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Gymnopeptides A and B, Cyclic Octadecapeptides from the Mushroom *Gymnopus fusipes*

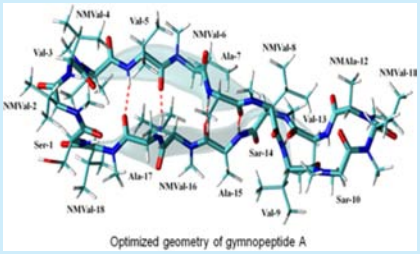
Attila Ványolós,[†,∥] Miklós Dékány,[‡] Bernadett Kovács,[†] Balázs Krámos,[‡] Péter Bérdi,[§] István Zupkó,[§] Judit Hohmann,[†] and Zoltán Béni*[,‡,∥]

[†]Department of Pharmacognosy and [§]Department of Pharmacodynamics and Biopharmacy, University of Szeged, Eötvös u. 6, 6720 Szeged, Hungary

[‡]Spectroscopic Research, Gedeon Richter Plc., Gyömrői út 19-21, 1103 Budapest, Hungary

Supporting Information

ABSTRACT: Mycochemical study of the mushroom *Gymnopus fusipes* led to the discovery of two new cyclopeptides. The two compounds, named as gymnopeptides A and B, are unprecedented highly *N*-methylated cyclic octadecapeptides. Detailed spectroscopic studies, Marfey's analysis, and a preliminary molecular modeling study suggested that both are natural cyclic β hairpins. The isolated compounds exhibited striking antiproliferative activity on several human cancer cell lines, with nanomolar IC_{50} values.



Mushrooms, defined as macroscopic fungi with distinct sporocarps visible with naked eye, represent an inexhaustible source of biologically active natural products. Since mushrooms as well as other fungi play an essential role in decomposition processes in nature, they have developed a specific and complex metabolism. Among the products of fungal metabolism, one can find compounds with high structural diversity, which have become indispensible tools of human therapy: the immunosuppressive cyclosporine A (cyclopeptide),[1] the hypocholesterolemic lovastatin (naphthalene)[2] and the emerging class of antibacterial compounds, the pleuromutilins (diterpenes),[3] among others.

In the last years, the number of publications dealing with the isolation and characterization of new cytotoxic compounds of mushroom origin has been increasing. Recent studies have revealed antitumor secondary metabolites with unusual structures: gloephylline A with a C-nor-D-homoergosteroid skeleton from *Gloephyllum abietinum*,[4] terreumols A–D, meroterpenoids with an unusual 10-membered ring from *Tricholoma terreum*,[5] and schizines A and B from *Schizophyllum commune*, representing the first iminolactones from natural sources.[6]

As part of our ongoing research aimed at the identification of fungal natural products with potential anticancer activity, novel cyclic octadecapeptides from the mushroom *Gymnopus fusipes* (Bull.: Fr.) Gray (syn. *Collybia fusipes*) have been discovered. Also known as spindleshank, *G. fusipes* is a basidiomycetes species of the Omphalotaceae family, native to woodlands of Europe and Asia, but considered an invasive species in North America. It is a parasitic mushroom that can cause serious damage to the roots of different oak species. Field mycologists in general consider *G. fusipes* an edible species with lower culinary value; however, the old fruiting bodies can produce gastrointestinal symptoms.[7a,b] Our literature survey on *G. fusipes* revealed that the chemistry and pharmacology of this species are practically unexplored. As a result of our screening program for Hungarian mushrooms for cytotoxic activity, it was observed that the chloroform extract of *G. fusipes* exerted outstanding antiproliferative activity on several human cancer cell lines. In order to identify the compounds responsible for the observed cytotoxic activity, the chloroform extract of *G. fusipes* was separated first by rotational planar chromatography, followed by preparative reversed-phase HPLC to obtain compounds **1** and **2**, named gymnopeptides A and B, respectively (Figure 1).

Gymnopeptides A and B showed highly similar ^{1}H NMR spectra in $CDCl_3$. The ^{1}H NMR features confirmed the peptidic nature of the two compounds and suggested the presence of a single conformer in both cases. Besides the eight doublet signals detected in the amide region, 10 singlet signals with 3H intensity (in case of gymnopeptide B two of these singlets were overlapping) were observed in the spectral region of 2.6–3.5 ppm. This suggested the presence of 18 amino acids (AA), ten of which being *N*-methylated. Consecutive analysis of ^{1}H, ^{13}C, 2D-TOCSY, heteronuclear 2D NMR correlation (HSQC and HMBC), and selective 1D-TOCSY data allowed us to overcome the difficulties caused by the severe overlaps of H_α, H_β, and methyl group resonances and permitted the identification and complete NMR assignments of the 18 amino acid (AA) residues (Table S1). This led to the conclusion that the two compounds differed only in the presence of a single AA; a serine (Ser) found in gymnopeptide A was replaced by a threonine (Thr) in gymnopeptide B. The other 17 AA were

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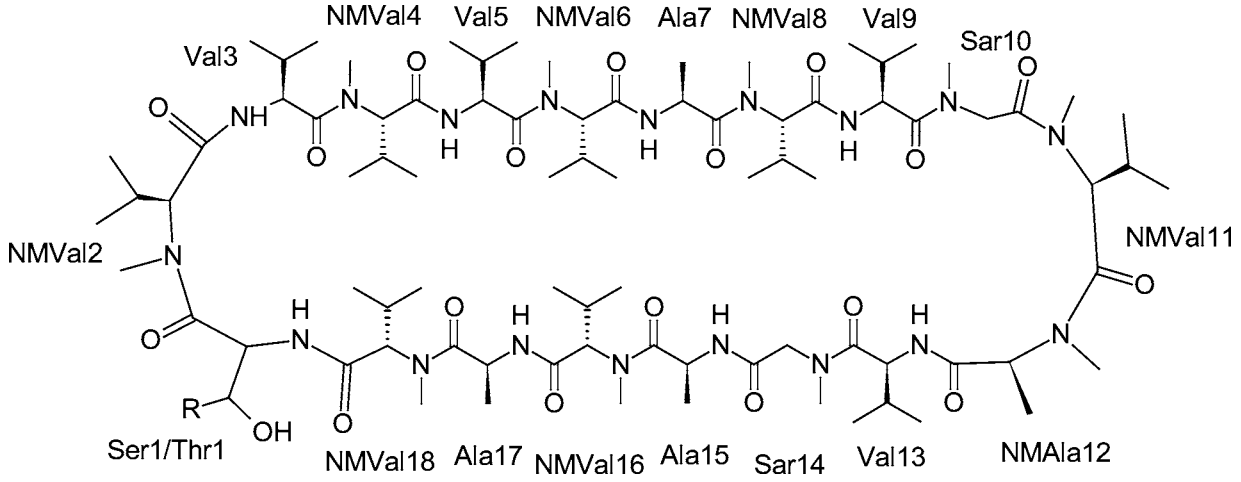

Figure 1. AA sequences of gymnopeptides A (R = H) and B (R = CH_3), all residues are in L configuration except Ser1/Thr1 for which configuration was not determined.

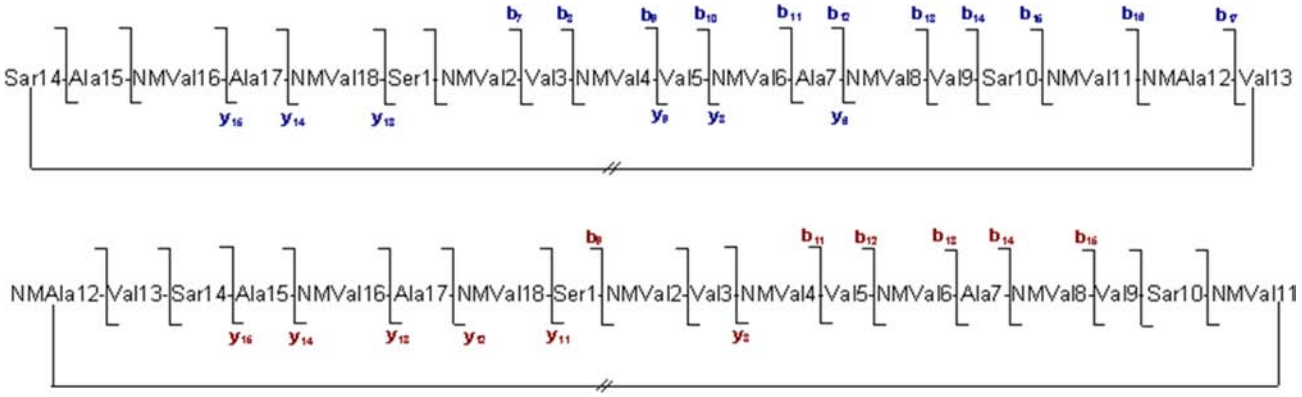

Figure 2. Most probable ring-opening positions b and y detected in the MS/MS spectrum of gymnopeptide A.

common in the two compounds; thus, three alanine (Ala), four valine (Val), two *N*-methyl glycine (sarcosyl, Sar), an *N*-methylalanine (NMAla), and seven *N*-methyl-valine (NMVal) residues were shown to be present in both cases. The ^{1}H and ^{13}C chemical shifts (Table S1) determined for the amino acids were similar to the appropriate values reported in organic solvents for a given AA found in highly *N*-methylated nonribosomal or synthetic peptides,[8a−f] except for the α methine group in NMVal2 where unusually upfield ^{1}H (doublet at 2.95 ppm in gymnopeptide A and 2.96 ppm B) and unusually downfield ^{13}C resonances (at 79.8 ppm in both peptides) were assigned to the α protons and carbons, respectively. A probable structural rational behind these values is given below. Following the identification of the amino acids, sequential assignments were accomplished. The usual NH(i)/CO(i−1), NCH_3(i)/CO(i−1), and Hα(i)/CO(i and/or i−1) HMBC correlations (Figure S1) allowed the deduction of the AA sequence shown in Figure 1 for the two analogues.

Both are octadecacyclopeptides, where natural and *N*-methylated amino acids alternate in the sequences, except in position 11, where instead of a natural amino acid an NMVal is present. NOE data obtained from ROESY and NOESY spectra confirmed the HMBC-derived sequences and the cyclic nature in case of both peptides. Thus, interresidue correlations were observed between Hα(i)/NH(i+1) and Hα(i)/NCH_3(i+1) (key correlations are shown in Figure S1) in all cases, except those involving the *N*-methyl protons of NMVal2 and NMAla12 and the amide proton of Val3. In the case of NMVAl2 and NMAla12 Hα(i)/Hα(i−1), correlations were observed instead of the NCH_3(i)/Hα(i-1), while the amide proton of Val3 showed NOE correlation to the β instead of the α methine proton of NMVal2. These findings suggested that the amide bonds between Ser1 (Thr1 in gymnopeptide B) and NMVal2 and that between NMVal11 and NMAla12 residues are in *cis*, while all other amide bonds are in the *trans* configuration. Besides the sequential NOEs, spatial proximity between the amide protons Ser1(Thr1)/Val3, Val5/Val17, Ala7/Val15, and Val9/Val13 were detected in both gymnopeptides. These correlations suggested that in $CDCl_3$ the compounds exhibit secondary structures stabilized by H-bonds centered on the listed residues. In accordance with this, all amide temperature coefficients ($\Delta\delta/\Delta T$) are in the range of −4.5 to −1.0 ppb (Table S1). These values are similar to those typically reported[9a,b] for amide protons involved in H-bonds in water as solvent. In addition, the observed ca. 9 Hz ^{3}J coupling constant values of the amide protons, based on the Karplus relationship,[9b−d] suggest that these residues are part of β strands. Taking all these into account, an 18mer cyclic β-hairpin structure (Figure 1) is suggested for both peptides, wherein two antiparallel β strands (stabilized by alternating natural and *N*-methyl AAs) are connected with two turns, both containing a *cis* amide bond (Ser1/Thr1-NMVal2 and NMAla11-NMVal12). The latter suggests the presence of β VI like turn regions,[10a,b] where the i+2 position is occupied by

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Table 1. Antiproliferative Activity of Gymnopeptides A (1) and B (2) Compared to Cisplatin (3) on Human Cancer Cell Lines

	calcd IC50 values (nM) ± SD				
	HeLa	A431	MCF7	MDA-MB-231	T47D
1	88.4 ± 3.3	66.4 ± 4.1	26.5 ± 3.2	37.4 ± 2.2	18.0 ± 3.0
2	42.5 ± 4.5	44.3 ± 2.7	18.5 ± 6.8	30.7 ± 3.0	14.0 ± 2.2
3 (μM)	12.4 ± 1.5	2.8 ± 0.9	5.8 ± 1.0	19.1 ± 2.1	9.8 ± 3.4

an *N*-methylated AA instead of the usual proline (Pro).[11a−d] Indeed, this "replacement" is in accordance with literature data[12] showing that in cyclic pentapeptides an *N*-methylated AA could successfully replace Pro by preserving the conformational preference. Although it needs to be further investigated, the above-noted unusual chemical shift values (observed for the α methine group of NMVal2) can most probably be associated with some steric stress this residue experiences in the *i*+2 position of the turn region.

FT-HR-ESI-MS analysis was in accordance with the constitution suggested by NMR spectroscopic analysis. Thus, the protonated molecular ions of gymnopeptides A and B were detected at 1716.13393 and 1730.15751 *m*/*z*, respectively. These values corresponded to the molecular formulas of $C_{84}H_{151}N_{18}O_{19}$ and $C_{85}H_{153}N_{18}O_{19}$ (protonated molecular ions), respectively, with high accuracy (Δ = −3.4 and 1.2 ppm, respectively) and were in accordance with the suggested sequences showing a single amino acid difference in the two gymnopeptides. Although MS/MS^n-based amino acid sequencing of cyclopeptides is usually complicated by the possibility of multiple and often indiscriminable ring-opening positions,[13] in the case of gymnopeptides A and B, the MS/MS data could unambiguously confirm the AA sequences suggested by NMR. In the MS/MS spectra of the cyclopeptides, the two largest fragment ions (SI) corresponded to linear peptides that could be derived from the parent ions by the loss of a Val and a Val plus an NMAla residue. Regarding the suggested sequence, only a single Val-NMAla peptide bond is present (Figure 1). On this basis, a ring opening between Val13 and Sar14 (or between NMAla12 and Val13) (Figure 2) could be envisaged. Analysis of the fragment ions detected in the MS/MS spectra with regard to the simulated (ProteinProspector 5.12.1) b and y ions derived from these linear peptides supported this ring opening and could already verify most of the suggested sequences in the case of both gymnopeptides. Further analysis of the largest unassigned fragment ions (b15 and y15 in Figure 2 and in the SI) suggested another ring opening position between NMVal11 and NMAla12. With the b and y ions derived from these linear peptides, another large set of fragment ions detected in the MS/MS spectra could be explained. Combining the fragmentation information on these two linear peptides, the suggested AA sequence could unambiguously be verified in case of both cyclopeptides.

Absolute configurations of the amino acid residues were investigated by HPLC−MS following the methods described in the literature.[14a,b] First, acidic hydrolysis of the cyclopeptides was undertaken. This was followed by derivatization of the resulted amino acid mixture with Marfrey's reagent.[15] Finally, HPLC−MS analysis was performed, and the retention times belonging to the accurate mass values of the derivatized amino acids in the extract ion chromatograms were compared to those observed for the derivatized L,D-amino acid pairs prepared and measured using similar experimental conditions (SI). On the basis of the observed data, all Ala, NMAla, Val, and NMVal residues were shown to have the L configuration. Indeed, this finding is in accordance with the conclusion of Gibbs et al.[9c] suggesting that a D amino acid residue in the β strand would prevent the formation of β hairpin structures in cyclic peptides. It should be noted that in most cases (Table S3) the epimeric derivatives were detected in low quantities (ca. 1 to 7%) as well. Detection of these derivatives was due to either racemization of the AAs during the long acidic hydrolysis[16a−c] or low-level peptidic contaminants of the isolated samples. The absolute configurations of serine and threonine could not be determined from the hydrolysates, since neither the serine nor the threonine derivatives could be detected by HPLC−MS. Unfortunately, the isolated amount of gymnopeptides A and B did not allow more detailed investigation; thus, we are bound to leave these configurations unassigned. However, according to a preliminary molecular modeling study on gymnopeptide A, with L-Ser in position 1 a geometry well agreeing with all NMR data could be reached by calculations. The calculations were performed within the Schrödinger software suite.[17a−d] First, an initial geometry was calculated for gymnopeptide A at the MM level using the key NOE-derived geometry restraints and conformational search (SI). Finally, a molecular dynamics simulation was performed to further optimize the geometry and to prove that no significant changes occur during the simulation when no restraints are applied. As mentioned above, the optimized structure (Figure S28) is in good agreement with the above-discussed NMR data. Even trends in ^{13}C chemical shifts in the case of α carbons (Figure S29) could be reproduced by DFT calculations within Jaguar 9.1[17d] following the method described by Bagno et al.[18] Taking all these data into account, the structures shown in Figure 1 can be proposed for gymnopeptides A and B.

The antiproliferative properties of the isolated peptides were determined by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on a panel of human adherent cancer cell lines including cervical (HeLa), skin epidermoid (A431), and breast (T47D, MCF7, and MDA-MB-231) cell lines. Both peptides exhibited potent cell growth inhibitory action on all of the studied cells (Table 1). In comparison, gymnopeptide B is more potent than gymnopeptide A, while both of them are at least 2 orders of magnitude more efficient than that of the reference agent cisplatin (Table 1).

Although the well-known mycotoxins, phalloidin, and amanitins were among the first cyclic peptides discovered,[19] these secondary metabolites are fairly rare in the world of mushrooms, and their occurrence seems to indicate a rather specific distribution. Besides (−)-ternatin isolated from fruiting bodies of *Trametes versicolor*,[20] cyclopeptides have only been shown to be present in few genera of poisonous species (*Amanita, Conocybe, Galerina, Lepiota*, and *Omphalotus*), which contain series of peptides including amatoxins, phallotoxins, virotoxins, omphalotins, and the nontoxic cycloamanides.[21]

Gymnopeptides A and B have now become new members of the group of mushroom cyclopeptides, possessing some interesting structural features which distinguish them from

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the above-mentioned fungal products. To the best of our knowledge, gymnopeptides A and B are the largest cyclic peptides of mushroom origin: they are constituted of 18 monomers, while for example, (−)-ternatin has only 7 and the cyclic peptides of the poisonous species have 7−12 monomers in their molecules. Both gymnopeptides are highly methylated: the number of *N*-methylated amino acids is 10 out of 18, and *N*-methylvaline contributes with 7 monomers to the overall structure.

Further biological studies are warranted to clarify some aspects of absorption and metabolism of gymnopeptides after consumption of fruiting bodies of *G. fusipes* as well as to explore the potential role of these metabolites in the parasitic lifestyle of the mushroom. The isolation of gymnopeptides A and B, unprecedented cyclic octadecapeptides with strong cytotoxic activity from *G. fusipes*, clearly demonstrates that the increased attention paid to fungal natural products is fully justified.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b01158.

Isolation and purification of compounds, selected NMR and MS spectra, table of NMR assignments of isolated compounds, full details of Marfey's analyses, and data on the preliminary modeling study and on antiproliferative activity (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: z.beni@richter.hu.

Author Contributions

∥A.V. and Z.B. contributed equally.

Notes

The authors declare no competing financial interest.

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