Synthetic and Cytotoxic and Antimicrobial Activity Studies on Annomuricatin B

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The first total synthesis of annomuricatin B (**8**) is described *via* coupling of the tripeptide Boc-L-asparaginyl(benzhydryl)-L-alanyl-L-tryptophan-OH and the tetrapeptide L-leucyl-glycyl-L-thryl-L-proline-OMe followed by cyclization of the linear heptapeptide fragment. On pharmacological investigation, it was observed that the cycloheptapeptide **8** displays moderate cytotoxicity against *Dalton's lymphoma ascites* and *Ehrlich's ascites carcinoma* cell lines with cytotoxic inhibitory concentration (50 %) values of 11.6 and 14.1 μM, in addition to potent antidermatophyte activity against *Trichophyton mentagrophytes* and *Microsporum audouinii* with a minimum inhibitory concentration of 6 μg mL^{-1}. Moreover, Gram-negative bacteria and *Candida albicans* were found to be moderately sensitive towards the newly synthesized peptide.

Key words: Annomuricatin B, Cycloheptapeptide, Solution-phase Synthesis, Macrocyclization, Pharmacological Activity

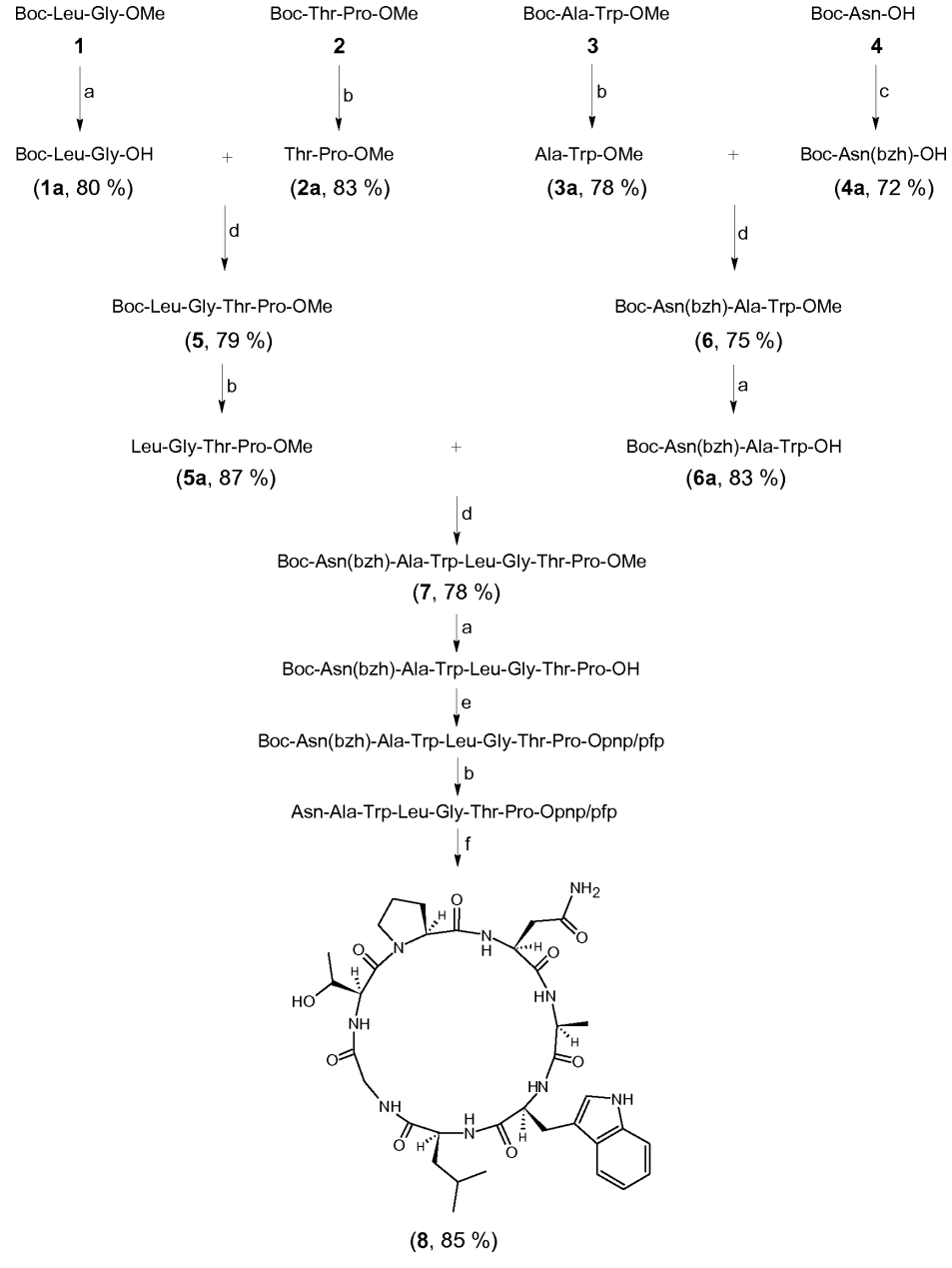
Introduction

During past years, a lot of work has been reported by various scientists which demonstrates the potential of higher plants to produce a wide array of natural products with interesting bioactivities [1 – 5]. Among these, cyclopolypeptides and related congeners [6] are emerging as novel organic compounds with unique structures and a wide pharmacological profile that may prove better candidates to overcome the problem of resistance towards conventional drugs. Plant-derived cyclic peptides possess a variety of biological activities including antitumor [7], vasorelaxant [8], immunosuppressive [9], tyrosinase and cyclooxygenase inhibitory [10, 11], antimalarial [12], and estrogen-like activity [13, 14]. A novel cyclic heptapeptide, annomuricatin B was isolated by column chromatography from seeds of *Annona muricata* (Annonaceae), and the structure was elucidated by chemical and spectral methods [15].

Prompted by the medicinal properties of plant-derived cyclopolypeptides as well as to obtain the natural peptide in good yield, the present study aimed at the synthesis of annomuricatin B employing solution-phase chemistry. The cytotoxic, antibacterial and antifungal activities of the synthesized peptide were also evaluated.

Results and Discussion

The cycloheptapeptide molecule was split into three dipeptide units Boc-L-Leu-Gly-OMe (**1**), Boc-L-Thr-L-Pro-OMe (**2**) and Boc-L-Ala-L-Trp-OMe (**3**) and a single amino acid unit Boc-L-Asn-OH (**4**). Dipeptide units **1** – **3** were prepared by coupling of Boc-amino acids such as Boc-L-Leu, Boc-L-Thr and Boc-L-Ala with the corresponding amino acid methyl ester hydrochlorides such as Gly-OMe·HCl, L-Pro-OMe·HCl and L-Trp-OMe·HCl by following the modified Bodanzsky and Bodanzsky method [16]. The carboxamide side chain of amino acid unit **4** was protected using benzhydrol to get Boc-L-Asn(bzh)-OH (**4a**). After deprotection at the carboxy terminus, dipeptide **1** was coupled with dipeptide **2** deprotected at the amino terminus, to get the tetrapeptide unit Boc-L-Leu-Gly-L-Thr-L-Pro-OMe (**5**). The Boc group of dipeptide **3** was removed using trifluoroacetic acid (TFA), and the deprotected peptide was coupled with the benzhydryl-protected amino acid unit **4a** utilizing three different carbodiimides to



Scheme 1. Synthetic route to the cycloheptapeptide (annomuricatin B) **8**. Reaction conditions: a = $LiOH{\cdot}H_2O$ (1 : 1), r. t., 1 h; b = TFA, $CHCl_3$, r. t., 1 h; c = benzhydrol, GAA, H_2SO_4, r. t., 30 min; d = EDC·HCl or DIPC, TEA or NMM, THF or DMF, r. t., 24 – 36 h; e = DIPC, pnp/pfp, r. t., 12 h; f = TEA or NMM or pyridine, 7 d, 0 °C.

get the tripeptide unit Boc-L-Asn(bzh)-L-Ala-L-Trp-OMe (**6**). After removal of the ester group of tripeptide **6** and the Boc group of tetrapeptide **5**, the deprotected units were coupled to get the linear heptapeptide unit Boc-L-Asn(bzh)-L-Ala-L-Trp-L-Leu-Gly-L-Thr-L-Pro-OMe (**7**). The methyl ester group of the linear peptide fragment was replaced by *p*-nitrophenyl or pentafluorophenyl (Pnp or Pfp) ester groups. The Boc and Bzh groups of the resulting compound were removed using TFA, and the deprotected linear fragment was now cyclized by keeping the whole contents at 0 °C for 7 d in the presence of catalytic amounts of

Table 1. Cytotoxic activity data.

Compd.	Conc. (μg mL^{-1})	— DLA cells — Live cells counted	No. of dead cells	% GI[a]	CTC_{50} (μM)[b]	— EAC cells — Live cells counted	No. of dead cells	% GI	CTC_{50} (μM)
1	62.5	0	38	100.0		0	28	100.0	
	31.25	6	32	84.21		4	24	83.33	
	15.63	16	22	57.89	12.8	15	13	46.43	16.5
	7.81	26	12	31.58		23	5	17.86	
	3.91	31	7	18.42		27	1	3.57	
2	62.5	0	38	100.0		0	28	100.0	
	31.25	2	36	94.74		1	27	96.43	
	15.63	10	28	73.68	11.6	8	20	71.43	14.1
	7.81	20	18	47.37		17	11	39.28	
	3.91	24	14	36.84		22	6	21.43	
Control	62.5	38	0	–		28	0	–	
	31.25	38	0	–		28	0	–	
	15.63	38	0	–	–	28	0	–	–
	7.81	38	0	–		28	0	–	
	3.91	38	0	–		28	0	–	
Standard (5-FU)	62.5	0	38	100.0		0	28	100.0	
	31.25	0	38	100.0		0	28	100.0	
	15.63	10	28	73.68	37.4	11	17	60.71	90.6
	7.81	13	25	65.79		19	9	32.14	
	3.91	22	16	42.11		23	5	17.86	

[a] % Growth inhibition (GI) = $100 - [\{(Cell_{total} - Cell_{dead}) \times 100\}/Cell_{total}]$; [b] CTC_{50} = cytotoxic concentration inhibiting 50 % of percentage growth.

TEA or NMM or pyridine to get the cyclic product **8** (Scheme 1).

The structure of the newly synthesized cyclic heptapeptide as well as that of the intermediate tri/tetra/heptapeptides were confirmed by FTIR and ^{1}H NMR spectroscopy, and elemental analysis. In addition, ^{13}C NMR and mass spectra were recorded for the linear as well as for the cyclic heptapeptide.

The synthesis of cyclopeptide **8** was accomplished with 85 % yield, and pyridine proved to be an effective base for cyclization of the linear heptapeptide fragment. Cyclization of the linear peptide was indicated by the disappearance of absorption bands at 1748, 1270 and 1388, 1371 cm^{-1} (C–O_{str}, ester and C–H_{def}, *tert*-butyl groups) and the presence of additional amide I and II bands of the -CO–NH- moiety at 1637 – 1634 cm^{-1} and 1527 – 1525 cm^{-1} in the IR spectrum of **8**. Deprotection of asparagine was confirmed by the presence of amide I and II bands (1653, 1628 cm^{-1}) and bands at 3350, 3178 and 1405 cm^{-1} due to N–H_{str} and C–N_{str} of the -$CONH_2$ moiety, and the disapppearance of strong out-of-plane deformation bands at 733 – 731 cm^{-1} and 699 – 694 cm^{-1} due to the aromatic rings of the bzh group, in the IR spectra and the disapppearance of the multiplet at 7.23 – 7.16 and 7.09 – 7.03 ppm due to 10 protons of phenyl rings

Table 2. Antifungal activity data[a].

Compd.	Diameter of zone of inhibition (mm) Fungal strains				
	C. albicans	*A. niger*	*Ganoderma sp.*	*M. audouinii*	*T. mentagrophytes*
7	12(6)	9(50)	11(25)	19(6)	22(6)
8	14(6)	13(50)	14(25)	23(6)	26(6)
Control	–	–	–	–	–
Griseofulvin	20(6)	18(12.5)	22(6)	17(6)	19(6)

[a] Values in parentheses are MIC values (μg mL^{-1}).

of the benzhydryl (Bzh) group in the ^{1}H NMR spectrum of **8**. The formation of the cyclopeptide was further confirmed by the disappearance of singlets at 3.63 and 1.54 ppm corresponding to three protons of the methyl ester group and nine protons of the *tert*-butyl group of Boc in the ^{1}H NMR spectrum and the disappearance of the singlets at 154.6, 79.9 and 53.9, 28.3 ppm corresponding to carbon atoms of ester and *tert*-butyl groups in the ^{13}C NMR spectrum of **8**. Furthermore, the ^{1}H NMR and ^{13}C NMR spectra of the synthesized cyclic heptapeptide showed characteristic peaks confirming the presence of all the 49 protons and 35 carbon atoms. The appearance of the pseudomolecular ion peak $[M + 1]^+$ at *m/z* = 740 corresponding to the molecular formula $C_{35}H_{49}N_9O_9$ in the mass spectrum of **8**, along with other fragment

Table 3. Antibacterial activity data[a].

Compd.	Diameter of zone of inhibition (mm) Bacterial strains					
	C. pyogenes	*S. aureus*	*B. subtilis*	*K. pneumoniae*	*P. aeruginosa*	*E. coli*
7	10(50)	12(25)	9(50)	16(6)	13(6)	11(6)
8	14(50)	17(25)	13(50)	20(6)	19(6)	15(6)
Control	–	–	–	–	–	–
Gatifloxacin	20(12.5)	28(6)	18(12.5)	25(6)	24(6)	19(6)

[a] Values in parentheses are MIC values ($\mu g\,mL^{-1}$).

ion peaks resulting from cleavage at 'Gly-Thr', 'Thr-Pro' and 'Pro-Asn' amide bonds showed the exact sequence of the attachment of all the seven amino acid moieties in a chain. In addition, the presence of the immonium ion peaks at *m/z* = 159 (Trp), 87 (Asn), 86 (Leu), 74 (Thr), 70 (Pro), 44 (Ala), and 30 (Gly) further confirmed all the seven amino acid moieties in the cyclopeptide structure. Furthermore, the elemental analysis of **8** afforded values with tolerances of ± 0.03 in strict accordance with the molecular composition.

The synthesized cyclopeptide **8** exhibited moderate cytotoxic activity against *Dalton's lymphoma ascites* (DLA) and *Ehrlich's ascites carcinoma* (EAC) cell lines with CTC_{50} values of 11.6 and 14.1 μM, respectively, in comparison to the standard drug 5-fluorouracil (5-FU) (CTC_{50} values – 37.4 and 90.6 μM) (Table 1). The possible mechanism of the cytotoxic action of **8** might be through apoptosis *via* induction of early cell death, nuclear fragmentation and internucleosomal DNA scission. Comparison of the antifungal activity data suggested that **8** possessed potent bioactivity against dermatophytes *M. audouinii* and *T. mentagrophytes* and moderate antifungal activity against pathogenic *Candida albicans* with MIC values of 6 μg/mL when compared to the reference drug griseofulvin (Tables 2 and 3). Moreover, a moderate level of activity was observed against Gram-negative bacteria *K. pneumoniae*, *P. aeruginosa* and *E. coli* for the newly synthesized cyclopeptide, in comparison to the standard drug gatifloxacin. However, **8** displayed no significant activity against neither Gram-positive bacteria nor pathogenic *Ganoderma sp*. and *Aspergillus niger*. In addition, the analysis of the pharmacological activity data revealed that cycloheptapeptide **8** displayed a higher bioactivity against pathogenic microbes and cell lines than its linear form **7**. This is possibly because cyclization of peptides reduces the degree of freedom for each constituent within the ring and thus substantially leads to reduced flexibility, increased potency and selectivity of cyclic peptides. Further, the inherent flexibility of linear peptides leads to different conformations which can bind to more than one receptor molecule, resulting in undesirable adverse effects.

Conclusion

The present study presents the successful synthesis of the natural peptide annomuricatin B (**8**) in good yield *via* coupling reactions utilizing different carbodiimides. The DIPC/TEA coupling method proved to be yield-effectice, in comparison to methods utilizing EDC·HCl and NMM, providing 10 – 12 % additional yield. The pentafluorophenyl ester was shown to be better for the activation of the acid functionality of the linear heptapeptide unit when compared to the *p*-nitrophenyl ester. Pyridine was found to be a good base for the intramolecular cyclization of the linear peptide fragment in comparison to TEA or NMM. The synthesized cycloheptapeptide displayed moderate cytotoxicity as well as potent antidermatophyte activity. In comparison, Gram-negative bacteria were found to be more sensitive than Gram-positive bacteria towards the newly synthesized peptide. On passing toxicity tests, cyclopeptide **8** may prove as a good candidate for clinical studies and can be a new antifungal and cytotoxic drug of future.

Experimental Section

General methods

Melting points were determined using a Jindal Scientific melting point apparatus (Jindal, Delhi, India) by the open capillary method and are uncorrected. IR spectra were recorded on an FTIR-8400S Fourier transform spectrophotometer (Shimadzu, Kyoto, Japan) using a thin film supported on KBr pellets for solids and $CHCl_3$ as solvent for intermediate semisolids. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC 300 spectrometer at 300 MHz (Bruker, IL, USA) using $CDCl_3$ as solvent and tetramethylsilane as internal standard. Mass spectra were recorded on a JMS-DX 303 spectrometer (Jeol, Tokyo, Japan) operating at 70 eV using the fast atom bombardment technique. Elemental analyses of the cyclopeptide as well as of the intermediates were performed on a Vario EL III elemental analyzer (Elementar Vario EL III, Hanau, Germany). Optical rotation of the synthesized peptides was measured on an Optics Tech-

nology automatic polarimeter (OpticsTech, Delhi, India) in a 2 dm tube at 25 °C using a sodium lamp and methanol as solvent. Purity of the synthesized cyclopeptide and the intermediates was checked by TLC on precoated silica gel G plates (Kieselgel 0.25 mm, 60G F_{254}, Merck, Germany) utilizing $CHCl_3$·MeOH as developing solvent in different ratios. Brown spots were detected on exposure to iodine vapors in a tightly closed chamber.

*Protection of the carboxamide side chain of Boc-L-asparagine (**4**)*

To the solution of Boc-L-asparagine (2.32 g, 0.01 mol) in glacial acetic acid (GAA, 25 mL), benzhydrol (1.84 g, 0.01 mol) was added with stirring at room temperature (r. t.) for a time period of 30 min. Concentrated sulfuric acid (0.05 mL) was added to the mixture which was allowed to stand overnight. The reaction mixture was poured in water (75 mL). An oily product separated which soon solidified. The crude product was finally purified with ethyl acetate to provide 3 g (72 %) of the pure product as a white solid. M. p. 145 – 146 °C. – $[\alpha]_D = -11.7°$ (c = 0.2, MeOH). – IR (KBr): ν = 3295 – 2515 (O–H_{str}, CO*OH*), 3134 (N–H_{str}, amide), 3075, 3044 (C–H_{str}, rings), 2926, 2853 (C–H_{str}, CH_2), 1710 (C=O_{str}, *CO*OH), 1642 (C=O_{str}, 2° amide), 1535 (N–H_{def}, 2° amide), 1574, 1482, 1477 (skeletal bands), 1409 (C–OH_{def}, COOH), 1395, 1372 (C–H_{def}, *tert*-butyl), 738, 733, 695 – 692 (C–H_{def}, out-of-plane, rings) cm^{-1}. – ^{1}H NMR ($CDCl_3$): δ = 11.25 (br. s, 1 H, O*H*), 7.82 (br. s, 1 H, α-N*H*), 7.22 – 7.10 (m, 10 H, *o*-H′s, *m*-H′s and *p*-H′s, phenyl rings), 6.75 (br. s, 1 H, δ-N*H*), 6.02 (d, J = 5.5 Hz, 1 H, α-H, Bzh), 4.37 – 4.32 (m, 1 H, α-H, Asn), 2.95 (d, J = 4.9 Hz, 2 H, β-H′s, Asn), 1.55 (s, 9 H, *tert*-butyl).

*General procedure for the synthesis of linear tri/tetrapeptide segments **5** and **6***

To the solution of the amino acid methyl ester hydrochloride or dipeptide methyl ester (0.01 mol) in DMF (25 mL), NMM (0.021 mol) was added at 0 °C, and the reaction mixture was stirred for 15 min. The Boc-dipeptide (0.01 mol) in DMF (25 mL) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 0.01 mol) were added with stirring. Stirring was first done for 1 h at 0 – 5 °C and then for another 24 – 36 h at r. t. After the completion of the reaction, the reaction mixture was diluted with an equal amount of water. The precipitated solid was filtered, washed with water and recrystallized from a mixture of chloroform and petroleum ether (b. p. 40 – 60 °C) followed by cooling at 0 °C to get the title compounds.

*tert-Butyloxycarbonyl-L-asparaginyl(bzh)-L-alanyl-L-tryptophan methyl ester (**5**)*

Semisolid mass. Yield 79 %. – $[\alpha]_D = -93.4°$ (c = 0.25, MeOH). – R_f = 0.59 ($CHCl_3$-MeOH = 8 : 2). – IR ($CHCl_3$): ν = 3492 (N–H_{str}, ring), 3127, 3122 (N–H_{str}, amide), 3077, 3056 – 3052 (C–H_{str}, rings), 2969, 2925 (C–H_{str}, asym, CH_3 and CH_2), 2872, 2846 (C–H_{str}, sym, CH_3 and CH_2), 1744 (C=O_{str}, ester), 1646, 1640 (C=O_{str}, amide), 1575 – 1569, 1480 – 1476 (skeletal bands), 1537, 1532 (N–H_{def}, amide), 1387, 1372 (C–H_{def}, *tert*-butyl), 1267 (C–O_{str}, ester), 739 – 732, 697 – 693, 675 (C–H_{def}, out-of-plane, rings) cm^{-1}. – ^{1}H NMR ($CDCl_3$): δ = 8.95 (br. s, 1 H, N*H*, indole), 8.83 (br. s, 1 H, N*H*), 8.52 (br. s, 1 H, N*H*), 7.53 (d, J = 7.85 Hz, 1 H, β-H, indole), 7.25 – 7.18 (m, 6 H, *m*-H′s and *p*-H′s, phenyl rings, Bzh), 7.15 – 7.06 (m, 4 H, δ–η-H′s, indole), 7.05 – 6.98 (m, 4 H, *o*-H′s, phenyl rings, Bzh), 6.89 (br. s, 1 H, δ-N*H*, Asn), 6.77 (br. s, 1 H, N*H*), 5.98 (d, J = 5.45 Hz, 1 H, α-H, Bzh), 4.46 – 4.42 (m, 1 H, α-H, Asn), 4.41 – 4.37 (m, 1 H, α-H, Ala), 4.24 – 4.18 (m, 1 H, α-H, Trp), 3.56 (s, 3 H, OCH_3), 3.22 (d, J = 4.85 Hz, 2 H, β-H′s, Asn), 2.98 (d, J = 5.65 Hz, 2 H, β-H′s, Trp), 1.48 (d, J = 5.9 Hz, 3 H, β-H′s, Ala), 1.55 (s, 9H, *tert*-butyl). – $C_{37}H_{43}N_5O_7$ (669.77): calcd. C 66.35, H 6.47, N 10.46; found C 66.38, H 6.45, N 10.45.

*tert-Butyloxycarbonyl-L-leucyl-glycyl-L-thryl-L-proline methyl ester (**6**)*

Off-white solid. M. p. 112 – 113 °C. Yield 75 %. – $[\alpha]_D$ = +13.4° (c = 0.25, MeOH). R_f = 0.73 ($CHCl_3$-MeOH = 8 : 2). – IR (KBr): ν = 3342 (O–H_{str}), 3129, 3124 (N–H_{str}, amide), 2998 – 2992 (C–H_{str}, CH_2, Pro), 2968, 2927, 2923 (C–H_{str}, asym, CH_3 and CH_2), 2854, 2849 (C–H_{str}, sym, CH_2), 1747 (C=O_{str}, ester), 1665, 1647 – 1642 (C=O_{str}, 3° and 2° amide), 1538 – 1533 (N–H_{def}, 2° amide), 1389, 1370 (C–H_{def}, *tert*-butyl), 1383, 1362 (C–H_{def}, *iso*-propyl), 1269, 1095 (C–O_{str}, ester and C–OH) cm^{-1}. – ^{1}H NMR ($CDCl_3$): δ = 9.35 (br. s, 1 H, N*H*), 8.25 (br. s, 1 H, N*H*), 6.52 (br. s, 1 H, N*H*), 4.49 (br. s, 1 H, O*H*), 4.28 (dd, J = 6.2 Hz, 4.9 Hz, 1 H, α-H, Thr), 4.13 (d, J = 5.45 Hz, 2 H, α-H, Gly), 3.93 (t, 1 H, J = 6.85 Hz, α-H, Pro), 3.89 – 3.85 (m, 1 H, β-H, Thr), 3.84 – 3.78 (m, 1 H, α-H, Leu), 3.62 (s, 3 H, OCH_3), 3.40 (t, 2 H, J = 7.2 Hz, δ-H, Pro), 2.05 – 1.98 (m, 4 H, β-H and γ-H, Pro), 1.89 (t, 2 H, J = 5.9 Hz, β-H, Leu), 1.66 – 1.59 (m, 1 H, γ-H, Leu), 1.56 (s, 9 H, *tert*-butyl), 1.27 (d, 3 H, J = 5.8 Hz, γ-H, Thr), 0.99 (d, 6 H, J = 6.25 Hz, δ-H, Leu). – $C_{23}H_{40}N_4O_8$ (500.59): calcd. C 55.19, H 8.05, N 11.19; found C 55.22, H 8.03, N 11.20.

Deprotection of the tripeptide unit at the carboxyl terminal

To a solution of the tripeptide **5** (6.7 g, 0.01 mol) in THF-H_2O (1 : 1, 36 mL), LiOH (0.36 g, 0.015 mol) was added at 0 °C. The mixture was stirred at r. t. for 1 h and then acidified to pH = 3.5 with 1 N H_2SO_4. The aqueous layer was extracted with Et_2O (3 × 25 mL). The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was finally crystallized from methanol and ether to get the pure deprotected compound **5a**.

*tert-Butyloxycarbonyl-L-asparaginyl(bzh)-L-alanyl-L-tryptophan (**5a**)*

White solid. M. p. 91 – 93 °C. Yield 87 %. – $[\alpha]_D$ = −69.7° (*c* = 0.25, MeOH). R_f = 0.77 ($CHCl_3$-MeOH = 8 : 2). – IR (KBr): *v* = 3489 (N–H_{str}, ring), 3297 – 2509 (O–H_{str}, CO*OH*), 3129, 3126 (N–H_{str}, amide), 3075, 3055 – 3052 (C–H_{str}, rings), 2968, 2927 (C–H_{str}, asym, CH_3 and CH_2), 2874, 2845 (C–H_{str}, sym, CH_3 and CH_2), 1713 (C=O_{str}, *CO*OH), 1645, 1639 (C=O_{str}, amide), 1576 – 1568, 1482 – 1478 (skeletal bands), 1538 – 1533 (N–H_{def}, amide), 1386, 1370 (C–H_{def}, *tert*-butyl), 737 – 732, 698 – 692, 678 (C–H_{def}, out-of-plane, rings) cm^{-1}. – ^1H NMR ($CDCl_3$): δ = 10.48 (br. s, 2 H, N*H*, indole and O*H*, COO*H*), 8.87 (br. s, 1 H, N*H*), 8.50 (br. s, 1 H, N*H*), 7.55 (d, *J* = 7.9 Hz, 1 H, β-H, indole), 7.52 (br. s, 1 H, N*H*), 7.22 – 7.17 (m, 6 H, *m*-H′s and *p*-H′s, phenyl rings, Bzh), 7.16 – 7.08 (m, 4 H, δ–η-H′s, indole), 7.06 – 6.99 (m, 4 H, *o*-H′s, phenyl rings, Bzh), 6.87 (br. s, 1 H, δ-N*H*, Asn), 5.99 (d, *J* = 5.5 Hz, 1 H, α-H, Bzh), 5.43 – 5.37 (m, 1 H, α-H, Ala), 4.48 – 4.43 (m, 1 H, α-H, Trp), 4.42 – 4.38 (m, 1 H, α-H, Asn), 3.28 (d, *J* = 5.7 Hz, 2 H, β-H′s, Trp), 3.02 (d, *J* = 4.9 Hz, 2 H, β-H′s, Asn), 1.53 (s, 9H, *tert*-butyl), 1.49 (d, *J* = 5.85 Hz, 3 H, β-H′s, Ala). – $C_{36}H_{41}N_5O_7$(655.75): calcd. C 65.94, H 6.30, N 10.68; found C 65.95, H 6.27, N 10.70.

Deprotection of the tetrapeptide unit at the amino terminal

Tetrapeptide **6** (5.0 g, 0.01 mol) was dissolved in $CHCl_3$ (15 mL) and treated with CF_3COOH (2.28 g, 0.02 mol). The resulting solution was stirred at r. t. for 1 h and washed with a saturated $NaHCO_3$ solution (25 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by crystallization from $CHCl_3$ and petroleum ether (b. p. 40 – 60 °C) to get the pure deprotected compound **6a**.

*L-Leucyl-glycyl-L-thryl-L-proline methyl ester (**6a**)*

Semisolid mass. Yield 83 %. – $[\alpha]_D$ = +2.8° (*c* = 0.25, MeOH). R_f = 0.55 ($CHCl_3$-MeOH = 8 : 2). – IR (KBr): *v* = 3379, 3268 (N–H_{str}, amine), 3371 (O–H_{str}), 3133, 3126 (N–H_{str}, amide), 2997 – 2992 (C–H_{str}, CH_2, Pro), 2966, 2929, 2922 (C–H_{str}, asym, CH_3 and CH_2), 2855, 2848 (C–H_{str}, sym, CH_2), 1748 (C=O_{str}, ester), 1667, 1649 – 1644 (C=O_{str}, 3° and 2° amide), 1624 (N–H_{def}, amine), 1539, 1536, 1533 (N–H_{def}, 2° amide), 1382, 1364 (C–H_{def}, *iso*-propyl), 1272, 1092 (C–O_{str}, ester and C–OH), 1139 (C–N_{str}, amine) cm^{-1}. – ^1H NMR ($CDCl_3$): δ = 9.33 (br. s, 1 H, N*H*), 8.19 (br. s, 1 H, N*H*), 4.30 (dd, *J* = 6.15 Hz, 4.85 Hz, 1 H, α-H, Thr), 4.07 (d, *J* = 5.5 Hz, 2 H, α-H, Gly), 3.92 (t, 1 H, *J* = 6.9 Hz, α-H, Pro), 3.88 – 3.83 (m, 1 H, β-H, Thr), 3.64 (s, 3 H, OCH_3), 3.42 (t, 2 H, *J* = 7.15 Hz, δ-H, Pro), 3.30 – 3.26 (m, 1 H, α-H, Leu), 2.07 – 1.99 (m, 4 H, β-H and γ-H, Pro), 1.82 (t, 2 H, *J* = 5.95 Hz, β-H, Leu), 1.25 (d, 3 H, *J* = 5.75 Hz, γ-H, Thr), 1.15 – 1.07 (m, 1 H, γ-H, Leu), 0.98 (d, 6 H, *J* = 6.2 Hz, δ-H, Leu), 0.11 (br. s, 3 H, O*H* and NH_2). – $C_{18}H_{32}N_4O_6$ (400.47): calcd. C 53.99, H 8.05, N 13.99; found C 54.01, H 8.02, N 14.02.

*Procedure for the synthesis of the linear heptapeptide unit **7***

Compound **6a** (4.0 g, 0.01 mol) was dissolved in THF (35 mL). To this solution, TEA (2.8 mL, 0.021 mol) was added at 0 °C, and the resulting mixture was stirred for 15 min. Compound **5a** (6.6 g, 0.01 mol) was dissolved in THF (35 mL), and DIPC (1.26 g, 0.01 mol) was added to the mixture with stirring. Stirring was continued for 24 h, after which the reaction mixture was filtered, and the filtrate was washed with 5 % $NaHCO_3$ and saturated NaCl solutions (30 mL each). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated in vacuum. The crude product was recrystallized from a mixture of chloroform and petroleum ether (b. p. 40 – 60 °C) followed by cooling at 0 °C.

*tert-Butyloxycarbonyl-L-asparaginyl(bzh)-L-alanyl-L-tryptophyl-L-leucyl-glycyl-L-thryl-L-proline methyl ester (**7**)*

Semisolid mass. Yield 78 %. – $[\alpha]_D$ = −51.8° (*c* = 0.35, MeOH). R_f = 0.63 ($CHCl_3$-MeOH = 9 : 1). – IR ($CHCl_3$): *v* = 3486 (N–H_{str}, ring), 3374 (O–H_{str}), 3132 – 3125 (N–H_{str}, amide), 2998 – 2991 (C–H_{str}, CH_2, Pro), 3077, 3056, 3053 (C–H_{str}, rings), 2969, 2928, 2922 (C–H_{str}, asym, CH_3 and CH_2), 2875, 2847, 2844 (C–H_{str}, sym, CH_3 and CH_2), 1748 (C=O_{str}, ester), 1669, 1646 – 1639 (C=O_{str}, amide), 1578 – 1569, 1484 – 1479 (skeletal bands), 1539, 1535, 1530 (N–H_{def}, amide), 1388, 1371 (C–H_{def}, *tert*-butyl), 1381, 1363 (C–H_{def}, *iso*-propyl), 1270, 1094 (C–O_{str}, ester and C–OH), 736 – 731, 699 – 694, 676 (C–H_{def}, out-of-plane, rings) cm^{-1}. – ^1H NMR ($CDCl_3$): δ = 8.90 (br. s, 1 H, N*H*), 8.75 (br. s, 1 H, N*H*), 8.63 (br. s, 1 H, N*H*), 8.50 (br. s, 1 H, N*H*), 8.19 (br. s, 1 H, δ-N*H*, Asn), 8.16 (br. s, 1 H, N*H*), 7.82 (br. s, 2 H, N*H*, indole and O*H*, Thr), 7.69 (br. s, 1 H, N*H*), 7.25 (d, *J* = 7.85 Hz, 1 H, β-H, indole), 7.23 – 7.16 (m, 6 H, *m*-H′s and *p*-H′s, phenyl rings, Bzh), 7.15 – 7.10 (m, 4 H, δ–η-H′s, indole), 7.09 – 7.03 (m, 4 H, *o*-H′s, phenyl rings, Bzh), 5.98 (d, *J* = 5.45 Hz, 1 H, α-H, Bzh), 4.73 – 4.68 (m, 1 H, α-H, Ala), 4.45 – 4.41 (m, 1 H, α-H, Asn), 4.24 – 4.19 (m, 1 H, α-H, Trp), 4.09 (dd, *J* = 6.2 Hz, 4.85 Hz, 1 H, α-H, Thr), 4.02 (d, *J* = 5.45 Hz, 2 H, α-H′s, Gly), 3.90 (t, *J* = 6.85 Hz, 1 H, α-H, Pro), 3.77 – 3.72 (m, 1 H, β-H, Thr), 3.63 (s, 3 H, OCH_3), 3.52 – 3.48 (m, 1 H, α-H, Leu), 3.45 (t, *J* = 7.2 Hz, 2 H, δ-H′s, Pro), 3.22 (d, *J* = 4.85 Hz, 2 H, β-H′s, Asn), 3.18 (d, *J* = 5.65 Hz, 2 H, β-H′s, Trp), 2.06 – 1.98 (m, 4 H, β-H′s and γ-H′s, Pro), 1.71 (t, *J* = 5.9 Hz, 2 H, β-H′s, Leu), 1.54 (s, 9 H, *tert*-butyl), 1.50 – 1.43 (m, 1 H, γ-H, Leu), 1.32 (d, *J* = 5.8 Hz, 3 H, γ-H′s, Thr), 1.22 (d, *J* = 5.8 Hz, 3 H, β-H′s, Ala), 0.99 (d, *J* = 6.15 Hz, 6 H, δ-

H′s, Leu). – ^{13}C NMR ($CDCl_3$): δ = 173.8 (γ-C=O, Asn), 172.1 (C=O, Pro), 170.5 (C=O, Ala), 169.8 (C=O, Asn), 168.7 (C=O, Leu), 167.3 (C=O, Trp), 166.5 (C=O, Gly), 163.3 (C=O, Thr), 154.6 (C=O, Boc), 151.4 (2 C, β-C′s, Bzh), 137.7 (2′-C, indole), 129.6 (2 C, *o*-C′s, Ph-1, Bzh), 128.3 (2 C, *o*-C′s, Ph-2, Bzh), 127.8 (3′-C, indole), 127.2 (2 C, *m*-C′s, Ph-1, Bzh), 126.5 (2 C, *p*-C′s, Bzh), 125.9 (2 C, *m*-C′s, Ph-2, Bzh), 125.0 (5-C, indole), 124.4 (6-C, indole), 122.9 (2-C, indole), 119.4 (4-C, indole), 111.6 (7-C, indole), 110.7 (3-C, indole), 79.9 (α-C, *tert*-butyl), 72.2 (α-C, Asn), 69.5 (β-C, Thr), 66.8 (α-C, Thr), 60.3 (α-C, Pro), 56.8 (α-C, Bzh), 54.5 (α-C, Trp), 53.9 (O*C*H$_3$), 53.2 (α-C, Leu), 51.8 (α-C, Ala), 50.4 (β-C, Asn), 45.8 (δ-C, Pro), 44.5 (β-C, Leu), 42.7 (α-C, Gly), 34.4 (β-C, Trp), 29.0 (β-C, Pro), 28.3 (3 C, β-C′s, *tert*-butyl), 26.6 (γ-C, Leu), 24.8 (γ-C, Pro), 23.2 (2 C, δ-C′s, Leu), 22.0 (γ-C, Thr), 18.3 (β-C, Ala). – $C_{54}H_{71}N_9O_{12}$ (1038): calcd. C 62.47, H 6.89, N 12.14; found C 62.49, H 6.92, N 12.15.

*Synthesis of the cyclic heptapeptide – annomuricatin B (**8**)*

To synthesize compound **8**, the linear heptapeptide unit **7** (5.2 g, 0.005 mol) was deprotected at the carboxyl end using LiOH (0.18 g, 0.0075 mol) to get Boc-L-Asn(bzh)-L-Ala-L-Trp-L-Leu-Gly-L-Thr-L-Pro-OH. The deprotected heptapeptide unit (5.12 g, 0.005 mol) was now dissolved in $CHCl_3$ (50 mL) at 0 °C. To this solution, 0.0067 mol of *p*-nitrophenol or pentafluorophenol (0.94 g or 1.23 g) and DIPC (0.63 g, 0.005 mol) were added, and stirring was done at r. t. for 12 h. The reaction mixture was filtered, and the filtrate was washed with 10 % $NaHCO_3$ solution (3 × 25 mL) and finally with 5 % HCl (2 × 30 mL) to get the corresponding *p*-nitrophenyl or pentafluorophenyl esters Boc-L-Asn(bzh)-L-Ala-L-Trp-L-Leu-Gly-L-Thr-L-Pro-Opnp or Boc-L-Asn(bzh)-L-Ala-L-Trp-L-Leu-Gly-L-Thr-L-Pro-Opfp. To this compound (4.58 g or 4.76 g, 0.004 mol) dissolved in $CHCl_3$ (35 mL), CF_3COOH (0.91 g, 0.008 mol) was added, and the mixture was stirred at r. t. for 1 h and washed with 10 % $NaHCO_3$ solution (2 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4 to get L-Asn-L-Ala-L-Trp-L-Leu-Gly-L-Thr-L-Pro-Opnp or L-Asn-L-Ala-L-Trp-L-Leu-Gly-L-Thr-L-Pro-Opfp which was dissolved in $CHCl_3$ (25 mL), and TEA/NMM/C_5H_5N (2.8 mL or 2.21 mL or 1.61 mL, 0.021 mol) was added. Then the mixture was kept at 0 °C for 7 d. The reaction mixture was washed with 10 % $NaHCO_3$ (3 × 25 mL) and 5 % HCl (2 × 30 mL) solutions. The organic layer was dried over anhydrous Na_2SO_4, and the crude product was crystallized from $CHCl_3$-*n*-hexane to get the pure cyclic product **8**.

*Cyclo (L-Asn-L-Ala-L-Trp-L-Leu-Gly-L-Thr-L-Pro) (**8**)*

White crystals. M. p. 212 – 213 °C (213 °C for natural annomuricatin B [15]). Yield 85 % (C_5H_5N), 78 % (NMM), 69 % (TEA). – $[\alpha]_D$ = −37.3° (*c* = 0.5, MeOH) (−37.25° for natural annomuricatin B [15]). R_f = 0.81 ($CHCl_3$-MeOH = 9 : 1). – IR (KBr): ν = 3488 (N–H$_{str}$, ring), 3372 (O–H$_{str}$), 3350, 3178 (N–H$_{str}$, 1° amide), 3136 – 3127 (N–H$_{str}$, 2° amide), 2998 – 2993 (C–H$_{str}$, CH_2, Pro), 3074 (C–H$_{str}$, ring), 2967, 2929, 2920 (C–H$_{str}$, asym, CH_3 and CH_2), 2878, 2844, 2842 (C–H$_{str}$, sym, CH_3 and CH_2), 1667, 1653, 1645 – 1634 (C=O$_{str}$, amides), 1628 (NH$_{2(def)}$, 1° amide), 1572, 1480 (skeletal bands), 1538 – 1532, 1529 – 1525 (N–H$_{def}$, amide), 1405 (C–N$_{str}$, 1° amide), 1383, 1362 (C–H$_{def}$, *iso*-propyl), 1095 (C–O$_{str}$, C–OH), 735, 677 (C–H$_{def}$, out-of-plane, ring) cm^{-1}. – ^{1}H NMR ($CDCl_3$): δ = 11.78 (br. s, 1 H, N*H*), 8.25 (br. s, 2 H, N*H*, indole and O*H*, Thr), 8.20 (br. s, 1 H, N*H*), 8.05 (br. s, 1 H, N*H*), 7.93 (br. s, 1 H, N*H*), 7.28 (d, *J* = 7.9 Hz, 1 H, β-H, indole), 7.25 (d, *J* = 7.65 Hz, 1 H, δ-H, indole), 7.14 (br. s, 1 H, N*H*), 7.13 – 7.10 (m, 3 H, ε–η-H′s, indole), 6.97 (br. s, 1 H, N*H*), 6.75 (br. s, 2 H, N*H*$_2$, Asn), 6.28 – 6.24 (m, 1 H, α-H, Leu), 5.98 – 5.93 (m, 1 H, α-H, Ala), 5.78 (dd, *J* = 6.15 Hz, 4.9 Hz, 1 H, α-H, Thr), 5.74 – 5.69 (m, 1 H, α-H, Trp), 5.28 (d, *J* = 5.5 Hz, 2 H, α-H′s, Gly), 4.99 – 4.95 (m, 1 H, α-H, Asn), 3.94 (t, *J* = 6.9 Hz, 1 H, α-H, Pro), 3.81 – 3.75 (m, 1 H, β-H, Thr), 3.26 (t, *J* = 7.15 Hz, 2 H, δ-H′s, Pro), 2.95 (d, *J* = 4.9 Hz, 2 H, β-H′s, Asn), 2.88 (d, *J* = 5.7 Hz, 2 H, β-H′s, Trp), 2.67 – 2.63 (m, 2 H, β-H′s, Pro), 1.88 (t, *J* = 5.85 Hz, 2 H, β-H′s, Leu), 1.85 – 1.79 (m, 2 H, γ-H′s, Pro), 1.47 (d, *J* = 5.75 Hz, 3 H, β-H′s, Ala), 1.42 (d, *J* = 5.7 Hz, 3 H, γ-H′s, Thr), 0.98 (d, *J* = 6.2 Hz, 6 H, δ-H′s, Leu), 0.86 – 0.78 (m, 1 H, γ-H, Leu). – ^{13}C NMR ($CDCl_3$): δ = 175.2 (γ-C=O, Asn), 174.8 (C=O, Trp), 174.0 (C=O, Thr), 172.6 (C=O, Ala), 171.4 (C=O, Asn), 169.8 (C=O, Gly), 168.9 (C=O, Pro), 165.7 (C=O, Leu), 137.3, 129.6 (2 C, 2′-C and 3′-C, indole), 125.5 (5-C, indole), 122.3 (2-C, indole), 119.2 (4-C, indole), 117.4 (6-C, indole), 112.0 (7-C, indole), 110.3 (3-C, indole), 68.4 (β-C, Thr), 59.7 (α-C, Pro), 57.2 (α-C, Thr), 56.0 (α-C, Asn), 55.7 (α-C, Trp), 50.2 (α-C, Ala), 49.5 (α-C, Leu), 48.8 (δ-C, Pro), 44.5 (α-C, Gly), 43.6 (β-C, Asn), 39.2 (β-C, Leu), 33.0 (β-C, Pro), 30.3 (γ-C, Leu), 26.8 (β-C, Trp), 25.0 (γ-C, Pro), 23.7 (2 C, δ-C′s, Leu), 23.1 (γ-C, Thr), 18.9 (β-C, Ala). – MS (FAB, 70 eV): *m/z* (%) = 740 (100) $[M + 1]^+$, 712 (11) $[740–CO]^+$, 683 (72) [Thr-Pro-Asn-Ala-Trp-Leu]$^+$, 582 (39) [Pro-Asn-Ala-Trp-Leu]$^+$, 570 (27) [Thr-Pro-Asn-Ala-Trp]$^+$, 554 (14) $[582–CO]^+$, 542 (18) $[570–CO]^+$, 485 (48) [Asn-Ala-Trp-Leu]$^+$, 384 (55) [Thr-Pro-Asn-Ala]$^+$, 372 (29) [Asn-Ala-Trp]$^+$, 356 (14) $[384–CO]^+$, 344 (10) $[372–CO]^+$, 313 (32) [Thr-Pro-Asn]$^+$, 283 (19) [Pro-Asn-Ala]$^+$, 199 (39) [Thr-Pro]$^+$, 171 (10) $[199–CO]^+$, 159 (16) $[C_{10}H_{11}N_2]^+$, 130 (9), 116 (11), 115 (8) [Asn]$^+$, 102 (15) [Thr]$^+$, 98 (13) [Pro]$^+$, 87 (11) $[C_3H_7N_2O]^+$, 86 (16) $[C_5H_{12}N]^+$, 74 (14) $[C_3H_8NO]^+$, 70 (10) $[C_4H_8N]^+$, 58 (7), 57 (13), 45 (8), 44 (8) $[C_2H_6N]^+$, 43 (16), 42 (12), 30 (9) $[CH_4N]^+$, 17 (4), 15 (6) $[CH_3]^+$. – $C_{35}H_{49}N_9O_9$(739): calcd. C 56.82, H 6.68, N 17.04; found C 56.79, H 6.70, N 17.05.

Pharmacological activity studies

Cytotoxicity screening

The synthesized linear and cyclic heptapeptides (**7**, **8**) were subjected to a short term *in vitro* cytotoxicity study [17] against *Dalton's lymphoma ascites* (NCRC 101) and *Ehrlich's ascites carcinoma* (NCRC 69) cell lines at 62.5 – 3.91 μg mL^{-1} using 5-fluorouracil (5-FU) as reference compound. The activity was assessed by determining the percentage inhibition of DLA and EAC cells. CTC_{50} values were determined by the graphical extrapolation method (Table 1).

Antimicrobial screening

The synthesized linear and cyclic heptapeptides (**7**, **8**) were evaluated for their antimicrobial activity against six bacterial strains *Corynebacterium pyogenes* (MUMC 73), *Staphylococcus aureus* (MUMC 377), *Bacillus subtilis* (MUMC 408), *Klebsiella pneumoniae* (MUMC 95), *Pseudomonas aeruginosa* (MUMC 266) and *Escherichia coli* (MUMC 106), and five fungal strains *Candida albicans* (MUMC 29), *Aspergillus niger* (MUMC 77), *Ganoderma sp*. (MUMC 218), *Microsporum audouinii* (MUMC 545) and *Trichophyton mentagrophytes* (MUMC 665) at 50 – 6 μg mL^{-1} using the Kirby-Bauer disk diffusion method [18]. MIC values of the test compounds were determined by the tube dilution technique. The solvents DMF and DMSO were used as negative controls, and gatifloxacin and griseofulvin were used as antibacterial and antifungal standards (Tables 2 and 3).

Experimental details of the biological activity test procedures are given in our previously published reports [19].

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