoniella echinata*, *Stachybotrys* species have also been found to produce phenylspirodrimane derivatives closely related to L-671,776: the stachybocins[5], the antibiotic K-76 (from *Stachybotrys complementi*)[6], the Mer-NF5003 series[7], stachybotrin A and stachybotral (from *Stachybotrys alternans*)[8], the spirodihydrobenzofuran lactams,[9] and stachyflin[10]. Memnobotrin A, the stachybocins, stachybotrin A, spirodihydrobenzofuran lactam, and stachyflin all contain a 5-membered lactam structure, a nitrogen-containing subunit which—the quite different alkaloids aside—is comparatively rare in terpenes.

In the course of target-oriented screening for inhibitors of glucose-6-phosphate translocase[11] we also investigated nutrient media used to culture *Memnoniella echinata* and detected weak inhibitors of this enzyme. The unusual properties and high nitrogen content of one of the natural products we isolated led us to study the substance class detected more closely. Described herein is the isolation, structure determination, and some biological properties of the novel terpene peptide, which we have named memnopeptide A (**1**), whose unusual composition allows us to draw some conclusions as to the mechanism of its origin. An attempt is also made to provide an explanation for the common occurrence of terpene lactams in cultures of the fungus *Memnoniella echinata* and of *Stachybotrys* species.

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Results

Our screening program for glucose-6-phosphate translocase inhibitors was carried out using the imperfect fungus *Memnoniella echinata* FH 2272, which was derived from the strain *Memnoniella echinata* ATCC 20928. The fungal strain was fermented at 28°C for 44 hours in a liquid nutrient medium containing 1% glucose, 0.5% casein peptone, 0.17% cornsteep liquor, and trace elements. At the time of harvest, all the free phosphate and all the carbohydrate capable of determination with anthrone had been consumed, and the free ammonium in solution—determined by the Kjeldahl method—had fallen to one third of its original concentration.

Routine check HPLC analysis of the harvested culture fluid revealed—by comparison with over a hundred other microbial cultures which are mostly likewise rich in secondary metabolites—an extraordinary diversity of fermentation products. Such a plethora of metabolic products has up to now been seen only in cultures of the related fungus *Stachybotrys atra*, which was investigated in a different context.

The culture filtrate (180 liters) from the *Memnoniella echinata* FH 2272 fermentation was worked up. The test used to screen for inhibitors during the purification process was the glucose-6-phosphate translocase assay. The enrichment process consisted of solid-phase extraction on an adsorption resin with gradient elution of the active compounds. Activity was detectable across the entire eluate, peaking in the 25~30% 2-propanol and 45~50% 2-propanol fractions. Repeated preparative HPLC of the nonpolar eluate led to the isolation of a number of neutral inhibitors. Also isolated was the inositol monophosphatase inhibitor L-671,776[1], which was found to be inactive in the G-6-P translocase assay. L-671,776 was identified on the basis of its molecular weight of 388.51, its mass spectrum, and NMR measurements.

To obtain the G-6-P translocase-inhibiting components of the polar 25~30% fraction of the adsorption resin eluate, the basic metabolic products, which also showed inhibitory activity, were isolated using a cation exchange column. We succeeded in further purifying this still complex mixture of basic secondary metabolites by preparative HPLC, which yielded both colorless and yellowish red compounds, each containing a terpenoid moiety plus an amino acid component. The amounts obtained varied. We investigated an active main component, memnopeptide A, of which 140 mg was obtained. Its UV absorption spectrum, which showed a maximum at 269 nm and a shoulder at 300~310 nm, provided further evidence of the presence of a sesquiterpenylphenol substructure. Amino acid analysis after hydrolysis in constant boiling hydrochloric acid revealed the constituents Glu (2), Pro (4), His (2), and Leu (1).

The ESI mass spectrum shows an intense protonated molecular ion MH^+ at *m/z* 1565.5 and a doubly charged ion at *m/z* 783.3 to give a monoisotopic molecular mass of 1564.5. From the molecular mass, it can be deduced that the number of nitrogen atoms is even.

Table 1 lists the ^1H and ^{13}C NMR chemical shifts, correlations, and assignments for **1**. Analysis of the NMR spectra (^1H, ^{13}C, HSQC[12], NOESY[13], HMBC[14], COSY[15], and TOCSY[16,17]) of memnopeptide A suggested that **1** represents a novel conjugation of terpene and peptide moieties. The terpenoid moiety resembles the topology proposed for L671,776[1], but the stereochemistry of the phenylspirodrimane ring system, as established by NOE measurements and analysis of homonuclear coupling constants (Fig. 2), is identical with that of Mer-NF-5003 E[7].

The relative stereochemistry within the terpenoid moiety as depicted in Fig. 1 is deduced from the analysis of ^1H-^1H vicinal coupling constants, indicating axial ($^3J_{HH}$ >8 Hz) or equatorial ($^3J_{HH}$ <3.5 Hz) orientation within the chair conformation. The stereospecific assignment is listed in Table 1.

The amide linkage of a L-671,776 terpenoid to lysine, glutamic acid, and δ-aminopropionic acid has been reported[9,18]. Despite the perfect agreement in the assigned chemical shifts of the terpenoid moieties of spirodihydrobenzofuran lactam VI[9,18] and **1**, ^1H-^{13}C long range correlations and NOE effects (Fig. 2) indicate that **1** represents a different regioisomer of the spirodihydrobenzofuran-lactam.

Two down-field resonances at 155.73 and 153.6 ppm point to the presence of oxygen substituents within the aromatic moiety. Long-range correlations to the resonances at 4.33 ppm and to the two resonances at 3.154 and 2.792, assigned to C-8′ and C-11 respectively, place this center at the 6′-position. The other oxygen-substituted center showed long-range correlations to H-3′(6.59 ppm) and H-11 (3.154 and 2.792 ppm) placing it at C-2′. Any other substitution pattern would require these strong long-range correlations to be across 4 bonds or more. An additional NOE correlation between the phenolic OH signal (at 9.75 ppm) and the sole phenyl proton signal (H-3′ at 6.598) indicates the proximity of these two protons.

The sequence of the 10 amino acid residue peptide was determined from the mass spectrometric fragmentation pattern (see below), significant overlap of the C_α proton-

Table 1. NMR chemical shifts and correlations of memnopeptide A (8 mg in 480 μl DMSO-d_6), 310 K.

δ-^{13}C	m	δ-^{1}H	m	$^{n}J_{HH}$	$^{n}J_{CH}$ (10 Hz)	assignment
23.90	t	1.764 eq. 0.962 ax.	m m	0.962, 1.416, 1.871	0.978, 1.416, 3.227	Terpen-1
24.77	t	1.840 ax. 1.416 eq.	m m	1.416, 1.740, 0.962 1.840, 0.926	-	Terpen-2
73.50 73.51	d	3.227 eq.	dd	1.840, 1.416	0.903, 0.824	Terpen-3
37.22	s	-	-	-	0.903, 0.824	Terpen-4
28.50	q	0.918 eq.	s	0.827	0.827	Terpen-4-Me
22.25	q	0.827 ax.	s	0.918	0.918, 2.035	Terpen-4-Me
39.45	d	2.035 ax.	dd	1.409, 1.460	0.903, 0.824, 0.971	Terpen-5
20.37	t	1.409 ax. 1.460 eq.	m	2.035, 1.538, 1.460 2.035,	2.035	Terpen-6
30.63	t	1.538 eq. 1.430 ax.	m m	1.430, 1.460, (1.409), (1.802) 1.538, 1.460, 1.802	0.667	Terpen-7
36.43	d	1.802 ax.	ddq	1.538, 1.430, 0.667	0.667, 2.790, 3.156	Terpen-8
15.42	q	0.668 eq.	d	1.802	1.802	Terpen-8-Me
97.93 97.91	s	-	-	-	3.154, 2.792, 0.972, 0.668	Terpen-9
41.71	s	-	-	-	3.156, 2.790, 0.977, (1.764), 2.035	Terpen-10
15.72	q	0.978 ax.	s	-	2.035, 1.763	Terpen-10-Me
31.62	t	3.154 2.792	d d	2.792 3.154	6.598	Terpen-11
116.92 116.90	s	-	-	-	3.154, 2.792, 6.597	Terpen-1′
153.60 153.62	s	-	-	-	6.597, 3.157, 2.792	Terpen-2′
OH		9.75	br		NOE : 6.598	Terpen-2′-OH
100.90 100.88	d	6.598 6.596	s	-	-	Terpen-3′
133.00 132.98	s	-	-	-	4.330	Terpen-4′
112.62 112.55	s	-	-	-	6.593, 4.330	Terpen-5′
155.73	s	-	-	-	(6.597), 3.154, 2.792, 4.330	Terpen-6′
168.11 168.07	s	-	-	-	6.597, 4.330, 4.892, 4.869	Terpen-7′
44.25	t	4.332	-	-	4.892, 4.869	Terpen-8′
53.30 53.62	d	4.892 4.869		2.162, 2.284 2.162, 2.284	2.162	Met^{1}-α
22.64 22.80	t t	2.182 2.307 2.19 2.31	ddt ddt ddt ddt	4.892, 2.788 2.632 4.869, 2.757, 2.635	4.892, 2.788, 2.632 4.869, 2.757, 2.635	Met^{1}-β
49.44 49.70	t t	2.682, 2.758 2.633, 2.783	ddt ddt ddt ddt	2.758, 2.162, 2.289 2.682, 2.162, 2.289 2.783, 2.289, 2.162 2.633, 2.289, 2.162	4.892, 2.550 4.869, 2.545	Met^{1}-γ
37.73 37.84	q	2.548 2.546	s	-	2.682, 2.758 2.633, 2.783	Met^{1}-ε
169.97	s	-	-	-	8.506, 4.869, 2.190	Met^{1}-CO

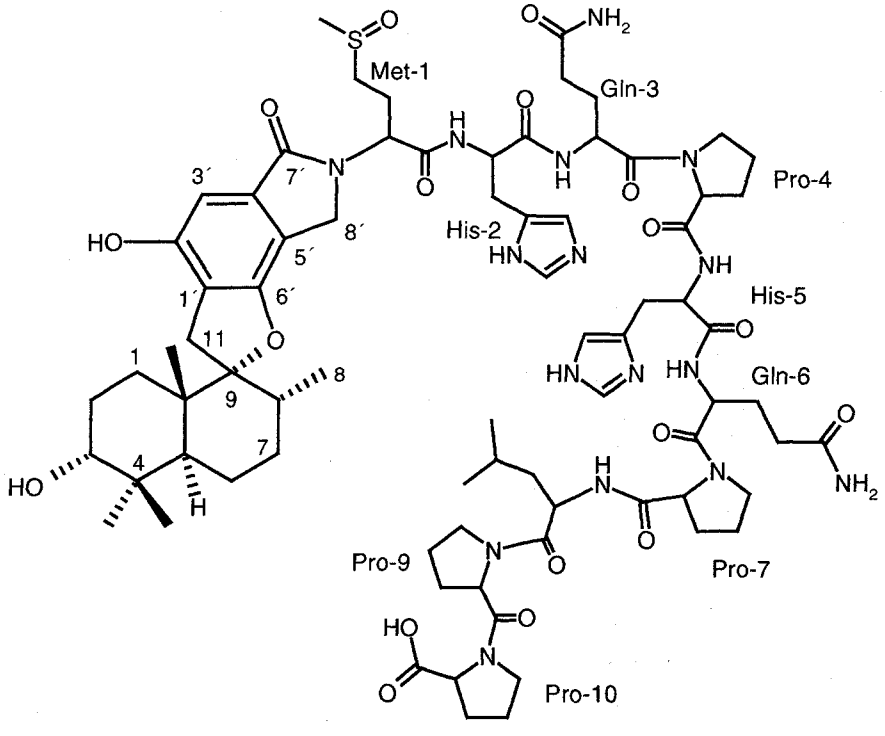
Table 1. (Continued)

δ-^{13}C	m	δ-^{1}H	m	$^{n}J_{HH}$	$^{n}J_{CH}$ (10 Hz)	assignment
169.89					8.496, 4.892, 2.182	
NH		8.506 8.496	d d	4.585 4.590	NOE: 4.869 /4.892	His^{2}-NH
51.68	d	4.590 4.585	ddd ddd	8.496, 2.934, 3.067 8.506, 2.934, 3.067	2.934, 3.067	His^{2}-α
27.09	t	2.937 3.067	dd dd	4.570	(4.570)	His^{2}-β
129.63	s	-	-	-	7.230, 3.064, 2.937, 4.592	His^{2}-γ
133.63	d	8.655	s	7.230	7.230	His^{2}-δ
116.95	d	7.230	s	8.655	(3.067), 2.937, 8.665	His^{2}-ε
169.74	s	-	-	-	8.207, 2.937, 4.585	His^{2}-CO
NH		8.206	d	4.484	NOE : 3.072, 2.948, 4.590 / 4.585	Gln^{3}-NH
50.31	d	4.484	dt	8.206, 1.935, 1.707	2.182	Gln^{3}-α
26.51	t	1.940 1.719	m m	1.719, 4.484, 2.198 1.719, 4.484, 2.198	2.198	Gln^{3}-β
30.65	t	2.192	t	1.940, 1.719	1.940, 1.719, 4.489, 6.789	Gln^{3}-γ
173.88 173.86	s	-	-	-	2.19, 1.93, 1.73	Gln^{3}-δ-CO
NH_2		7.230 6.789	s s	(6.789) (7.230)		Gln^{3}-NH_2
170.07	s	-	-	-	4.471, 1.733, 1.924, 4.356	Gln^{3}-CO
59.44	d	4.356	dd	2.031, 1.798	3.635, 1.880	Pro^{4}-α
28.86	t	1.798 2.031	m m	4.356, 2.031, (1.863)		Pro^{4}-β
24.32	t	1.863	m	2.031, 1.798, 3.625	4.356, 3.625, 2.153, 1.792	Pro^{4}-γ
46.87	t	3.644	m	1.863	-	Pro^{4}-δ
171.32	s	-	-	-	8.138, 4.356, 2.003, 1.805, 4.570	Pro^{4}-CO
NH		8.135	d	4.563		His^{5}-NH
51.37	d	4.570	ddd	8.135, 2.975, 3.082	2.975, 3.082	His^{5}-α
27.04 br	t	2.976 3.067	dd dd	4.598	-	His^{5}-β
129.32	s	-	-	-	8.783, 7.322, 3.083 2.976, 4.585	His^{5}-γ
133.68	d	8.771	s	7.320	7.320	His^{5}-δ
117.26	d	7.322	s	8.771	3.092, 3.000, 8.771	His^{5}-ε
169.59	s	-	-	-	8.110, (4.570)	His^{5}-CO
NH		8.110	d	4.470	NOE : 4.570, (4.466), 1.722, (1.927)	Gln^{6}-NH
50.18	d	4.466	dt	8.110, 1.927, 1.722	2.166	Gln^{6}-α
26.85	t	1.927 1.722	m m	1.722, 4.468, 2.166 1.927, 4.468, 2.166	2.166	Gln^{6}-β
30.64	t	2.166	t	1.927, 1.722	1.927, 1.722, 4.466, 6.807	Gln^{6}-γ
173.80 173.81	s	-	-	-	2.19, 1.93, 1.73	Gln^{6}-δ-CO
NH_2		7.260 6.807	s s	(6.807) (7.260)		Gln^{6}-NH_2
170.21	s	-	-	-	4.466, 1.722, 1.927, 4.388	Gln^{6}-CO
59.20	d	4.388	dd	2.012, 1.814	2.012	Pro^{7}-α
28.89	t	1.814 2.012	m m	2.012, 4.388, (1.927) 1.814, 4.388, (1.927)	4.388, 3.646	Pro^{7}-β
24.37	t	1.927	m	(2.153), (1.814), 3.624	4.388, 3.624, 1.814	Pro^{7}-γ
46.81	t	3.624	m	1.927	1.814, 4.388	Pro^{7}-δ
170.99	s	-	-	- -	4.534, 4.388, 2.012, 1.844, 7.918	Pro^{7}-CO
NH		7.918	d	4.534	NOE : 4.388, 1.827, 1.688, 1.427	Leu^{8}-NH
48.56	d	4.534	dt	7.918, 1.428	7.918, 1.428, 1.678	Leu^{8}-α

Table 1. (Continued)

δ-^{13}C	m	δ-^{1}H	m	$^{n}J_{HH}$	$^{n}J_{CH}$ (10 Hz)	assignment
40.07 40.03	t	1.428	dt	4.536, 1.678	0.890, 0.873, 4.536	Leu^{8}-β
23.88 br	d	1.678	m	0.87, 0.88		Leu^{8}-γ
23.08	q	0.890	d	1.678	1.678, 1.428, 0.873	Leu^{8}-δ′
21.43	q	0.873	d	1.678	1.678, 1.428, 0.890	Leu^{8}-δ
169.69	s	-	-	-	4.534, 1.444	Leu^{8}-CO
57.33	d	4.588	dd	2.144, 1.771	2.144, 1.771, 1.905, 1.945, 3.684	Pro^{9}-α
27.50	t	2.144 1.771	m m	1.771, 4.578,1.905, 1.945 2.144, 4.578,1.905, 1.945	1.945, 3.689, 3.467, 4.578	Pro^{9}-β
24.22	t	1.910	m	2.144, 4.588, 3.467	2.144, 1.771, 4.588, 3.467, 3.689	Pro^{9}-γ
46.53	t	3.689 3.467	m m	1.905, 1.936	4.588, 2.165, 1.781, 1.936	Pro^{9}-δ
169.57	s	-	-	-	4.588, (4.223)	Pro^{9}-CO
58.31	d	4.223	dd	2.144, 1.840	3.669, 3.553, 1.926, 2.144, (1.840)	Pro^{10}-α
28.34	t	2.144 1.840	m m	1.840, 4.223, 1.926 2.144, 4.223, 1.926	4.223, 3.669, 3.533	Pro^{10}-β
24.43	t	1.926	m	2.144, 1.840, 3.655, 3.538	4.224, 3.655, 3.538	Pro^{10}-γ
46.10	t	3.669 3.533		1.934	4.223, 2.147, 1.934, 1.851	Pro^{10}-δ
173.10	s	-	-	-	4.225, 2.144, 1.840	Pro^{10}-CO

Fig. 1. Structure of memnopeptide A (**1**).



and carbonyl resonances having prevented determination of the sequence by NMR methods. However, once the correct peptide sequence had been established in this way, the sequential assignment of all resonances was possible (Table 2).

An interesting feature is the *S*-oxide of methionine in position 1, initially identified by mass spectrometric analysis (see below). In the ^{13}C NMR spectrum this gives rise to remarkable chemical shift differences (C-γ: 49.44 ppm and C-ε: 37.73 ppm) as well as to diastereotopic splitting of neighboring resonances, the *S*-oxide being tetrahedral and thus stereogenic[19].

All MS/MS spectra show predominantly the formation of B-type and Y″ type ions (nomenclature according to ROEPSTORFF[20]).

The fragmentation of the pseudomolecular ion at *m/z* 1565.5 is dominated strongly by an unusual neutral loss of 64 Da to give an ion at *m/z* 1501. However, the MS^3 spectrum of *m/z* 1501 reveals the whole B-series from B_1 to B_{10}. In addition to the corresponding Y''_5 to Y''_9- fragments, this allows the sequence His-Gln-Pro-His-Gln-Pro-Leu-Pro-Pro to be deduced.

Table 3 lists all expected B-fragments, (B-64 Da)-fragments, and Y″-fragments. The fragments present in the MS^3 spectrum (Fig. 3) are shown in bold.

The terpenoid moiety of the memnopeptide A, which has been fully characterized by NMR, shows no significant mass spectrometric fragments. The neutral loss of 64 Da must therefore arise from the first amino acid, which cannot be cleaved because the *N*-terminus forms part of an imide structure.

Fig. 2. NOE effects (solid curved arrows) within the terpenoid moiety which establish the stereochemistry and ^1H-^{13}C long-range correlations (double arrows) which support the regioisomerism depicted in Fig. 1.

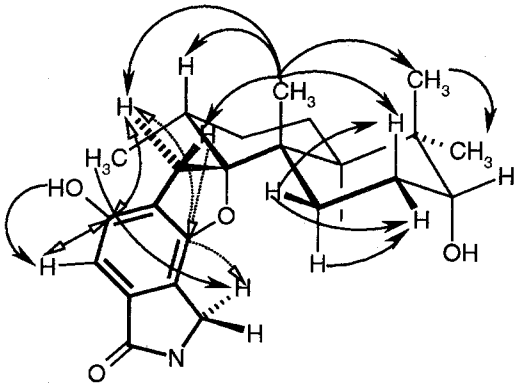


Table 3. Normal B-fragments of memnopeptide A in the first and second columns, (B-CH_4SO)- and Y″-fragments in the third column; observed ions are shown in bold.

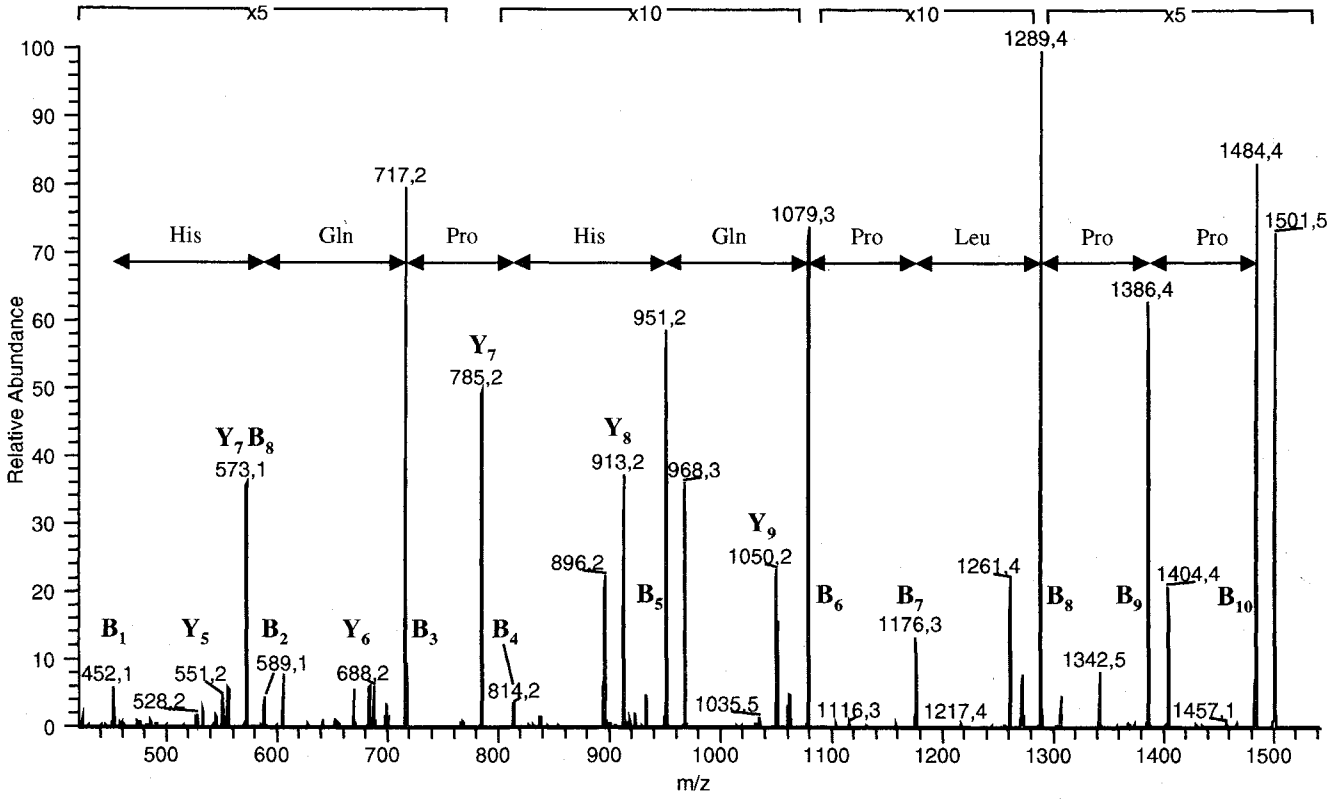
B_1	516,2	B_1	**452,2**	Y''_1	116,1
B_2	653,3	B_2	**589,3**	Y''_2	231,1
B_3	**781,4**	B_3	**717,4**	Y''_3	326,2
B_4	878,4	B_4	**814,4**	Y''_4	423,3
B_5	**1015,5**	B_5	**951,5**	Y''_5	**551,3**
B_6	**1143,5**	B_6	**1079,5**	Y''_6	**688,4**
B_7	**1240,6**	B_7	**1176,6**	Y''_7	**785,4**
B_8	**1353,6**	B_8	**1289,6**	Y''_8	**911,5**
B_9	**1450,7**	B_9	**1386,7**	Y''_9	**1048,5**
B_{10}	**1548,7**	B_{10}	**1484,7**	Y''_{10}	1197,6

Table 2. Sequence information as established by NMR correlations.

Sequence	Terpene	Met[1]	His[2]	Gln[3]	Pro[4]	His[5]	Gln[6]	Pro[7]	Leu[8]	Pro[9]	Pro[10]
δ NH	-		8.506 8.496	8.206	-	**8.135**	**8.110**	-	7.918	-	-
δ H_α	-	4.892 4.869	**4.590** **4.585**	4.484	4.356	4.570	4.466	4.388	4.534	**4.588**	4.223
δ CO	168.11	169.97 169.89	**169.74**	170.07	171.31	**169.59**	170.21	170.99	**169.69**	**169.57**	173.10
$^nJ_{CH}$ CO_i-NH_{i+1}											
CO_i-$C_\alpha H_{i+1}$											
NOE NH_i-$H_{\alpha i-1}$											
$H_{\alpha i}$-$H_{\alpha i-1}$											

ambiguous correlation: ⋯▸ unambiguous correlation: ⟶

Fig. 3. MS^3 spectrum of *m/z* 1501.5 (pseudomolecular ion after loss of CH_4SO).



High-resolution accurate mass measurements by fast atom bombardment (FAB-MS) were also performed, and gave a protonated molecular mass of 1565.7819, which is in good agreement with the theoretical mass of 1565.7827 calculated for $C_{76}H_{109}N_{16}O_{18}S$.

Deducting the masses of all known substructures from the measured pseudomolecular ion, we arrive at a mass of 91.0210 Da for the missing amino acid residue. The only reasonable composition that would account for this mass difference is C_3H_7SO. This can be rationalized by an oxidized methionine residue, which also explains the observed loss of 64 Da (as CH_4SO).

The final amino acid sequence therefore reads (ox)Met-His-Gln-Pro-His-Gln-Pro-Leu-Pro-Pro. On the basis of the above peptide sequence and from the phenylspirodrimane structure inferred from the NMR experiments, the structure of memnopeptide A is as shown in Fig. 1.

A comparison of the determined amino acid sequence Met-His-Gln-Pro-His-Gln-Pro-Leu-Pro-Pro in memnopeptide A (**1**) with those of published peptides and proteins confirms a 100% homology with a subsequence of β-casein[21] [BLAST[22] search on NBCI[23] portal for available protein sequences]. No similarly close agreement with other protein sequences was observed.

The *N*-terminus of the decapeptide in memnopeptide A is linked to the phenylspirodrimane subunit in the form of a lactam. The unusual properties of the peptide are a consequence not only of the blocking of the *N*-terminus by the sesquiterpenylphenyl moiety, but also of the oxidation of the methionine to methionine oxide.

Biological Properties

Memnopeptide A is a weak inhibitor of G-6-P-translocase[11], with an IC_{50} of 78 μM. Surprisingly, **1** shows antibacterial activity against Gram-positive bacteria, including resistant forms, and activity against other pathogenic fungal species was not observed. The determined minimum inhibitory concentrations are listed in Table 4.

Memnopeptide A was also found to cause activation of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase[24] (EC 3.6.1.38, Ca^{2+}-transporting ATPase). The contraction process of the heart muscle involves shortening of the

Table 4. Inhibitory effect of memnopeptide A (**1**) on Gram-positive bacteria (μg/ml).

Strain	Resistant against	MIC (μg/ml)
Staphylococcus aureus SG 511	-	16
Staphylococcus aureus 285	penicillin	16
Staphylococcus aureus 503	penicillin	16
Staphylococcus aureus FH 1982	methicillin	16
Staphylococcus aureus 701E	methicillin	16
Staphylococcus aureus 707E	methicillin	16
Staphylococcus aureus 9 Tüb	ofloxacin	16
Staph. epidermidis ZH 2c	-	16
Staph. epidermidis 763	methicillin	> 16
Staph. epidermidis 5747IIW	methicillin	32
Staph. epidermidis 291	ofloxacin	8
Staph. epidermidis 799	ofloxacin	8
Enterococcus faecium Md8B	-	8
Enterococcus faecium VR1	vancomycin	> 64
Enterococcus faecium VR2	vancomycin	> 64
Streptococcus pyogenes VR3	vancomycin	> 64
Streptococcus pyogenes 308A	-	8
Streptococcus pyogenes 77A	-	4

Table 5. Stimulation of SERCA2 activity in the presence of memnopeptide A; Ca^{2+} serves as control.

Memnopeptide A (μM)	SERCA 2 Activation (%)
50	- 152
25	- 140
12.5	- 103
6.25	- 48
3.1	- 32
1.6	- 35
0.8	0
Ca^{2+} (μM)	SERCA 2 Activation (%)
0.3	0
3.0	- 200

myofibrils. This is initiated by motor nerve impulses, which within a few milliseconds trigger an influx of extracellular calcium ions (Ca^{2+}) into the sarcoplasmic space, emptying the calcium depots. In myocardial insufficiency (heart failure), Ca^{2+} concentration in the myofibrils is reduced. Ca^{2+} ions are, however, essential for the activation of the

contractile apparatus. Increased demand causes Ca^{2+} to be pumped into the sarcoplasmic reticulum in a process mediated by the membrane-bound Ca^{2+}-dependent enzyme Mg^{2+}-ATPase[25], also termed sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA2). In the unphosphorylated state SERCA2 is inhibited by phospholamban. Under conditions of physiological stress, phosphorylation of phospholamban occurs, resulting in an increase in the affinity of SERCA2 for Ca^{2+}, and hence in an increase in the rate of transportation of Ca^{2+} ions into the sarcoplasmic reticulum. SERCA2 activators would therefore be expected to have a beneficial effect in heart failure. Semi-maximal activation of SERCA2 is achieved at a memnopeptide A (**1**) concentration of 12.5 μM (Table 5). The molecular mechanism of this activation is not known.

Discussion

In a number of previous publications[1~10] *Memnoniella echinata* and *Stachybotrys* species have been reported to form a large number of sesquiterpene polyketides with structures containing—in addition to the terpene subunit—substituted aromatic *ortho*-dialdehydes, 2-hydroxy-methylbenzaldehydes, and benzoic acids, the latter normally being present in the lactone form as phthalides, *i.e.* as 1(3H)-isobenzofuranones.

Some of these earlier papers reported the detection, in cultures of *Memnoniella echinata* and *Stachybotrys* species, of phenylspirodrimane derivatives[5,8~10] in which the nitrogen atoms of amino acids and ethanolamine are incorporated in the form of a γ-lactam (dihydroisoindolone) structure. The new memnopeptide A is likewise a member of this sesquiterpene isoindolone series. The detection of the phenylspirodrimane lactam **1** containing the noteworthy peptide subunit only present in β-casein suggests that the new natural product is not a secondary metabolite, but a metabolic product formed from a sesquiterpenyl 2-hydroxy-methylbenzaldehyde or sesquiterpenyl 2-hydroxy-methylbenzoic acid and a peptide from the nutrient medium. This proposal fits with the structures of the phenylspirodrimane lactams previously described, which can similarly be regarded as simple reaction products of the same phenylspirodrimane derivatives with amino acids or ethanolamine. The sesquiterpenyl 2-hydroxy-methylbenzaldehydes, sesquiterpenyl phthalides, and numerous regioisomers that have been detected could thus be serving as convenient vehicles for the binding, and hence sequestration, of easily digestible amines. The diverse, but not very specific effects observed for the phenylspirodrimane derivatives so far known suggest that their importance to the producing organism has yet to be recognized. The benefit to the fungus of safeguarding supplies of vital nitrogen building blocks would represent one plausible explanation of their function. The multitude of sesquiterpene derivatives and their metabolic products with a diversity of nitrogen building blocks from the nutrient media could account for the observed complex composition of *Memnoniella* and *Stachybotrys* cultures.

A parallel with the discussed sesquiterpene polyketides is seen with the azaphilones[26]. Azaphilones, for example sclerotiorin[27], are yellow pigments obtained from *Penicillium* spp. and other various fungal species. These compounds contain isochromane rings as a characteristic common structural element and generate red reaction products with amines[26], even at ambient temperature. A diversity of relatively weak biological effects has likewise been reported for the azaphilones, though a structure-activity relationship remains unclear, with the exception of a high reactivity with lysine[28]. The obvious inference here too is that azaphilones, like the sesquiterpene polyketides, perform the function of nitrogen sequestration for the generator organism.

Further experiments are necessary for unambiguous clarification of the genesis of memnopeptide A and other memnopeptides. Biological studies to establish the physiological significance of sesquiterpenyl phenols are still required.

Materials and Methods

General

Quantitative ultraviolet absorption spectra were recorded using a Cary 118 B spectrophotometer (Varian, Darmstadt, Germany); for all other purposes, including the performance of HPLC analyses, Hewlett-Packard series 1100 equipment fitted with diode array detectors was used. Preparative HPLC was carried out using Pharmacia equipment (Uppsala, Sweden).

Fermentation

Memnopeptide A and other terpene derivatives were produced by fermentation in a 10 liter Braun Fermentation stainless steel stirred vessel. 300 ml conical flasks containing 100 ml of seed medium were inoculated with frozen vegetative mycelium of *Memnoniella echinata* at a concentration of 1%. The seed medium consisted of 20 g/liter malt, 2 g/liter yeast extract, 10 g/liter glucose, and

0.5 g/liter $(NH_4)_2HPO_4$. The seed flasks were incubated for 120 hours at 25°C on a rotary shaking machine at 140 rpm. The fermenter was charged with 8 liters of a medium consisting of 10 g/liter glucose, 5 g/liter casein peptone, 1.7 g/liter of cornsteep liquor, and traces of KCl, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, and $MnSO_4 \cdot H_2O$. The nutrient solution was sterilized in the fermenter at 121°C for 1 hour. The fermenter was inoculated with the seed flask culture at a concentration of 1%. The temperature during fermentation was maintained at 28°C. The stirring rate was 80 rpm and the air flow rate was 1 v/v/minute. Antifoam (Desmophen) was added initially at a concentration of 0.01%. The fermentation was terminated after 44 hours.

Isolation

At the end of the fermentation of *Memnoniella echinata* FH 2272, the culture solution from the fermenter (200 liters) was filtered with the addition of approx. 2% filtration aid (*e.g.* Celite®) and the cell mass (22 liters) was extracted with 66 liters methanol. The methanolic solution containing the desired products was filtered from the mycelium and concentrated under vacuum. The concentrate was diluted with water and loaded together with the culture filtrate (180 liters) onto a prepared MCI gel, CHP20P column (capacity 17 liters), which was eluted with a gradient of water to 60% 2-propanol in water. The eluate was collected in 10 liters fractions at a flow rate of 25 liters/hour and the memnopeptide-containing fractions (from 25 to 30% 2-propanol) were pooled. The fractions eluted with 45~50% 2-propanol were likewise pooled. Concentration of the memnopeptide fractions under vacuum yielded 20 liters of a brown solution. The 20 liters of concentrate was loaded onto a column (125×500 mm) packed with 6 liters of a cation exchange resin (Fractogel® EMD SO_3^-) equilibrated with pH 7 potassium-phosphate buffer. The column was eluted with a gradient of 10 mM pH 7 potassium-phosphate buffer to 1 M NaCl in 10 mM pH 7 potassium-phosphate buffer in methanol - water (1 : 1), with the eluate collected in 1 liter fractions at a flow rate of 12 liters/hour. Memnopeptide A was eluted from the column with 0.75 M NaCl (fractions 31 and 32). These fractions were pooled and concentrated under vacuum to approximately 500 ml. This salt-containing enriched solution of memnopeptide A was chromatographed on a 500 ml Nucleosil® 100-7 C_{18}AB column (Macherey & Nagel, Düren, Germany), which was eluted at a flow rate of 50 ml/minute with a gradient of 25~50% acetonitrile in 0.05% trifluoroacetic acid/water. The eluate was collected in 50 ml fractions, with memnopeptide A eluting in fractions 71~88. Repeated purification of the pooled fractions at a constant solvent concentration of 28% acetonitrile in 0.05% trifluoroacetic acid gave, after freeze drying, >95% of pure memnopeptide A (140 mg).

The purity of the product was investigated using the following HPLC system:

Eluent: 0.1% trifluoroacetic acid in 32% acetonitrile.

Column: Nucleosil $100C_{18}$AB 5 μ, 250/4, Macherey-Nagel, Düren, Germany.

Flow rate: 1.0 ml/minute.

Detection: ultraviolet absorbance at 210 nm.

Under the above conditions, memnopeptide A has a retention time of 7 minutes.

NMR Spectroscopy

All spectra were recorded on a Bruker DRX 500 spectrometer operating at 500 MHz for ^1H. The data were processed on an indigo2 station (Silicon Graphics) using Bruker XWINNMR software. The sample contained 8 mg of the compound in 450 μl Me_2SO-d_6. The temperature was set to 310 K for all measurements. The following spectra were recorded for **1**:

^1H and ^{13}C spectra; heteronuclear single-quantum correlation spectroscopy (HSQC)[12]; nuclear Overhauser effect spectroscopy (NOESY)[13]; heteronuclear multiple-bond correlation (HMBC)[14], optimized for a coupling constant of 4 Hz and 8 Hz respectively; as well as folded for carbonyl-region, optimized for coupling constants of 4 Hz and 8 Hz; double quantum filtered homonuclear correlated spectroscopy (COSY)[15]; total correlated spectroscopy (TOCSY)[16,17]. Mixing times of 100 and 200 msec were used for the NOESY spectra and 30 msec and 100 msec for the TOCSY spectra.

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 a 40 V potential was applied. The conversion dynode was set at 15 kV, the electron multiplier at 1.0 kV, and the spray voltage at 5.0 kV.

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

For the MS/MS analysis, a 2 μ wide window was used for isolation of the precursor. A relative excitation energy of 35% was applied to dissociate the precursor ions (MS/MS). The daughter ions were unit resolved across the

scan range. Three microscans were acquired over the mass range of 420~2000, 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 2 μl/minute. The sample 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.

The high-resolution fast atom bombardment (FAB) measurements were recorded on a VG ZAB 2-SEQ instrument (Micromass).

Sarco(endo)plasmic Reticulum Ca^{2+}-ATPase (SERCA2) Assay

The assay was performed in the 96-well microtiter plate format as a single-point determination. The final assay volume was 100 μl and the final extract dilution was 1 : 50.

Frozen sarcoplasmic reticulum microsomes from canine heart were thawed at ambient temperature and centrifuged for 10 minutes at 4400 *g*. The supernatant was diluted with buffer 1 (25 mM MOPS-K, 120 mM KCl, 2 mM $MgCl_2$, pH 7.0) to a concentration of 0.4 mg protein/ml.

In a typical assay, 15 μl (6 μg protein) of the microsomal preparation was preincubated with 10 μl of the microbiological extract (prediluted 1 : 5) for 15 minutes at 37°C. SERCA2 activity was induced by adding 75 μl of buffer 2 (buffer 1 plus 2 mM ATP, 5 mM NaN_3, 0.5 mM EGTA, 1.5 μM Ionophore A 23187, and 0.3 μM free Ca^{2+}). After incubating at 37°C for 10 minutes the reaction was stopped by adding 25 μl ascorbic acid (10%, pH 5.0) and 100 μl ammonium molybdate (15 mM)/zinc acetate (100 mM) solution. The color reaction, which is based on the formation of an ammonium molybdate π-complex, was measured spectrophotometrically in a microtiter plate reader (Dynatech) at 620 nm after incubating for 15 minutes at ambient temperature.

To exclude false positives caused by nonspecific effects of phosphate or Ca^{2+} in the crude extracts, blank plates without microsomes (phosphate blanks) and blank plates without Ca^{2+} were run in parallel to the assay plates.

The stimulating effect of the extract samples was calculated according to the following equation (negative values indicate activation):

$$\text{Activation} = [OD_{620\,nm}\,(\text{sample}) - OD_{620\,nm}\,(\text{low control, } 0.3\ \mu\text{M } Ca^{2+})] / [OD_{620\,nm}\,(\text{high control, } 3\ \mu\text{M } Ca^{2+}) - OD_{620\,nm}\,(\text{low control, } 0.3\ \mu\text{M } Ca^{2+})] \times (-200)\%$$

Maximum activation [200% over low enzyme control (+0.3 μM Ca^{2+})] was achieved by adding 3 μM free Ca^{2+}.

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