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A Novel β-Amino Acid in Cytotoxic Peptides from the Cyanobacterium *Tychonema* sp.

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The cyclic dodecapeptides tychonamide A (**1**) and B (**2**) isolated from the methanolic extract of the cyanobacterium *Tychonema* sp. contain the novel β-amino acid 3-amino-2,5,7-trihydroxy-8-phenyloctanoic acid (Atpoa). Compounds **1** and **2** have cytotoxic activity towards cancer cell lines in a monolayer assay with mean IC_{50} values of 0.9 and 3.3 $\mu g\,mL^{-1}$, respectively. Compound **2** was shown to be active against tumor cell suspensions derived from solid tumor xenografts in a clonogenic assay (mean IC_{50} 2.4 $\mu g\,mL^{-1}$). Additionally, antiprotozoal activity was observed (*Trypanosoma b. rhodesiense*, IC_{50} 0.1 $\mu g\,mL^{-1}$) for compound **1**.

Introduction

Cyanobacteria are the source of a diverse array of compounds of pharmaceutical interest. A specific feature of their secondary metabolite spectrum is the occurrence of cyclic peptides containing unusual amino acids, such as β-amino acids.[1] Previously, we isolated three new cyclic hexapeptides with MptpB (*Mycobacterium tuberculosis* proteine tyrosine phosphatase B) inhibitory activity: the brunsvicamides A–C from the cyanobacterium *Tychonema* sp. These peptides are related to the sponge-derived mozamides and therefore support the suggestion that secondary metabolites of certain marine invertebrates are produced by associated microorganisms.[2]

In the current study, the cytotoxic fraction of the same cyanobacterial extract was investigated and yielded cyclic dodecapeptides **1** and **2** (tychonamide A, B). They are structurally related to some other cyclic peptides, for example, schizotrin A, isolated from the cyanobacterium *Schizotrix* sp.[3] and pahayokolide A from *Lyngbya* sp.[4] Tychonamide A and B contain the novel β-amino acid 3-amino-2,5,7-trihydroxy-8-phenyloctanoic acid (Atpoa). The compounds are thus further members of the β-amino acid containing peptides, such as microcystins,[5] nodularins,[6] nostophycin,[7] scytonemin A,[8] calophycin,[9] cryptophycin,[10] puwainaphycins,[11] schizotrin A,[3] and pahayokolide A and B.[4] Tychonamide A and B exhibit cytotoxic activity towards several cancer cell lines (mean IC_{50} value of 0.9 and 3.3 $\mu g\,mL^{-1}$, respectively). Tychonamide B inhibited anchorage independent growth and in vitro colony formation of tumor cells in a concentration-dependent and tumor-type selective manner (mean IC_{50} value of 2.4 $\mu g\,mL^{-1}$). Additionally, antiprotozoal activity was observed (*Trypanosoma b. rhodesiense*, IC_{50} value of 0.1 $\mu g\,mL^{-1}$) for tychonamide A.

Results

The cyanobacterial strain *Tychonema* sp. was collected from a pond of a sugar factory (waste water) near Braunschweig (Germany). After cultivation in a photobioreactor, cells were lyophylized and subsequently extracted with dichloromethane, ethyl acetate, and methanol. The methanol extract was dissolved in water and repeatedly extracted with diethyl ether and butanol. The latter phase was purified by repeated preparative reverse-phase HPLC to obtain compounds **1** and **2** as white amorphous materials.

Compound **1** showed a positive ESI quasimolecular ion signal at m/z = 1486.8 $[M + H]^+$. Its molecular formula, $C_{73}H_{107}N_{13}O_{20}$, is based on HRFTICR mass measurements (calcd. 743.8950; observed 743.8951 for $[M + 2\,H]^{2+}$). Signals in the ^1H NMR spectra were very broad, which is indicative of the presence of different conformers in solution. NMR spectra were thus measured at 75 °C and

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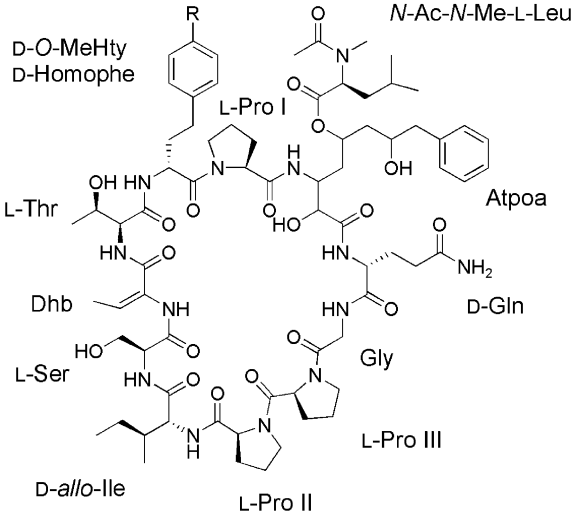
showed characteristic resonances for α-CH groups between δ = 3.9–5.0 ppm and exchangeable NH resonances between δ = 7.3–9.2 ppm, which provides evidence for the peptidic nature of compound **1** (Scheme 1). Resonance signals between δ = 6.8–7.3 ppm evidenced the presence of aromatic amino acids. In addition, the ^{1}H NMR spectrum featured three sharp signals at δ = 1.96, 2.80, and 3.71 ppm for an *N*-acetyl group (72-CH_3), an *N*-methyl group (73-CH_3), and a -OCH_3 (30-CH_3) functionality, respectively. The ^{13}C NMR spectrum revealed signals for 9 methyl, 20 methylene, 26 methine, and 18 quaternary carbon atoms.



Scheme 1. Compounds **1** and **2**.

From the fragmentation pattern obtained from a MALDI-TOF MS–MS experiment, the immonium ions ($^+NH_2$=CH–R) of three amino acids could be detected in the low-mass region: Pro at *m*/*z* = 70 (19%), Ile at *m*/*z* = 86 (2%), and MeHty (*O*-methylhomotyrosine) at *m*/*z* = 164 (4%). After assignment of all protons to their directly bonded carbon atoms by ^{1}H–^{13}C HSQC, it was possible to establish all amino acid residues by ^{1}H–^{1}H COSY, HMBC, HSQC–TOCSY, and TOCSY experiments and to deduce fragments A–C (Figure 1). Proline was present in triplicate as indicated by a HSQC–TOCSY spectrum that exhibited three almost-identical spin systems with typical ^{1}H and ^{13}C NMR shifts and 2D correlations in the HMBC spectrum for this amino acid.

^{1}H–^{1}H COSY correlations and HSQC–TOCSY data allowed us to deduce the presence of a spin system beginning with 2-CH (δ_C = 71.9 ppm and δ_H = 3.91 ppm) and continuing through to 8-CH_2 (δ_C = 43.6 ppm and δ_H = 2.63, 2.68 ppm). The HMBC correlations between 10/11-H (δ_H = 7.18 ppm) and C-8 led us conclude that an aromatic moiety was connected to this long aliphatic chain. On the basis of the chemical shifts, one nitrogen-bearing methine carbon atom, that is, C-3 (δ = 48.1 ppm), and three oxygen-bearing carbon atoms, that is, C-2 (δ_C = 71.9 ppm), C-5 (δ_C = 69.4 ppm), and C-7 (δ_C = 67.5 ppm), were identified. Furthermore, HMBC correlations from 8-H_2 (δ_H = 2.63, 2.68 ppm) to C-7 (δ_C = 67.5 ppm) and to C-6 (δ_C = 41.5 ppm) and NOESY correlations from 5-H (δ_H = 5.03 ppm) to the neighboring CH_2 groups (6-CH_2 with δ_C = 41.5 ppm and δ_H = 1.58 ppm; 4-CH_2 with δ_C = 35.2 ppm and δ_H = 1.79 ppm) and to 3-CH (δ_C = 48.1 ppm and δ_H = 4.04 ppm), as well as from 3-H to 2-H (δ_H = 3.91 ppm) were indicative for the novel β-amino acid Atpoa (3-amino-2,5,7-trihydroxy-8-phenyloctanoic acid, Figure 1). The second amino acid in fragment A could easily be determined by COSY, HMBC, and TOCSY experiments as *N*-acetyl-*N*-methylleucine. The *N*-methyl group 73-CH_3 (δ_C = 31.7 ppm and δ_H = 2.80 ppm) possessed, besides its correlation to C-66 (δ_C = 53.9 ppm), a correlation to the carbonyl group at

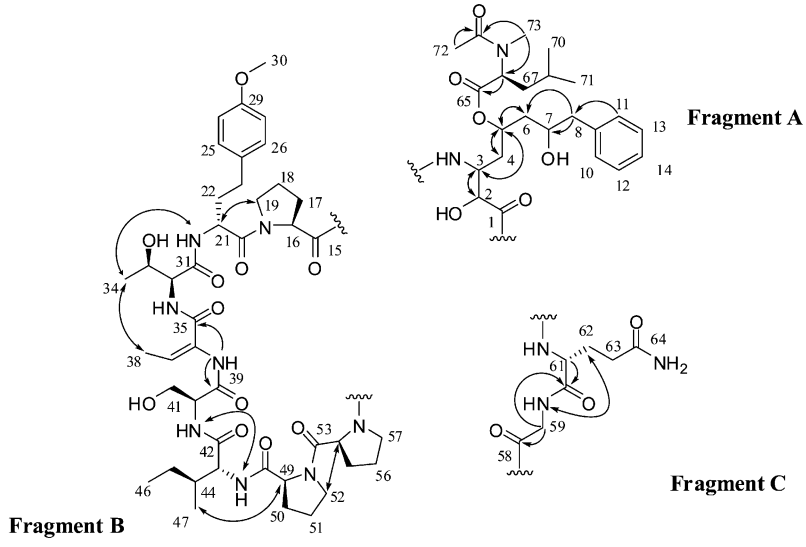


Figure 1. Structure of fragments A–C and selected HMBC and NOE correlations for compound **1** (single-headed arrow = HMBC; double-headed arrow = NOESY).

C-71 (δ_C = 170.0 ppm), which is part of an acetyl residue. A very prominent fragment at *m/z* = 1299 (62%) in the MALDI-TOF MS–MS experiment suggested the loss of the *N*-acetyl-*N*-methylleucine (M_r = 187.2) from **1** by elimination, which led to the assumption that *N*-acetyl-*N*-methylleucine is attached through an ester linkage and probably forms an exocyclic side chain.[4,8] The characteristic ^{1}H NMR shift of 5-H (δ_H = 5.03 ppm) of Atpoa was a strong indication for the presence of an acyl moiety at this position. It was thus concluded that *N*-acetyl-*N*-methylleucine is attached to C-5 (δ_C = 69.4 ppm) through an ester bridge. Because of the overlapping ^{1}H NMR signals of 5-H and 66-H (δ_H = 5.03 and 5.00 ppm, respectively) an HMBC correlation between 5-H and C-65 (δ_C = 170.4 ppm) could not be detected unambiguously. However, all experimental data and comparison with literature data[3,4,8] led us to conclude that *N*-acetyl-*N*-methylleucine is bound through an ester functionality to C-5 of the β-amino acid Atpoa, which gives rise to fragment A.

^{1}H and ^{13}C NMR spectroscopic data indicated a second aromatic moiety, which consisted of a 1,4-disubstituted benzene ring as shown by characteristic resonances for 25/26-H (δ_H = 7.10 ppm) and 27/28-H (δ_H = 6.80 ppm). In the HMBC experiment, a strong correlation between 25/26-H and C-23 (δ_C = 29.9 ppm), which is part of the 21-CH, 22-CH_2, and 23-CH_2 spin system, was detectable. An HMBC correlation between 30-H_3 (δ_H = 3.71 ppm) and C-29 (δ_C = 157.3 ppm) gave proof for a methoxy group to be connected to C-29. Thus, this aromatic amino acid could be determined as *O*-methylhomotyrosine (MeHty). The 19-H_2 (δ_H = 3.35 ppm) proton of proline (I) showed an NOE correlation to 21-H (δ_H = 4.31 ppm) of MeHty, which made clear that both amino acids were attached to each other as illustrated in Figure 1. The spin system 32-CH, 33-CH, and 34-CH_3, with its characteristic shift of the β-33-CH group (δ_H = 4.15 ppm and δ_C = 65.9 ppm), belongs to a threonine residue. The protons 37-H (δ_H = 5.69 ppm) and 38-H_3 (δ_H = 1.88 ppm) possessed an HMBC correlation to C-36 (δ_C = 129.8 ppm). In addition, 37-H showed an HMBC correlation to the carbonyl functionality at C-35 (δ_C = 163.9 ppm). Thus, the unusual amino acid Dhb (dehydrobutyrine) was assigned. The methyl group of threonine (34-H_3, δ_H = 1.11 ppm) had NOE correlations to the NH functionality of *O*-methylhomotyrosine (21-NH, δ_H = 7.92 ppm) and to the methyl group of Dhb (38-H, δ_H = 1.88 ppm). This way, the fragment could be extended in the N-terminal direction to form the short sequence -Dhb-Thr-MeHty-Pro(I)-. The α-40-CH (δ_C = 56.3 ppm and δ_H = 4.25 ppm) proton showed HMBC correlations to the oxygen atom bearing C-41 (δ_C = 61.3 ppm) and to the carbonyl functionality at C-39 (δ_C = 169.5 ppm), which is indicative of a serine residue. The 36-NH resonating at δ = 9.14 ppm showed clear HMBC correlations to both the carbonyl carbon atoms of serine (C-39, δ_C = 169.5 ppm) and Dhb (C-35, δ_C = 163.9 ppm), in which the intensity of the correlation between 36-NH and C-39 was much stronger. Thus, it was obvious that serine was attached to Dhb as shown in Figure 1. The 44-H (δ_H = 2.12 ppm) proton showed ^{1}H–^{1}H-COSY correlations to 43-H (δ_H = 4.38 ppm), 45-H_2 (δ_H = 1.19, 1.25 ppm), and 47-H_3 (δ_H = 0.83 ppm). The ^{1}H–^{1}H-COSY correlation between 45-H_2 and 46-H_3 (δ_H = 0.84 ppm) completed the isoleucine residue. The NH functionality of serine (40-NH, δ_H = 7.87 ppm) had an NOE correlation to the NH group of isoleucine (43-NH, δ_H = 8.27 ppm). Finally, 47-H_3 (δ_H = 0.83 ppm) showed an NOE correlation to the α-CH of proline (II) (49-H, δ_H = 4.36 ppm), and 52-H_2 (δ_H = 3.52, 3.83 ppm) of this proline residue showed an NOE correlation to the α-CH of proline (III) (54-H, δ_H = 4.61 ppm). These correlations connected serine to isoleucine, which was extended by two proline residues and completed fragment B (Figure 1).

Fragment C finally consisted of two further amino acids. The 59-CH_2 (δ_C = 40.7 ppm and δ_H = 3.85, 3.93 ppm) methylene group had no ^{1}H–^{1}H-COSY correlations and no correlations in the HSQC–TOCSY spectrum. An HMBC correlation between 59-H_2 and the carbonyl group at C-58 (δ_C = 166.2 ppm) clearly delineated the amino acid glycine. The 62-H_2 (δ_H = 1.81, 1.97 ppm) protons offered ^{1}H–^{1}H-COSY correlations to 61-H (δ_H = 4.26 ppm) and 63-H_2 (δ_H = 2.10, 2.14 ppm). The 63-H_2 protons had HMBC correlations to the carbonyl group at C-64 (δ_C = 173.7 ppm), whereas 61-H exhibited an HMBC correlation to the carbonyl functionality at C-60 (δ_C = 170.6 ppm), which provided evidence for the glutamine residue. A short sequence consisting of glutamine and glycine could be assigned through the heteronuclear long-range correlations between the CH_2 group of glycine (C-59, δ_C = 40.7 ppm and δ_H = 3.85, 3.93 ppm) and the carbonyl functionalities of both glycine (C-58, δ_C = 166.2 ppm) and glutamine (C-60, δ_C = 170.6 ppm). NOE correlations between 62-H_2 (δ_H = 1.81, 1.97 ppm) and the NH group of glycine (59-NH, δ_H = 7.99 ppm) confirmed the connection of these two amino acids and secured fragment C (Figure 1).

Connection of the three fragments was achieved by analyzing MALDI-TOF MS–MS measurements. Two amino acid sequences, that is, -Pro-Pro-Ile-Ser-Dhb-Thr-MeHty (*m/z* = 770) and -Pro-(Atpoa-AcMeLeu)-Gln-Gly- (*m/z* = 717), were identified as outlined in Table 1. Both of these sequences start with a proline residue at the N-termini, which is in accordance with the proposal of Schilling et al.[12] that states that cyclic and proline-containing peptides favorably break in such a manner that the proline residue is positioned at the N-terminated end of the resulting linear peptide. Both sequences taken together give the linear peptide -Pro-(Atpoa-AcMeLeu)-Gln-Gly-Pro-Pro-Ile-Ser-Dhb-Thr-MeHty-. The only possibility to get a cyclic structure from this linear peptide was the connection of the carboxyl group of glycine and the amine group of proline (III). The presence of further fragment ions (see Table 1), for example, a fragment for -Gly-Pro-Pro- (*m/z* = 252), confirmed this proposal. Thus, the planar structure of compound **1** was completed.

For the assignment of the absolute configuration by chiral GC–MS and chiral HPLC, the unusual amino acid *O*-methylhomotyrosine was synthesized in its D and L enantiomers as described previously by Yamada et al.[13] This two-

Table 1. MALDI-TOF-PSD fragment ions of **1**.

Fragment	*m/z* (%)[b]
[M[a] – 187 (AcMeLeu)]	1299 (62)
[M – 191 (MeHty)]	1295 (10)
[M – 101 (Thr) – 191 (MeHty)]	1194 (100)
[M – 83 (Dhb) – 101 (Thr) – 191 (MeHty)]	1111 (19)
[M – 87 (Ser) – 83 (Dhb) – 101 (Thr) – 191 (MeHty)]	1024 (22)
[83 (Dhb) +101 (Thr) + 191 (MeHty) + 97 (Pro) + H]$^+$	473 (4)
[97 (Pro) + 434 (Atpoa-AcMeLeu) + 128 (Gln) + 57 (Gly) + H]$^+$	717 (4)
[97 (Pro) + 434 (Atpoa-AcMeLeu) + 128 (Gln) + H]$^+$	660 (17)
[97 (Pro) + 434 (Atpoa-AcMeLeu) + H]$^+$	532 (1)
[434 (Atpoa-AcMeLeu) + 128 (Gln) + H]$^+$	563 (1)
[57 (Gly) + 97 (Pro) + 97 (Pro) + 113 (Ile) + 87 (Ser) + 83 (Dhb) + 101 (Thr) + 191 (MeHty) + 97 (Pro) + H]$^+$	924 (7)
[97 (Pro) + 97 (Pro) + 113 (Ile) + 87 (Ser) + 83 (Dhb) + 101 (Thr) + 191 (MeHty) + H]$^+$	770 (19)
[97 (Pro) + 97 (Pro) + 113 (Ile) + 87 (Ser) + 83 (Dhb) + 101 (Thr) + H]$^+$	579 (4)
[97 (Pro) + 97 (Pro) + 113 (Ile) + 87 (Ser) + 83 (Dhb) + H]$^+$	478 (20)
[97 (Pro) + 97 (Pro) + 113 (Ile) + 87 (Ser) + H]$^+$	395 (16)
[97 (Pro) + 97 (Pro) + 113 (Ile) H]$^+$	308 (3)
[97 (Pro) + 97 (Pro) + H]$^+$	195 (3)
[97 (Pro) + 113 (Ile) + 87 (Ser) + 83 (Dhb) + H]$^+$	381 (2)
[97 (Pro) + 113 (Ile) + 87 (Ser) + H]$^+$	298 (2)
[97 (Pro) + 113 (Ile) +H]$^+$	211 (3)
[128 (Gln) + 57 (Gly) +H]$^+$	186 (1)
[57 (Gly) + 97 (Pro) + 97 (Pro) + H]$^+$	252 (4)
[57 (Gly) + 97 (Pro) + H]$^+$	155 (8)

[a] M = [1485 + H]$^+$. [b] Values were rounded down.

step procedure involved diastereoselective aza-Michael addition of enantiopure 1-phenylethylamine to the commercially available 3-(4-methoxybenzoyl)acrylic acid, followed by hydrogenolysis of the carbon–heteroatom bonds in the benzylic positions (for details, see Supporting Information). The absolute configuration of the amino acids was determined as: L-serine, L-threonine, D-glutamine, D-*O*-methylhomotyrosine, L-proline, and D-*allo*-isoleucine. D/L-*N*-methylleucine resulting from *N*-acetyl-*N*-methylleucine, which is deacetylated during hydrolysis, could not be separated by chiral GC–MS. Thus, we determined the absolute configuration by chiral HPLC and showed that the L isomer is part of compound **1**. A strong NOE correlation between the NH group (36-NH, δ_H = 9.14 ppm) and the olefinic proton of Dhb (37-H, δ_H = 5.69 ppm) indicated the *E* configuration for the $\Delta^{36,37}$ double bond. Thus, with the exception of the four chiral centers in Atpoa, the structure was defined. The determination of the configuration of Atpoa would include several derivatization steps and excessive NMR spectroscopic analysis.[7,8] These investigations were beyond the scope of this study but represent issues of future research.

Compound **2** (Scheme 1) showed a positive ESI quasi-molecular ion at *m/z* = 1456.8 [M + H]$^+$. Its HRFTICR mass (calcd. 728.8903, observed 728.8898 for [M + 2 H]$^{2+}$) led us to conclude that there is a mass difference of 30 between compound **1** and **2**, which corresponds to a -CH_2O- group. The molecular formula was therefore $C_{72}H_{105}N_{13}O_{19}$. In the NMR spectra, no signal for a methoxy group was discernible and an obvious change in the aromatic region of the ^{1}H NMR spectrum could be observed. Therefore, we concluded that in compound **2**, which was named tychonamide B, the *O*-methylhomotyrosine of **1** is replaced by a homophenylalanine residue.

Both compounds were tested in a cytotoxicity assay against 37 human cancer cell lines. Compound **1** inhibited the growth of all 37 cell lines with similar potency; the mean IC_{50} value was 0.9 μg mL^{-1} and the IC_{50} values ranged from 0.3 to 1.3 μg mL^{-1}. Compound **2** was more selective than **1** and the IC_{50} values ranged from 1.0 (LXF H460; lung) to 14.9 μg mL^{-1} (PRXF LNCAP; prostate). The mean IC_{50} value was 3.3 μg mL^{-1}. Inhibition of clonogenicity of tumor cells was evaluated in additional tumor models by using a clonogenic assay. The antiproliferative activity of **2** was evaluated in cell suspensions prepared from 22 human tumor xenografts of 11 different tumor types. In addition, **2** was tested in a preparation of hematopoietic stem cells as a model system for nonmalignant tissue. Compound **2** inhibited anchorage-independent growth and in vitro colony formation in a concentration-dependent and tumor-type-selective manner. The mean IC_{50} value in this assay

Table 2. Antiprotozoal activity of compounds **1** and **2**.[a]

Compound	*T. b. rhod.* IC_{50} [μg mL^{-1}] (drug[b])	*T. cruzi* IC_{50} [μg mL^{-1}] (drug[c])	*L. don.* IC_{50} [μg mL^{-1}] (drug[d])	*P. falc.* IC_{50} [μg mL^{-1}] (drug[e])	Cytotox· L6 IC_{50} [μg mL^{-1}] (drug[f])
1	0.116 (0.003)	3.51 (0.563)	1.76 (0.101)	1.47 (0.078)	4.11 (0.009)
2	0.241 (0.002)	5.48 (0.512)	2.38 (0.116)	1.81 (0.078)	4.83 (0.009)

[a] *T. b. rhod.* = *Trypanosoma brucei rhodesiense* (strain STIB 900), *T. cruzi* = *Trypanosoma cruzi* (strain Tulahuen C4), *L. don.* = *Leishmania donovani* (strain MHOM-ET-67/L82), *P. falc.* = *Plasmodium falciparum* (strain K1). [b] Melarsoprol. [c] Benznidazole. [d] Miltefosine. [e] Chloroquine. [f] Podophyllotoxin.

was 2.4 μg mL^{-1} and the IC_{50} values ranged from 0.2 (MEXF 989, melanoma) to 4.0 μg mL^{-1} (PAXF 736, pancreas). Colony formation of hematopoietic stem cells was inhibited by **2** with an IC_{50} value of 2.8 μg mL^{-1}.

Tychonamide A and B were tested against several protozoans: *Plasmodium falciparum*, *Trypanosoma cruzi*, *Leishmania donovani*, and sleeping sickness causing *Trypanosoma brucei rhodesiense*. Both compounds were active against all tested protozoans, and **1** (IC_{50} value towards *T. b. rhod.* 0.1 μg mL^{-1}) seems to be more potent than **2** (see Table 2).

Discussion

The most unusual structural feature of compounds **1** and **2** is the presence of a novel β-amino acid. One of the natural functions of such β-amino acids could be the enhancement of the proteolytic stability towards peptidases,[14] which supports the ecological function of these secondary metabolites. The most common β-amino acids in cyanobacterial peptides is Adda [(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-(4*E*,6*E*)-dienoic acid], which is part of the hepatotoxic microcystins and nodularins. Adda, Ahoa [(2*S*,3*R*,5*R*)-3-amino-2,5-dihydroxy-8-phenyloctanoic acid[7]], Ahda [(2*S*,3*R*,5*S*)-3-amino-2,5,9-trihydroxy-10-phenyldecanoic acid[8]], and the novel amino acid Atpoa are all aromatic β-amino acids, in contrast to, for example, Aound (3-amino-2,7,8-trihydroxy-10-methyl-5-oxyundecanoic acid[3]), which is aliphatic. For Adda, the biosynthetic pathway and the corresponding gene cluster was described.[5,15] Most unusual is the phenylacetate starter unit, which is prolonged with four malonyl CoA units to form Adda. On the basis of this biosynthesis, a similar pathway (phenylacetate as starter prolonged with three-to-four malonyl CoA units) can be suggested for Ahoa, Ahda, and Atpoa (Figure 2). The main difference lies in the hydroxylation and the methylation pattern on one hand and the length of the side chain on the other.

Tychonamide A (**1**) showed cytotoxic potency with a mean IC_{50} value of 0.9 μg mL^{-1} in a panel consisting of 37 tumor cell lines. However, antitumor selectivity, that is, differential antitumor activity, was only slightly pronounced, which indicates a rather unspecific and nonselective activity. Tychonamide B (**2**) showed less cytotoxic potency (mean IC_{50} = 3.3 μg mL^{-1}), but selectivity was more pronounced, that is, for nonsmall-cell lung cancer, melanoma, and ovarian cancer. Furthermore, **2** displayed activity and selectivity in clonogenic assays against tumor cell suspensions from 22 solid tumors grown in semisolid medium. Those tumor cells, which represent 11 different tumor types, were freshly prepared from solid human tumor xenografts grown in nude mice. Cells that show anchorage independent growth in semisolid medium contain, to a certain extent, tumor stem cells that are considered to be responsible for the metastatic and infiltrative potential of a tumor.[16–18] Tychonamide B (**2**) selectively inhibited colony formation of melanoma cells. On the basis of IC_{50} values, these sensitive tumor models were on average about sixfold more sensitive than the mean IC_{50} of all models tested. Moreover, these sensitive tumor models were on average about sevenfold more sensitive than hematopoietic stem cells as representative model system for nonmalignant tissue, which is indicative of the satisfactory therapeutic index of the compound.

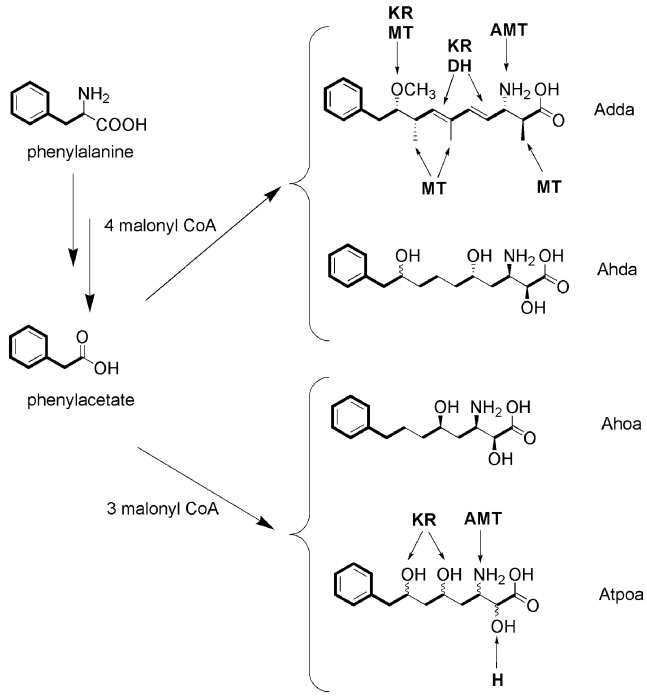


Figure 2. Proposed biosynthesis of cyanobacterial aromatic β-amino acids on the basis of literature.[5,15] KR = ketoreductase, MT = methyltransferase, DH = dehydratase, AMT = aminotransferase, H = hydroxylation.

Compound **1** showed pronounced antiprotozoal activity towards *Trypanosoma brucei rhodesiense* in the nanomolar range (IC_{50} value of about 100 ng mL^{-1}). This result has to be interpreted with care because of the cytotoxicity of the compound. The antitrypanosomal activity is, however, approximately 40-fold higher than the cytotoxicity [IC_{50} value of 4.1 μg mL^{-1}; rat skeletal myoblasts (L6-cells)].

Experimental Section

General Procedure: HPLC was performed with a Merck–Hitachi system equipped with an L-6200A pump, an L-4500A photodiode array detector, a D-6000A interface with D-7000 HSM software, and a Rheodyne 7725i injection system to get the first separation step and the chiral HPLC runs. A Waters system, controlled by Waters millenium software, consisting of a 717 plus autosampler, 600 controller pump with in-line degasser, and a 996 photodiode array detector was then used to purify peptide **1** and **2**. ^{1}H, ^{13}C, COSY, HSQC, HMBC, NOESY, and TOCSY NMR spectra were recorded in [D_6]DMSO by using either a Bruker Avance 500 DRX or a 600 DRX spectrometer operating at 500 or 600 MHz for proton and at 125 or 150 MHz for ^{13}C. Spectra were calibrated to residual solvent signals with resonances at $\delta_{H/C}$ = 2.5/39.5 ppm

([D_6]DMSO). Chiral GC–MS analyses were performed with a Perkin–Elmer Turbomass mass spectrometer by using an Alltech Capillary Chirasil-Val column. UV and IR spectra were measured with Perkin–Elmer Lambda 40 and Perkin–Elmer Spectrum BX instruments. Optical rotations were obtained by using a Jasco DIP 140 polarimeter. The high-resolution mass spectra were recorded with a Bruker Apex II FTICR (7 tesla magnet). The MS–MS MALDI-TOF spectra were recorded with an Applied Biosystems 4700 Proteomics Analyzer 7021 with an α-cyanocinnamic-acid matrix.

Biological Material: The cyanobacterial strain (for taxonomy see ref.[19]) was isolated from a sample collected from a pond of a sugar factory in Wierthe, Germany, and deposited in the Culture Collection of Algae and Protozoa (CCAP 1462/13) in Oban (Scotland, United Kingdom). The cyanobacterium was cultivated in a 25-L photobioreactor (Planktotec system Pluto) with sterile filtered BG-11 medium (Sigma Aldrich, C3061 BG-11) over a period of 9 months. During cultivation at 25 °C, the culture was constantly illuminated with white fluorescent light (Osram L 58W/11–869). Oxygen was supplied by a continuous stream of sterile air from the lower end of the bioreactor. The pH was titrated to a value between 7 and 10 by using an adequate volume of carbon dioxide.

Isolation Procedure: The lyophilized biomass from a 42-L culture (240 g) was exhaustively extracted with dichloromethane (8 L), ethyl acetate (6 L), and methanol (4 L) in three steps. The dried methanol extract amounted to 20.6 g. The ^{1}H NMR and MALDI-TOF spectroscopic measurements of this extract indicated the presence of several peptidic compounds. 12 g of the methanol extract were dissolved in water and repeatedly extracted with diethyl ether (1.5 L) and butanol (4.5 L), which resulted in 3.0 g of butanol extract with a high peptidic content. The crude butanol extract was fractionated by reverse-phase HPLC (Macherey–Nagel, Nucleodur 100, C_{18}, 5 μm, 250 × 10 mm) by using a gradient elution (MeOH/H_2O, 2:3 to MeOH over 30 min, 2 mL min^{-1}) to yield six fractions. Further reverse-phase HPLC separation of fraction 4 (307.1 mg) (Knauer, Eurospher 100, C_{18}, 5 μm, 250 × 8 mm, MeOH/H_2O, 72:28, 1 mL min^{-1}) led to the isolation of 74 mg of compound **1** and 23 mg of compound **2** (0.62 and 0.19% of the crude methanol extract, respectively).

Compound 1: White amorphous solid. $[a]_D^{24}$ = –2.4 (*c* = 2.48, MeOH). UV (MeOH): λ (ε, M^{-1} cm^{-1}) = 203 (79238), 228 sh (28970), 250 sh (6034), 273 sh (2127) nm. IR (ATR): ṽ = 3321, 2958, 2360, 2341, 1733, 1618, 1511, 1449, 1244, 1202, 1024 cm^{-1}. ^{1}H and ^{13}C NMR spectroscopic data see Table 3. HRMS (FTICR): calcd. for $C_{73}H_{109}N_{13}O_{20}$, [M + 2 H$^+$] 743.8950; found 743.8951.

Compound 2: White amophous solid. $[a]_D^{24}$ = –7.7 (*c* = 1.52, MeOH). UV (MeOH): λ (ε, M^{-1} cm^{-1}) = 203 (81991), 228 sh (19681), 250 sh (6234) nm. IR (ATR): ṽ = 3327, 2934, 2362, 2340, 1620, 1541, 1453, 1247, 1202, 1024 cm^{-1}. HRMS (FTICR): calcd. for $C_{72}H_{107}N_{13}O_{19}$, [M + 2 H$^+$] 728.8898; found 728.8903.

Chiral GC-MS: Peptide **1** (3.5 mg) was dissolved in HCl (6 N, 1 mL) and heated for 16 h at 110 °C in a closed vial. After this procedure, the solvent was removed by a stream of nitrogen. The dried hydrolysate was treated with isopropyl alcohol (500 μL) and acetyl chloride (150 μL) at 110 °C for 1 h to create the isopropyl esters of the amino acids. After removal of the solvent, the dry residues were acylated [trifluoroacetic anhydride (0.4 mL) in CH_2Cl_2 (0.4 mL); heating for 15 min at 110 °C]. The reaction mixture was dried and dissolved in EtOAc (1 mL). A 1 μL portion of this solution was analyzed by GC–MS by using an Alltech Capillary Chirasil-Val column (25 m × 0.25 mm; 0.16 μm; program rate: column temperature held at 50 °C for 3 min; 50 °C–180 °C at 4 °C min^{-1}; flow: 0.6 mL min^{-1}; injector temperature: 250 °C).

Table 3. ^{13}C and ^{1}H NMR spectral data for **1** in [D_6]DMSO at 75 °C.

Amino acid	No	δ_H [ppm][a] (mult., *J* [Hz])	δ_C [ppm]
Atpoa	1	–	[b]
	2	3.91	71.9
	3	4.04	48.1
	3-NH[c]	7.30	–
	4	1.79	35.2
	5	5.03	69.4
	6	1.58	41.5
	7	3.67	67.5
	8	2.63, 2.68	43.6
	9	–	138.8
	10	7.18	128.9
	11	7.18	128.9
	12	7.23	127.5
	13	7.23	127.5
	14	7.15	125.3
L-Proline (I)	15	–	[b]
	16	4.28	59.7
	17	1.86	27.8
	18	1.75, 1.81	23.9
	19	3.35, 3.47	46.3
D-*O*-Methyl-homotyrosine	20	–	[b]
	21	4.31	50.9
	21-NH	7.92	–
	22	1.92	32.4
	23	2.52, 2.59	29.9
	24	–	133.0
	25	7.10 (d, 7.8)	128.9
	26	7.10 (d, 7.8)	128.9
	27	6.80 (d, 7.8)	113.5
	28	6.80 (d, 7.8)	113.5
	29	–	157.3
	30	3.71 (s)	54.7
L-Threonine	31	–	[b]
	32	4.29	58.8
	32-NH	7.64	–
	33	4.15	65.9
	34	1.11 (d, 6.6)	19.4
Dhb	35	–	163.9
	36	–	129.8
	36-NH	9.14	–
	37	5.69 (q, 7.3)	124.5
	38	1.88 (d, 7.3)	12.7
L-Serine	39	–	169.5
	40	4.25	56.3
	40-NH	7.87	–
	41	3.70, 3.78	61.3
D-*allo*-Isoleucine	42	–	[b]
	43	4.38	54.7
	43-NH	8.27	–
	44	2.12	34.8
	45	1.19, 1.25	25.5
	46	0.84	11.0
	47	0.83	14.0
L-Proline (II)	48	–	[b]
	49	4.36	60.3
	50	2.16	28.4
	51	2.09	24.7
	52	3.52, 3.83	46.7
L-Proline (III)	53	–	[b]
	54	4.61	57.6
	55	1.76, 2.22	27.1
	56	1.89	23.9
	57	3.51	45.5

Table 3. *(Continued)*

Amino acid	No	δ_H [ppm][a] (mult., *J* [Hz])	δ_C [ppm]
Glycine	58	–	166.2
	59	3.85, 3.93	40.7
	59-NH	7.99	–
D-Glutamine	60	–	170.6
	61	4.26	52.2
	61-NH	7.65	–
	62	1.81, 1.97	28.1
	63	2.10, 2.14	31.3
	64	–	173.7
	64-NH_2	n.d.[d]	–
N-acetyl-*N*-methyl-leucine	65	–	170.4
	66	5.00	53.9
	67	1.63	36.5
	68	1.45	24.1
	69	0.88 (d, 6.6)	22.4
	70	0.84	21.1
N-Acetyl	71	–	170.0
	72	1.96 (s)	21.0
N-Methyl	73	2.80 (s)	31.7

[a] The multiplicity of many ^1H resonances was not determined due to broad and overlapping signals. [b] Chemical shifts of the remaining unassigned carbonyl carbon atoms: 169.7, 170.3, 170.5, 170.9, 171.0, 171.2, 171.7 ppm. [c] The assignment of the chemical shifts of the amide protons was done at room temperature (apart from 36-NH). [d] n.d. = not determined.

Derivatization of the amino acids was performed by using 5–10 mg of D- and L-amino acids (for Ile and Thr the configurations D-, D-*allo*, L- and L-*allo* were taken) and processed as described above. Before being analyzed by GC–MS, the solutions of standard amino acids were diluted with EtOAc (1:50). The retention times of the *N*-trifluoroacetylisopropyl ester of the amino acids were compared with those of the derivatized standards. In the case of Pro, the sample was spiked with both standards (because of the small difference in the retention times: D-Pro 19.98 min, L-Pro 20.05 min) to show that the L enantiomer is present. L-Thr and Gly could not be separated from the complex amino acid mixture of the hydrolysate with this method. By spiking experiments and comparison of the mass spectra it could be shown that both amino acids were present in the hydrolysate and eluted with t_R = 14.80 min. [Retention times for the standards: D-Thr 14.26 min, L-Thr 14.80 min, D-*allo*-Thr 17.34 min, L-*allo*-Thr 17.88 min, Gly 14.97 min, D-Ile 16.50 min, L-Ile 17.03 min, D-*allo*-Ile 16.06 min, L-*allo*-Ile 16.69 min, *N*-Me-Leu 17.36 min (L and D not to separate), D-Ser 17.03 min, L-Ser 17.53 min, D-Pro 19.98 min, L-Pro 20.09 min, D-Gln 28.00 min, L-Gln 28.38 min, D-*O*-MeHtyr 37.82 min, L-*O*-MeHtyr 38.11 min; compound **1**: L-Thr 14.80 min, glycine 14.80 min, D-*allo*-Ile 15.97 min, *N*-Me-Leu 17.28 min, L-Ser 17.40 min, L-Pro 20.09 min, D-Gln 27.87 min, D-*O*-MeHtyr 37.73 min].

Chiral HPLC: Compound **1** (600 μg) was hydrolyzed in HCl (6 N) at 110 °C for 16 h. The hydrolysate was dried with a stream of nitrogen. The residue was dissolved in the mobile phase used for the chiral HPLC (2 mM $CuSO_4$ in H_2O/MeCN, 95:5; flow rate 1 mL min^{-1}). The chiral HPLC was performed by using a Chirex 3126 (D)-penicillamine column (Phenomenex; 250 × 4.60 mm). In this experiment the retention times of the standards were 40.15 (*N*-Me-L-Leu) and 59.89 min (*N*-Me-D-Leu). In the chromatogram of the hydrolysate, two signals were obtained: one strong signal belonging to the L enantiomer and a smaller one belonging to the D enantiomer (ratio: 4:1). We proposed that an epimerization process took place during hydrolysis. Therefore, hydrolysis under milder conditions (500 μg peptide; 6 N HCl; 88 °C for 12 h) was done to minimize epimerization. The HPLC chromatogram of the latter experiment showed that the ratio *N*-Me-L-Leu to *N*-Me-D-Leu was shifted in favor of *N*-Me-L-Leu (ratio: 12:1). Consequently it was concluded that the L form is present in **1**.

Antitumor Testing. Monolayer Assay: A modified propidium iodide assay was used to determine the cytotoxic activity of the compounds against human tumor cell lines. The test procedure was described elsewhere.[20] Cell lines tested were derived from patient tumors engrafted as a subcutaneously growing tumor in NMRI nu/nu mice, or obtained from the American Type Culture Collection, Rockville, MD, USA, National Cancer Institute, Bethesda, MD, USA, or Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. Briefly, human tumor cells lines were grown at 37 °C in a humidified atmosphere (95% air, 5% CO_2) in monolayer cultures in RPMI 1640 medium supplemented with 10% FCS and phenol red (PAA, Cölbe, Germany). Cells were trypsinized and maintained weekly. Cells were harvested from exponentially growing cultures by trypsination, counted and plated in 96-well flat-bottomed microplates (140 μL cell suspension, 5×10^3 to 10×10^3 cells per well). After a 24 h recovery to allow cells to resume exponential growth, 10 μL of culture medium (6 control wells per plate) or medium containing the test drug were added to the wells. Each drug concentration was plated in triplicate. After 4 d of incubation the culture medium was replaced by fresh medium containing 6 μg mL^{-1} of propidium iodide. Microplates were then kept at –18 °C for 24 h, to give a total cell kill. After thawing of the plates, fluorescence was measured by using the Cytofluor 4000 microplate reader (Perseptive Biosystems) (excitation 530 nm, emission 620 nm). The amount of viable cells was proportional to the fluorescence intensity.

Clonogenic Assays with Human Tumor Xenografts and Hematopoietic Stem Cells: Effects of the test compound on clonogenicity of tumor cells were investigated in a clonogenic assay. Tumor xenografts were derived from patient tumors engrafted as a subcutaneously growing tumor in NMRI nu/nu mice obtained from Oncotest's breeding facility. Details of the test procedure have been described earlier.[21] Briefly, solid human tumor xenografts were removed from mice under sterile conditions, mechanically disaggregated and subsequently incubated with an enzyme cocktail consisting of collagenase type IV (41 U mL^{-1}), DNase I (125 U mL^{-1}), hyaluronidase type III (100 U mL^{-1}), and dispase II (1.0 U mL^{-1}) in RPMI 1640-Medium at 37 °C for 45 min. Cells were passed through sieves of 200 and 50 μm mesh size and washed twice with sterile PBS-buffer. The percentage of viable cells was determined in a Neubauer-hemocytometer using trypan blue exclusion. The bottom layer consisted of 0.2 mL per well Iscove's Modified Dulbecco's Medium (Invitrogen), supplemented with 20% (v/v) fetal calf serum (Sigma), 0.01% (w/v) gentamicin (Invitrogen) and 0.75% (w/v) agar (BD Biosciences). 1.5×10^4 to 4×10^4 cells were added to 0.2 mL of the same culture medium supplemented with 0.4% (w/v) agar and plated in 24-multiwell dishes onto the bottom layer. The test compounds were applied by continuous exposure (drug overlay) in 0.2 mL of culture medium. Every dish included six untreated control wells and drug-treated groups in triplicate at 6 concentrations. Cultures were incubated at 37 °C and 7.5% CO_2 in a humidified atmosphere for 7–20 d and monitored closely for colony growth using an inverted microscope. Within this period, in vitro tumor growth led to the formation of colonies with a diameter of >50 μm. At the time of maximum colony formation, counts were performed with an automatic image analysis system (OMNICON 3600, Biosys GmbH). 24 h prior to evaluation, vital colonies were stained with a sterile aqueous solution of 2-(4-iodophenyl)-3-(4-

nitrophenyl)-5-phenyltetrazolium chloride (1 mg mL^{-1}, 100 μL per well).

For testing hematopoietic stem cells, buffy coats or samples of human umbilical cord blood were diluted 2- to threefold with PBS containing 0.1% (w/v) BSA. Peripheral blood mononuclear cells were enriched from the respective samples by Ficoll Paque density gradient centrifugation and washed twice with PBS containing 0.1% (w/v) BSA. The resulting cell suspension was stored in aliquots in freezing medium at –80 °C. Aliquots were thawed for testing as appropriate. The colony forming test was performed using 6-well plates and HSC-CFU (Miltenyi Biotec) as culture medium. 1.25×10^4 to 1.5×10^4 cells per mL of the above mentioned preparation were seeded in a final volume of 1.0 mL per well. Solutions of the test article were added directly to the medium. Every 6-well dish contained one triplicate of one test concentration of one test compound. The other 3 wells of the test plate were filled with 2.0 mL of sterile water to ensure that maximum humidity is attained during the subsequent incubation period. Two 6-well plates received one triplicate of untreated cells each, which served as untreated control for the whole experiment. Cultures were incubated at 37 °C and 7.5% CO_2 in a humidified atmosphere for 14 d. Colony growth was evaluated by eye using an inverted microscope. For detailed results see the Supporting Information.

Antiprotozoal Activity: Antiplasmodial activity was determined against the K1 strain of *Plasmodium falciparum* by using a modified [^{3}H] hypoxanthine incorporation assay. Infected human erythrocytes were exposed to serial drug dilutions in microtiter plates for 48 h at 37 °C in a gas mixture with reduced oxygen and elevated CO_2. [^{3}H] hypoxanthine was added to each well and after further incubation for 24 h the wells were harvested on glass fiber filters and counted in a liquid scintillation counter. From the sigmoidal inhibition curve the IC_{50} value was calculated. Chloroquine was used as positive control.

Activity against *Trypanosoma brucei rhodesiense* (strain STIB 900) was evaluated according Räz et al.[22] Parasites were grown axenically in culture medium supplemented with horse serum. Following a 3-day exposure to test compounds, the viability of tryptomastigote parasites was quantified using the dye Almar Blue by monitoring the reductive environment of living cells. Fluorescence development was expressed as percentage of the control, and IC_{50} values were calculated. Melarsoprol was included as positive control.

Activity against *Trypanosoma cruzi* was determined according to Bruckner et al.[23] The strain Tulahuen C4 of *T. cruzi*, which had been transfected with the galactosidase *lac*-Z gene, was cultivated for 4 d on rat skeletal myoblasts (5% CO_2, 37 °C) in the presence of test compounds. For measurement of the IC_{50} value the substrate chlorophenol red-ß-D-galactopyranoside was added. The color reaction that developed during the following 2–4 h because of the β-galactosidase activity (change of color from yellow to red) was quantified photometrically employing an ELISA reader. As a positive control, benznidazole was included in each test series.

Evaluation of antileishmanial activity was carried out in mouse peritoneal macrophages. The ratio of infection with *Leishmania donovani* (strain MHOM-ET-67/L82) was determined microscopically after exposure to test compounds, incubation and staining with Giesma. IC_{50} values were calculated by linear regression. Miltefosine was used as positive control.

Cytotoxicity was evaluated in rat skeletal myoblasts (L6-cells) by using podophyllotoxin as positive control.

Supporting Information (see footnote on the first page of this article): Proposed MS elimination pattern of the exocyclic amino acid, description of the synthesis of the enantiomers of *O*-methylhomotyrosine, detailed cytotoxicity data, and NMR spectroscopic data and purity data of compound **1** and **2**.

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