Synthesis and Biological Evaluation of Analogues of the Peptaibol Ampullosporin A

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A series of analogues of the fungal peptaibol type metabolite ampullosporin A containing modifications in the C and N terminus as well as α-aminoisobutyric acid (Aib) substitutions in different positions of the peptide were synthesized by solid phase synthesis using the 9-fluorenylmethyloxycarbonyl strategy. Depending on the sequence position, couplings were performed with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole and tetramethylfluoroformamidinium hexafluorophosphate, respectively. The structures of the target peptides were analyzed by electrospray ionization mass spectrometry and chromatographic methods (high-performance liquid chromatography, thin-layer chromatography). The biological activities of these compounds have been evaluated by assaying their potencies for the induction of pigment formation on the fungus *Phoma destructiva* as well as for the induction of hypothermia and inhibition of locomotoric activity in mice and were compared to the naturally occurring ampullosporins. Native ampullosporin A and analogues with C-terminal Leu or Leu-NH₂ showed comparable activity in the pigmentation assay. Similarly, the ampullosporin A analogues with N-terminal aromatic amino acid residues, such as D-Trp and Tic, also have high potency for pigment formation. The peptides containing structural modifications of ampullosporin A by systematic replacement of Aib by Ala (Ala scan) displayed moderate or high activity in the pigmentation assay, whereas simultaneous substitution of all Aib residues by Ala and Ile, respectively, or by insertion of nonaromatic residues into position 1 resulted in a loss of the effect on *P. destructiva*. Most of the compounds with no or weak activity in the microbial assay were not active in the hypothermic test, too, except the compound with 1-amino-1-cyclohexane carboxylic acid in position 4 instead of Aib. However, only a few compounds with high potency for pigmentation induction were found to produce strong hypothermia in mice. Thus, in contrast to the native ampullosporins, we succeeded to a certain degree in differentiation of the bioactivities with our synthetic analogues.

Introduction

A variety of microbial metabolites are known to influence cytodifferentiation and morphogenesis of its producer or other microbes, e.g., by triggering different cellular processes or by acting as regulators in signal cascades. These properties have been widely used to identify new pharmacologically interesting substances in the course of screening peptides from fungal cultures.[1]

The phytopathogenic fungi *Phoma destructiva* has been shown to respond to the presence of different antibiotics, such as cyclosporin A[2] and the peptaibols chrysospermin A−D[3], by the formation of a brownish pigment, whereas other peptaibols, like alamethicin[4] and bergofungin,[5] showed no morphogenic effect on this fungal strain.[3,10] In the search for similar inducers of pigment formation, ampullosporin A (**1**) has been discovered as a new peptaibol type antibiotic displaying

moderate narrow spectrum antibacterial and antifungal activity. This peptide was the first of a series of isomers and analogues (ampullosporins A−E) isolated from the mycelium of *Sepedonium ampullosporum* HKI-0053 (DSM 10602).[1,6] With the high proportion of the nonproteinogenic amino acid Aib, an N-terminal acetyl group, and the presence of a C-terminal amino alcohol (Leuol), ampullosporin A belongs to the class of peptaibol antibiotics.[7]

In the above-described assay, employing *P. destructiva* ampullosporin A was more active in comparison to the chrysospermins A−D. To establish correlations of the effect on *P. destructiva* to pharmacological activities of drugs, it was found that known nonpeptide, commercial antipsychotics such as chlorpromazine (**2**), haloperidol (**3**), clozapine (**4**), and sertindole (**5**) are active in the same manner as cyclosporin A and ampullosporin A (Figure 1).[1,10]

Because many compounds differing in their chemical constitution possess identical or similar pharmacological effects, it became interesting to investigate vice versa the antipsychotic effect of ampullosporin A.[1,10] The proposed neuroleptic potential has thus been further

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(1) Ac-Trp-Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Gln-Leu-Aib-Gln-Leuol
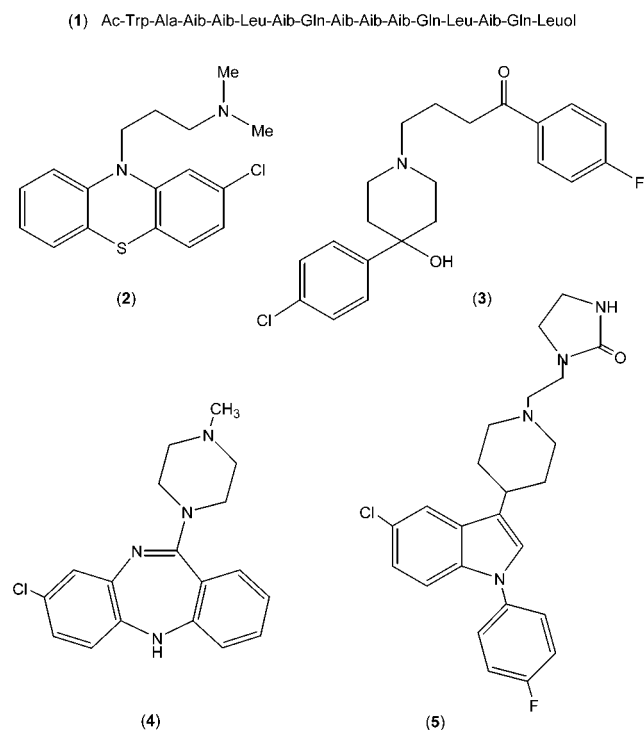
Figure 1. Structures of different inducers of pigment formation on *P. destructiva*: (**1**) ampullosporin A, (**2**) chlorpromazine, (**3**) haloperidol, (**4**) clozapine, and (**5**) sertindole.

evaluated in terms of its ability to inhibit locomotoric activity and to produce hypothermia in mice.

Hypothermia is an effect that is found for most neuroleptic drugs, such as chlorpromazine and clozapine, and is used among other tests to identify new antipsychotics.[8,9,11] Besides the described effect on *P. destructiva*, ampullosporin A was also found to induce hypothermia in mice.[1] Further investigations using specific behavioral tests have been undertaken to confirm the assumption of a neuroleptic activity of ampullosporin A. It was found that the peptide reduces locomotor functions in rats while it produces only low catalepsy.[10] These results supported the hypothesis that ampullosporin A has atypical neuroleptic activity.[1,6,10]

Further investigations concerning the mechanisms underlying the described effects as well as structure–activity relationship studies of ampullosporin A would be facilitated by the availability of related structures. Moreover, analogues of ampullosporin A may also represent interesting targets for the development of new antipsychotic drugs.

The present paper is focused on the efficient synthesis and characterization of ampullosporin A and analogues containing modifications in the N- and C-terminal region of the native peptide as well as substitutions of Aib in different sequence positions. We also report on the biological activities using the pigment formation assay of the fungus *P. destructiva* and the effects on reflex actions, locomotoric activity, and body temperature in mice. In a further paper, we will also report the conformational studies using circular dichroism to compare conformation and biological activity.

Results and Discussion

Peptide Structures and Syntheses. First, we were interested in finding an optimized synthetic strategy for the efficient preparation of ampullosporin A. We decided to use Fmoc strategy for the assembly of the peptides on the solid support.

Ampullosporin A like other naturally occurring peptaibols terminates in the form of an amino alcohol. For the first step, with respect to a convenient synthesis protocol, we preferred the method of Wenschuh et al.,[12] which is based on the direct anchoring of Fmoc-amino alcohols onto the solid support, e.g., 2-chlorotrityl chloride resin. Using the recommended mixture of dichloromethane (DCM) and dimethylformamide (DMF) as solvent and pyridine as base, we obtained loadings of about 0.4 mmol g^{-1}. This preloaded resin was used for the stepwise automated assembly of the peptide sequence.

The 15 peptide ampullosporin A contains, among the 15 amino acids, seven Aib residues with three of them occurring as adjacent units. Aib is known to cause difficulties during coupling in solid phase synthesis due to the much lower reactivity of this sterically hindered α,α-dialkyl amino acid as compared to that of usual α-amino acids. As reported for the synthesis of another difficult sequence, the peptaibol alamethicin,[4] the insertion of Aib is improved using the highly reactive amino acid halides, especially amino acid fluorides, instead of the bulky active esters formed from typical coupling agents, such as HBTU or PyBOP.[13] With this method, alamethicin could be obtained in high yields and good purity.[14] The disadvantage of the use of amino acid fluorides, although easy to obtain with cyanuryl fluoride[15] or (diethylamino)sulfur trifluoride (DAST),[16] is that one additional synthetic step is required for the preparation of these compounds prior to the coupling onto the solid phase. To avoid this, the onium reagent TFFH has been introduced as an useful alternative for the in situ generation of amino acid fluorides without the need of isolation and purification of the reactive intermediate.[17]

TFFH, which converts the amino acid into the reactive fluoride after a short preactivation time (12 min), was also reported to be suited for automated solid phase synthesis. Therefore, we also tested this coupling agent in comparison to conventional reagents, such as HBTU. These test syntheses of ampullosporin A have been carried out manually. Each step in the process was examined by the UV spectrophotometric determination of the Fmoc–piperidine adduct in order to evaluate the efficiency of the different coupling methods. Best results were obtained for the amino acid fluoride and TFFH couplings (yields are comparable), whereas the coupling yields for HBTU/HOBt dramatically decreased after the sixth coupling of Aib in position 9 onto Aib in position 10 (Figure 2).

These results prompted us to use HBTU/HOBt for the first five coupling steps and TFFH from position 9 as shown in Scheme 1.

This synthetic strategy was applied to all ampullosporin A analogues summarized in Table 1 with the exception of compounds **18** and **19** for which only HBTU/HOBt was used for all couplings in combination with "magic mixture" as the solvent. An appropriate resin was chosen for peptides terminating as carboxylic acid (Wang resin) and as amide (Rink amide MBHA resin), respectively.
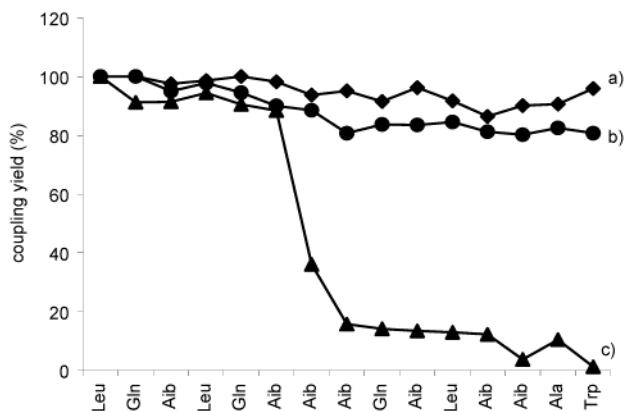
Figure 2. Monitoring of coupling yields obtained for the assembly of ampullosporin A on 2-chlorotrityl resin using (a) amino acid fluorides, (b) TFFH, and (c) HBTU.

Scheme 1. Synthetic Strategy for the Solid Phase Synthesis of the Aib-Containing Peptide Ampullosporin A
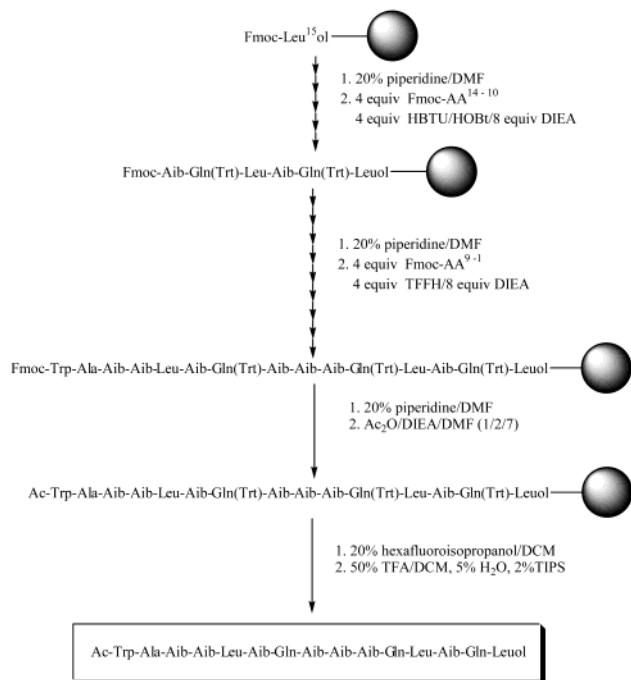
Our strategy for the first series of ampullosporin A analogues was to find structural features that are important for the biological activities of the peptide and for differentiation between these activities. For a better understanding of the roles of the N-terminal acetyl group, the Trp residue in position 1, the C-terminal Leuol, and the seven Aib residues, we synthesized analogues with modifications in these parts of the molecule (see Table 1).

Biological Assays. The peptides were tested for their in vitro and in vivo biological activity using the *P. destructiva* microbial assay and the induction of hypothermia, influence on reflex actions, and depression of locomotoric activity in mice. Each peptide was assayed three times or more. The biological results are presented in Table 2. The antipsychotic experiments with rats will be published separately.

Microbial Assay. In preceding studies, it was demonstrated that the fungus *P. destructiva* responds to the

presence of neuroleptics such as clozapine, chlorpromazine, sertindole, and haloperidol by the formation of brownish pigment halos (see Figure 3).[1,10] This indication is now used in a microbial assay for the screening of natural products, and it led to the disclosure of the peptaibol ampullosporin A, which showed a rather strong effect [++] in this test (see Table 2). It was therefore suggested that this peptide may have neuroleptic activity. The other naturally occurring ampullosporins (see Table 1) have similar (B, D) or lower (C) activity as compared to ampullosporin A.[6]

In the design of ampullosporin A analogues, our first intention was to find out which structural modifications of the N- and C-terminal amino acid in the sequence are accepted with respect to activity on *P. destructiva*. We found that the analogues containing Leu (**1**) or Leu-amide (**2**) in the C terminus were equally potent as compared to ampullosporin A, which contains an amino alcohol (Leuol) in this position. In the search for smaller active fragments, we also synthesized [des-Leu[15],Gln[14]]-ampullosporin A (**3**), which showed no effect in the microbial assay. Regarding the N terminus, we replaced the Trp residue by D-Trp and other aromatic amino acids such as Tic, Nal, Igl, and Nig. Only the analogues containing D-Trp (**7**) and Tic (**8**) had similar activity as compared to the native peptide, whereas the analogues with Nal, Igl, and Nig were found to have only about 50% of the activity of ampullosporin A (referred to as weak effect [+], see Table 2). These observations led us to speculate about the relative importance of an aromatic amino acid residue that is structurally related to Trp in position 1. To test this hypothesis, we also synthesized compound **9**, in which Trp is substituted by Oic and the analogues **5** and **6** lacking the N-terminal Trp. As was found for the C-terminal shortened peptide (**3**) also, the [des-Trp[1]]- and [des-Ac-Trp[1]]ampullosporin A showed no effect in the microbial assay. Interestingly, the same was observed for the Oic analogue. These findings suggest that only minor structural modifications in the N- and C-terminal region of the peptide are tolerated; hence, an aromatic Trp-related amino acid in position 1 is important for activity.

To find further structural requirements for inducing the effect on *P. destructiva* and also to evaluate the effect of the Aib-Ala replacements occurring in the natural ampullosporins B (Ala[8]), C (Ala[9]), and D (Ala[10]) as compared to ampullosporin A (Aib[8]-Aib[9]-Aib[10]), we decided to replace the remaining Aib residues by Ala, too. Thus, we synthesized compounds **13−16** as well as two analogues in which all Aib residues were replaced by Ala (**18**) and Ile (**19**), respectively. The biological results for these analogues differ strongly as do those for the natural ampullosporins. Whereas the ampullosporins A, B, and D and the analogues **13−16** showed a strong effect, ampullosporin C has only weak activity in the pigmentation assay. We cannot, however, explain the differences of this single Aib substitution on the biological activity, yet. Interestingly, replacing all Aib residues with Ala and Ile, respectively, led to a complete loss of the potency for inducing pigment formation.

Hypothermic Effect. Most of the neuroleptics that are in clinical use decrease the body temperature (hypothermia) and inhibit locomotoric activity; both

Table 1. Synthesized Ampullosporin A Analogues

peptide no.	modification	sequence
AmpA	native	Ac-Trp[1]-Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
1	[Leu[15]]ampullosporin A	Ac-Trp[1]-Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-**Leu[15]**
2	[Leu[15]-NH₂]ampullosporin A	Ac-Trp[1]-Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-**Leu[15]-NH₂**
3	[des-Leu[15], Gln[14]]ampullosporin A	Ac-Trp[1]-Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-**Gln[14]**
4	[des-Ac]ampullosporin A	**H**-Trp[1]-Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
5	[des-Trp[1]]ampullosporin A	**Ac**−**Ala[2]**-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
6	[des-Ac-Trp[1]]ampullosporin A	**H**-Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
7	[D-Trp[1]]ampullosporin A	Ac-**D-Trp[1]**−Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
8	[Tic[1]]ampullosporin A	Ac-**Tic[1]**−Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
9	[Oic[1]]ampullosporin A	Ac-**Oic[1]**−Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
10	[2-Nal[1]]ampullosporin A	Ac-**2-Nal[1]**−Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
11	[Igl[1]]ampullosporin A	Ac-**Igl[1]**−Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
12	[Nig[1]]ampullosporin A	Ac-**Nig[1]**−Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
13	[Ala[3]]ampullosporin A	Ac-Trp[1]-Ala[2]-**Ala[3]**-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
14	[Ala[4]]ampullosporin A	Ac-Trp[1]-Ala[2]-Aib[3]-**Ala[4]**-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
15	[Ala[6]]ampullosporin A	Ac-Trp[1]-Ala[2]-Aib[3]-Aib[4]-Leu[5]-**Ala[6]**-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
AmpB	native	Ac-Trp[1]-Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-**Ala[8]**-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
AmpC	native	Ac-Trp[1]-Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-**Ala[9]**-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
AmpD	native	Ac-Trp[1]-Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-**Ala[10]**-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
16	[Ala[13]]ampullosporin A	Ac-Trp[1]-Ala[2]-Aib[3]-Aib[4]-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-**Ala[13]**-Gln[14]-Leu[15]ol
17	[Ac₆c[4]]ampullosporin A	Ac-Trp[1]-Ala[2]-Aib[3]-**Ac₆c[4]**-Leu[5]-Aib[6]-Gln[7]-Aib[8]-Aib[9]-Aib[10]-Gln[11]-Leu[12]-Aib[13]-Gln[14]-Leu[15]ol
18	[(Ala)₇]ampullosporin A	Ac-Trp[1]-Ala[2]-**Ala[3]**-**Ala[4]**-Leu[5]-**Ala[6]**-Gln[7]-**Ala[8]**-**Ala[9]**-**Ala[10]**-Gln[11]-Leu[12]-**Ala[13]**-Gln[14]-Leu[15]ol
19	[(Ile)₇]ampullosporin A	Ac-Trp[1]-Ala[2]-**Ile[3]**-**Ile[4]**-Leu[5]-**Ile[6]**-Gln[7]-**Ile[8]**-**Ile[9]**-**Ile[10]**-Gln[11]-Leu[12]-**Ile[13]**-Gln[14]-Leu[15]ol

effects occur as an undesirable side effect of these drugs.[18−20] Some of these neuroleptics induce pigment formation in *P. destructiva*. It was supposed that there may be a correlation between these two effects.[1,10] Because ampullosporin A also produces hypothermia in mice,[1,10] we tested our analogues for their ability to modify the core body temperature of mice (Figure 4).

For most of the analogues, the degree of hypothermia induced was found to be correlated with the influence of the locomotoric activity of the mice. Moreover, by comparing the results with those of ampullosporin A, it is obvious that most analogues having high or moderate activity in the microbial assay induce hypothermia in mice, too.

For example, strong hypothermia was caused by compounds **2**, **13**, **14**, **16**, and **17**, and a moderate effect was found for **1** and **12**. Also, the natural ampullosporins B−D produce hypothermia. Ampullosporin B and D displayed a strong effect, but ampullosporin C evoked only a moderate effect. Among the synthetic analogues, **13**, **14**, and **17** increased the altitude and the duration of the hypothermic response significantly already 1 h

after injection, whereas for peptide **2**, hypothermia occurred only 3 h after injection. For these compounds, a decrease in temperature was still evident after at least 7 h, while peptide **16** decreased body temperature only for a short time period. Structurally, there are only minor differences between these analogues and ampullosporin A. For example, compound **2** contains Leu-NH₂ instead of Leuol in the carboxy terminus. With the exception of peptide **15** and ampullosporin C, the substitution of different Aib residues in positions 3, 4, 8, 10, and 13, respectively, by Ala obviously does not affect the biological activity as compared to ampullosporin A. Interestingly, incorporating 1-amino-1-cyclohexane carboxylic acid (Ac₆c) in position 4 instead of Aib resulted in a compound that showed a weak effect on *P. destructiva* but strong hypothermia. This finding may be due to the reduced conformational flexibility of peptide **17** as compared to ampullosporin A and peptide **14** containing Aib and Ala, respectively, in the considered position.

Concomitantly, the three shortened compounds (**3**, **5**, and **6**) as well as the [Oic[1]]-ampullosporin A

Table 2. Analytical Characterization and Biological Acitivities of Synthesized Ampullosporin A Analogues

peptide	$t_{R1}{}^a$ (min)	$t_{R2}{}^a$ (min)	$[M + Na]^{+b}$	$R_{f1}{}^c$	$R_{f2}{}^c$	pigment induction^d	hypothermic effect^e
AmpA	42.0	12.8	1645.1	0.55	0.68	++	++
1	41.8	12.1	1659.9	0.49	0.51	++	+
2	41.3	12.9	1658.5	0.50	0.62	++	++
3	32.5	10.6	1545.4	0.46	0.48	na	na
4	30.3	10.3	1602.7	0.46	0.64	++	na
5	35.5	11.6	1459.0	0.48	0.62	na	na
6	27.7	10.3	1418.1	0.37	0.58	n.a.	na
7	42.0	12.8	1645.1	0.55	0.70	++	+
8	44.5	13.5	1617.9	0.54	0.66	++	na
9	45.7	14.0	1602.7	0.50	0.63	na	na
10	46.6	13.7	1656.1	0.55	0.65	+	na
11	46.3	13.7	1632.1	0.53	0.63	+	na
12	46.7	13.8	1632.1	0.51	0.66	+	+
13	43.5	13.1	1631.0	0.48	0.61	++	++
14	42.1	12.7	1631.3	0.48	0.62	++	++
15	40.7	12.4	1632.1	0.52	0.63	++	na
AmpB	39.8	12.1	1630.9	0.55	0.67	++	++
AmpC	40.8	12.4	1631.2	0.55	0.67	+	+
AmpD	41.1	12.5	1630.9	0.55	0.71	++	++
16	39.9	12.2	1647.2 $[M + K]^+$	0.53	0.65	++	++
17	47.3	14.0	1685.6	0.60	0.69	+	++
18	31.7	10.7	1546.7	0.43	0.54	na	na
19^f			1857.8 $[M + K]^+$	0.82	0.83	na	na

^{a} HPLC retention times according to systems 1 and 2, purity >97% for both systems. ^{b} Molecular weight confirmed by ESI-MS. ^{c} TLC values according to systems 1 and 2. ^{d} Pigment induction capacity evaluated based on the *P. destructiva* halo color: strong effect (++), moderate effect (+), no activity (na). ^{e} Induction of hypothermia: strong effect (++), moderate effect (+), no activity (na). ^{f} HPLC analysis impossible due to very poor solubility; purity proved by TLC and MALDI-TOF MS.
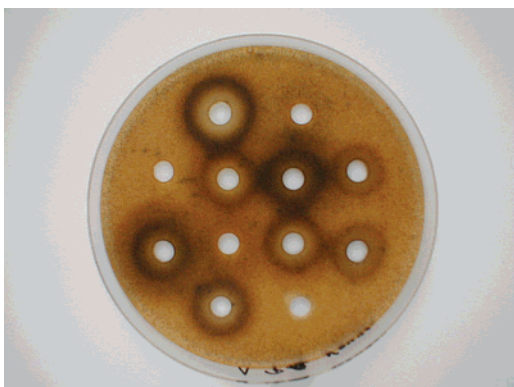
Figure 3. Induction of pigment formation on *P. destructiva* by ampullosporin A analogues.
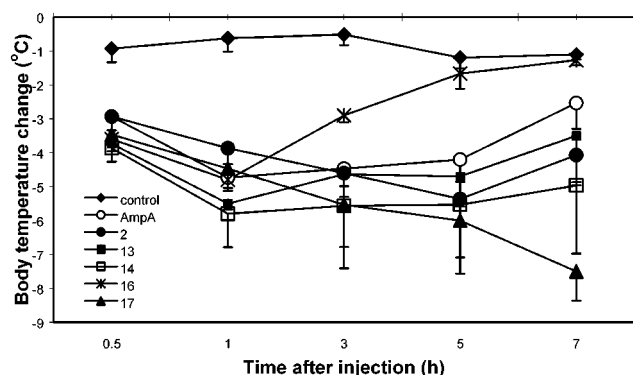
Figure 4. Hypothermic effect of selected ampullosporin A analogues.

analogue (**9**), which did not induce pigment formation on *P. destructiva*, showed no hypothermic effect in mice. Analogues **10**, **11**, **18**, and **19**, which showed no hypothermic response, were not active (**18** and **19**) or showed weak activity (**10** and **11**) on *P. destructiva*.

The most interesting results were obtained for compounds **4**, **7**, **8**, and **15**, which showed high potency for the pigment formation but no effect in the in vivo test. We think that these analogues and the active analogues (**2**, **13**, **14**, **16**, and **17**) may be good candidates for additional behavioral experiments.

Biodegradation Studies. Degradation of active peptides may limit their duration of action and can also change the pharmacological profiles.[21] Thus, we checked the biostability of ampullosporin A to find out whether the N-terminal Trp residue or other amino acid residues are enzymatically cleaved. Porcine liver and kidney homogenates were used to evaluate the in vitro stability against the soluble and membrane-bound peptidases present in these preparations. Additionally, analogue **18** was examined for its stability against proteolytic degradation, since this analogue does not contain nonproteinogenic Aib residues.

The proteolytic activity of the homogenates was estimated by a modified azocasein test,[22] and the protein concentration of the homogenate probes used for the incubation was determined by the Lowry method.[23] The enzymatic degradation of ampullosporin A and analogue **18** has been determined by incubating the peptides with homogenates at 37 °C and pH 6.5 for 5 min to 24 h. The process was followed by analyzing the remaining peptide concentration by high-performance liquid chromatography (HPLC). In addition, we tested the resistance to proteolytic degradation of ampullosporin A with purchaseable enzymes. The tests were performed with pepsin, chymotrypsin, and pronase. The incubation was executed as mentioned above. In the case of pepsin, we worked with pH 2.0. Tests with the other proteases were performed at their pH optimum.

The natural ampullosporin A turned out to be highly resistant to proteolytic degradation, at least for 24 h, whereas analogue **18** was slowly degraded by porcine liver and kidney homogenates. After 3 h, about 60% of

the peptide **18** was cleaved to fragments with reduced HPLC retention times.

From the comparison of ampullosporin A to alanine-containing analogue **18**, it seems reasonable to suggest that the Aib residues stabilize the peptide against proteolytic cleavage. Additionally, the N-terminal acetyl group and the C-terminal amino alcohol prevent the cleavage by amino- and carboxypeptidases, respectively.

Conclusions

Ampullosporin A, a peptaibol type antibiotic with neuroleptic activity, was the subject of our chemical modifications. Because of its difficult amino acid sequence containing several Aib residues, the challenge was to develop an optimized synthetic route and to synthesize the first series of analogues in order to obtain molecules with high activity and selectivity. We succeeded in the syntheses of the native compound and the analogues by using TFFH as a coupling reagent.

Replacements of N-terminal Trp by noncoded amino acids with similar chemical properties led in most cases to a loss of the hypothermic response, with the exception of the Nig[1] analogue for which a moderate effect was found. However, only analogues containing an aromatic amino acid in the Trp position displayed high activity in the pigmentation assay on *P. destructiva.* Analogues with modification of the C-terminal Leuol[15] showed similar or moderate biological activities as compared to the native peptide, whereas the 14 peptide terminating with Gln was inactive in both assays. Systematic replacement of Aib by Ala resulted in compounds with very high or at least medium biological activity for pigment induction. Most of these analogues also showed a very strong hypothermic effect in vivo. This was true except for compounds with Aib substitution in positions 6 (Ala) and 4 (1-amino-1-cyclohexane carboxylic acid), respectively, for which a selectivity between both assays was found. The results suggest that there are different modes of action of ampullosporin analogues as inducers of fungal pigment formation (*P. destructiva* assay) and hypothermia (neurolepsy) in rodents. Because nothing is known about the molecular mechanism of action of ampullosporin A, synthetic analogues with selective biological activities may be valuable tools for further investigations. Studies are now in progress to correlate the physicochemical properties of ampullosporin A analogues, such as helix formation in solutions and crystals, and interactions with artificial membranes with the observed biological activities.

Experimental Section

Materials. Solid phase peptide synthesis (SPPS) was performed on an automated peptide synthesizer ECOSYN P (Eppendorf Biotronik, Germany) using 2-chlorotrityl chloride resin (Advanced ChemTec Europe, Bamberg, Germany), Rink amide MBHA resin (Novabiochem, Bad Soden, Germany), and Wang resin (RAPP Polymere, Tübingen, Germany), respectively. All chemicals, unless otherwise stated, were purchased from Fluka (Sigma-Aldrich Chemie, Seelze, Germany). Amino acid derivatives and coupling reagents were purchased from Advanced ChemTech Europe (Bamberg, Germany) and Novabiochem (Bad Soden, Germany); TFFH was purchased from PE Biosystems (Weiterstadt, Germany). All materials and solvents were of reagent grade and were used without further purification with the following exceptions: DMF was first dried over molecular sieves and distilled from phthalic anhydride,

and DCM was stored over molecular sieves. Thin-layer chromatography (TLC) was performed on precoated glass plates (silica gel 60 F$_{254}$, Merck, Darmstadt, Germany) with (i) *n*-butanol/acetic acid/water (48/18/24) and (ii) *n*-propanol/25% ammonia (7/3) as the mobile phase. TLC detection was accomplished with UV light, ninhydrin reagent, and Cl$_2$/tolidine solution. The crude peptides were purified by semi-preparative reversed-phase HPLC on a Shimadzu LC-8A equipped with a Knauer C8 (5 μm particle size, 100 Å pore size, 32 mm $\times$ 250 mm) using an elution gradient from 50 to 100% solvent B over 120 min at a flow rate of 10 mL/min, where A was 0.1% TFA in water and B 0.1% TFA in 90% acetonitrile. Detection was accomplished at 220 nm. Pure fractions were identified by two diverse analytical HPLC systems on (i) a Shimadzu LC-10AT chromatograph with a Vydac 218TP column (5 μm particle size, 300 Å pore size, 4.6 mm $\times$ 250 mm), gradient 20−90% B in 70 min at a flow rate of 1.0 mL/min, A 0.1% TFA in water and B 0.1% TFA in acetonitrile, with detection at 220 nm; and (ii) with a Grom C18 column (5 μm particle size, 100 Å pore size, 3 mm $\times$ 125 mm), gradient 0.5−99.5% B in 16 min with SPD-M10AVP Diode array detector. The peptides were also analyzed by ESI-MS on a triple quadrupole instrument Quattro (Fisons Biotech VG, Altrincham, England).

Peptide Synthesis. General Procedure for the Preparation of Ampullosporin A, Ampullosporin C, and Analogues 5 and 7−17. Ampullosporin analogues were synthesized by the Fmoc strategy. The coupling of the first amino acid was carried out manually. Fmoc-Leuol (3 equiv) was attached directly onto 2-chlorotrityl resin (1.5 mmol/g) with a binary mixture of DMF and DCM and 6 equiv of pyridine for 6 h. The peptide chains were elongated either manually or automatically on a peptide synthesizer ECOSYN P (Eppendorf Biotronik). Fmoc-protected amino acid (4 equiv) was activated by a combination of 4 equiv of HOBt, 4 equiv of HBTU, and 8 equiv of DIEA for the first five amino acids. Four equivalents of TFFH and 8 equiv of DIEA were used for the activation from position 9, with a preactivation time of 12 min. The N terminus was acetylated with acetic anhydride/DIEA/DMF (1/2/7) for 30 min. The peptide alcohol was cleaved from 2-chlorotrityl resin with 20% hexafluoro-2-propanol in DCM for 30 min, and subsequent cleavage of side chain protecting groups using 50% TFA in DCM, 5% water, and 2% triisopropylsilane was performed for 1 h. The crude peptides were precipitated in cold diethyl ether, centrifuged, dissolved in 80% *tert*-butanol, and lyophilized. The yields of crude and purified peptides (75 and 30−35%, respectively) were determined as the ratios between the obtained and the theoretical amounts of peptides; the latter were calculated using the loading of the first amino acid on the solid support. Purity of the peptides was evaluated by TLC in two solvent systems and by two diverse analytical HPLC systems (see Table 2 for details). Amino acid analyses were performed on an Eppendorf-Biotronik LC 3000 amino acid analyzer and gave acceptable results for all peptides.

Analogues **4** and **6** were synthesized using the general procedure for the preparation of ampullosporin A but without the acetylation step. Analogues **18** and **19** were synthesized using the general procedure for the preparation of ampullosporin A, but only HBTU (4 equiv) in combination with HOBt (4 equiv) as a coupling reagent and "magic mixture" as solvent were used. Analogues **1** and **3** were synthesized using the general procedure for the preparation of ampullosporin A, except that Wang resin was used. The peptides with free carboxylic acid were cleaved with TFA, 2.5% water, and 2.5% ethandithiole for 2 h. Analogue **2** was synthesized using the general procedure for the preparation of ampullosporin A, except that Rink amide MBHA resin was used. The peptide amide was cleaved with TFA, 2.5% water, and 2.5% ethandithiole for 2 h. Ampullosporin B and ampullosporin D were obtained from *S. ampullosporum* HKI-0053. Extraction, purification, and characterization were reported elsewhere.[6]

Biological Activity Studies. Induction of Pigment Formation. The induction of pigment formation on *P. de-*

structiva was determined as described in more detail elsewhere.[1,3,6,10] Two different concentrations of peptide dissolved in methanol (50 μg/50 μL and 100 μg/50 μL) were added to agar wells. After a time of 48–72 h of inoculation, the appearing brownish halos were analyzed.

Induction of Hypothermia. The hypothermic effect of the ampullosporin A analogues was determined according to BANSINATH.[24] One milligram of peptide was dissolved in 0.1 mL of 98% ethanol and diluted with 0.9 mL of saline. A dose of 20 mg/kg b.w. (body weight) was injected intraperitoneally into mice (strain NMRI, Central Breeding Laboratory for Animals, Beutenberg Campus, Jena, Germany). The rectal body temperature was measured by a thermistor (Checktemp 1) and recorded 5 min, 0.5, 1, 3, 5, 7, and 24 h after administration.

Locomotoric Activity. Behaviorial changes in mice were also controlled within 24 h after administration. Neuroleptic potential is recognizable by depression of locomotoric activity, inhibition of reflex action, and reduction of muscle tone. This observational screening procedure depends on the experience of the experimenter.

Enzymatic Stability. The proteolytic stability of the ampullosporin A and analogue **18** against proteases of porcine liver and kidney was measured according to the literature.[25] A 1.5 mg amount of peptide was dissolved in 50 μL of DMSO and 450 μL of phosphate buffer (pH 6.5) and incubated with 500 μL of the homogenate at 37 °C. Samples of 100 μL were taken off at 5 min, 0.5, 1, 3, 7, and 24 h and, after several workup procedures, prepared for HPLC analysis. The proteolytic activity of porcine liver homogenate determined by degradation of 0.2% azocasein was 0.42 U/mL. The protein concentration of a homogenate probe determined by the Lowry method was 12.9 mg/mL.

The proteolytic stability of ampullosporin A against purchaseable enzymes was carried out as recommended from the producer. To dissolve ampullosporin A, the solution was prepared with 10% of DMSO. Chymotrypsin was purchased from Sigma Aldrich Chemie GmbH (Deisenhofen, Germany), and pronase and pepsin were from Boehringer Mannheim (Mannheim, Germany).

Abbreviations Used

Ac$_6$c, 1-amino-1-cyclohexane-carboxylic acid; DIEA, *N*,*N*-diisopropylethylamine; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; Igl, indanyl glycine; MBHA, (4-methylbenzhydrylamino)resin; 2-Nal, β-(2-naphthyl)-alanine; Nig, N-indanyl glycine; Oic, octahydro-1H-indole-2-carboxylic acid; PyBOP, benzotriazol-1-yl-oxytris-pyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TFFH, tetramethylfluoroformamidinium hexafluorophosphate; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.

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