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ORIGINAL ARTICLE

Synthesis, structural aspects, antimicrobial activity and ion transport investigation of five new [1+1] condensed cycloheterophane peptides

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Abstract Five novel [1+1] condensed cycloheterophane peptides were synthesized via reaction of pyridine-2,6-bis(2-aminothiophenoxymethyl) with several diacid chlorides: glutaryl dichloride, adipoyl dichloride, 2,2′-thiodiacetyl chloride, dithiodiglycoloyl chloride and 3,3′-thiodipropionoyl chloride combinations (**L_1**–**L_5**). The compounds were characterized by elemental analyses, mass, FT-IR, ^{1}H, and ^{13}C NMR spectral data. The antimicrobial activities of the compounds were evaluated using the disk diffusion method in dimethyl sulfoxide as well as the minimal inhibitory concentration dilution method, against several bacteria and yeast cultures. The results were compared with those of commercial antibiotic and antifungal agents. Structure activity relationships were also discussed. Permeability of compound **L_5** against Na^+ and K^+ were also investigated.

Keywords Antimicrobial · Cycloheterophane · Diffusion · Ion transport · Permeability

Introduction

Crown ethers have evolved from simple macrocyclic ethers to more complicated, multifunctional cyclic compounds with a wide range of activities since their discovery [1, 2]. Cyclopeptides, systematically called cyclophane peptides, having many similarities with crown ethers, are very common natural products and found in the marine environment, as well as in higher plants. Marine cyanobacteria [3], green algae [4], marine sponges [5, 6], tropical phoma [7] are among the sources for naturally occurring cyclopeptides. Hydrophilic cavity and hydrophobic outer shell of these macrocycles enables them to carry cations from hydrophobic non polar media to aqueous environment and vice versa. Similar property has been observed in living organisms by carrying cations in and out of the cells by passing the membrane. Macrocyclic compounds containing both ether and amide functionalities show high selectivity towards earth alkaline metals [8] rather than alkali metals. The same type of affinity towards metals such as Hg^{2+}, Pb^{2+}, Cd^{2+} makes them extremely useful in environmental chemistry and biomedical research [9]. Another important aspect of these macrocyclic compounds is that they can be used as receptors for neutral molecules [10]. Hydrogen bonding in the cavity establishes complexation between neutral molecules and hetero macrocyclic compounds. Biological activity studies showed that large amount of macrocyclic peptides exhibit antimicrobial, antiviral, immunosuppressive, antitumor activities and inhibit fat accumulation and as a result, they are also very important synthetic targets [11–16].

The syntheses of the cycloheterophane peptides by cyclocondensation reactions are also well documented [17–19]. Corporation of pyridine unit may add interesting properties to cyclophane peptides due to their structural versatility and suitability for synthetic modifications. One possible application of these materials is in generating supramolecular arrays, which embody additional functional groups that are capable of molecular recognition, models for intercalation, building blocks for organic catalysts and

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metal complexation or interaction of biomolecules via internally or externally available networks.

Synthesis of similar mixed aza-oxo-thia macrocyclic tetrapeptides and their respective antimicrobial and cytotoxicity studies have recently been reported [20–22]. In this paper we focus on the synthesis, spectroscopic characterization, ion transportation and pharmacological investigation of the five new [1+1] condensed macrocycles, bearing nitrogen and sulfur donor centers. IUPAC Phane Nomenclature System has been used for naming the compounds (Fig. 1) [23, 24].

Chemistry

All the macrocyclic peptides were prepared through the main route depicted in Scheme 1, based on the condensation of diacid chlorides with diamines at low temperature in dry dichloromethane. The synthetic procedure to obtain the diacid chlorides was adapted from that of [25]. Esterification was carried out by using toluene and a Dean-Stark separator to remove water. The diols were prepared from related esters by reduction with $NaBH_4$ and purified by using chloroform and a soxhlet extractor. The synthetic procedure to obtain the tosylates was adapted from that of [26] with some modifications. They were then reacted with 2-aminothiophenol to generate diamine in a good yield 82 %. The full procedure for the preparation of the compounds is given in the "Experimental" section. The spectroscopic data from the mass, FT-IR, ^{1}H and ^{13}C NMR spectroscopy together with elemental analysis provide useful information about their formation and structural characterization.

Biological data

Standardized samples of penicillin G (blocking the formation of bacterial cell walls, rendering bacteria unable to multiply and spread); ampicillin (preventing the growth of

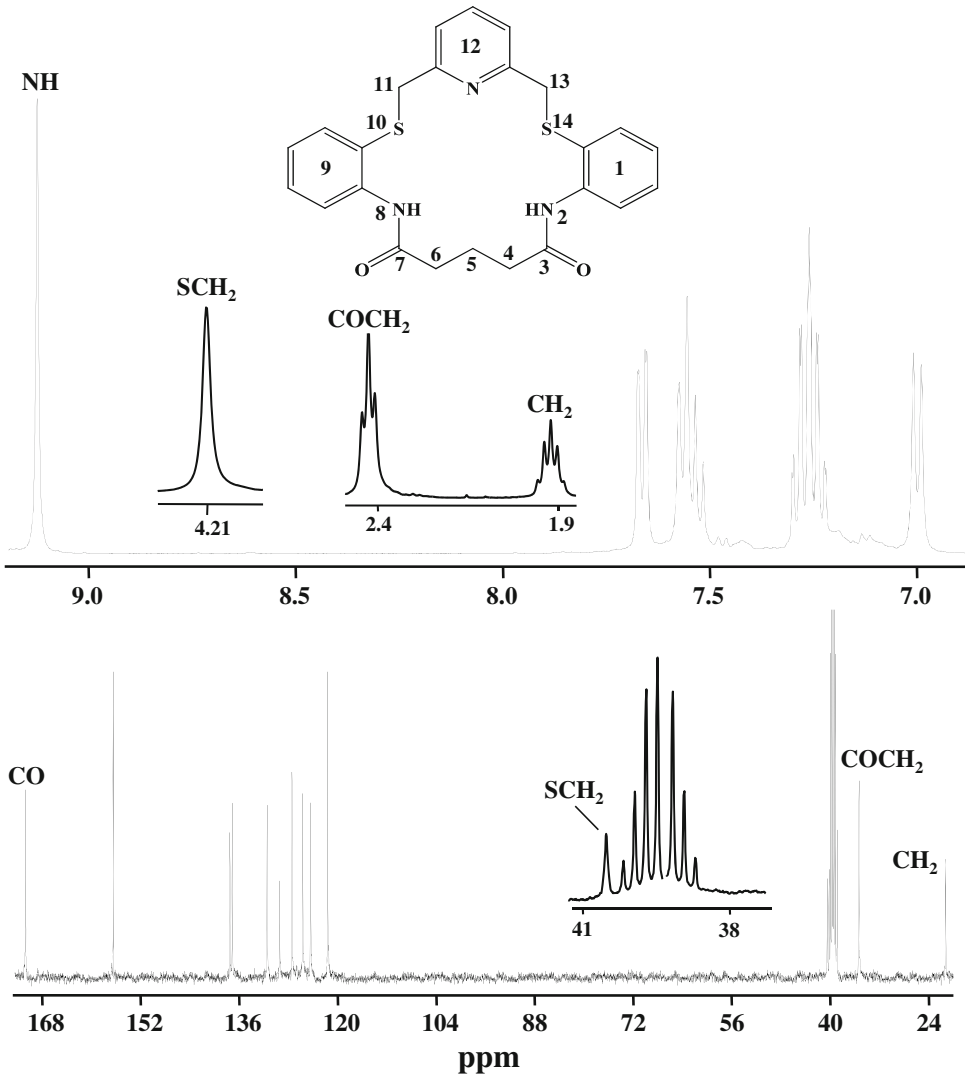
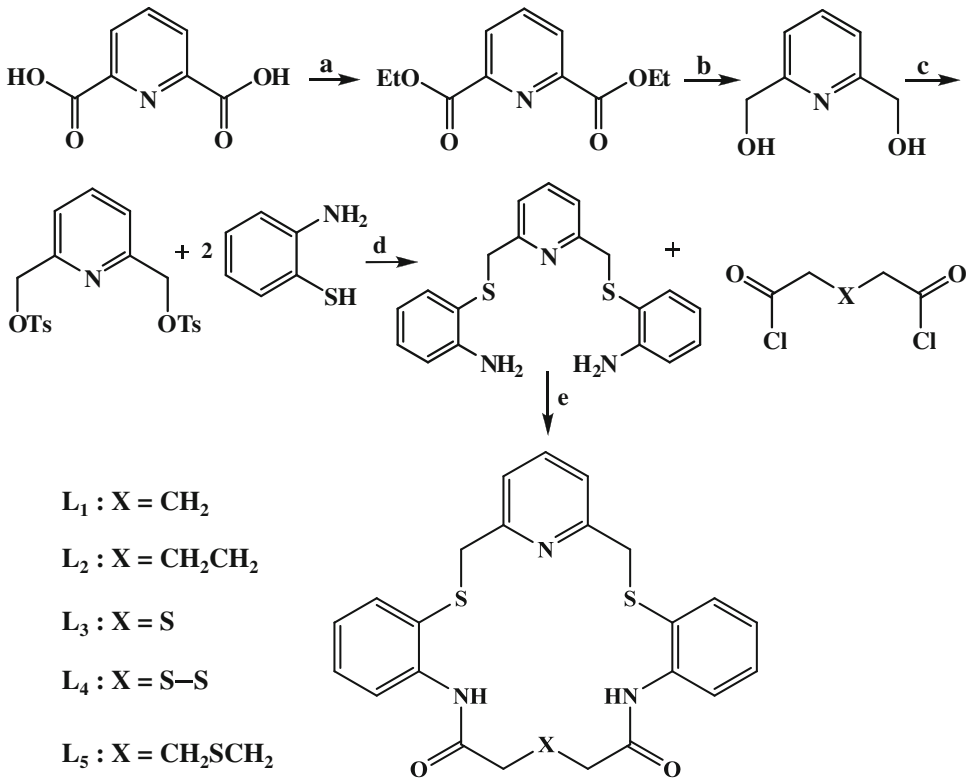


Fig. 1 1,9(1,2)-Dibenzena-2,8-diaza-3,7-dioxo-10,14-dithia-12(2,6)-pyridinacyclotetra decaphane($\mathbf{L_1}$) ^{1}H and ^{13}C NMR spectra in DMSO-d_6



Scheme 1 Reagents and conditions: **a** benzene, EtOH, H_2SO_4, Dean-Stark, 12 h, 87 %, **b** THF, $NaBH_4$, 12 h, 85 %, **c** TsCl, KOH, CH_2Cl_2, 87 %, **d** Na/EtOH, 60 °C, 3 h, 84 %, **e** CH_2Cl_2, pyridine, 0 °C 5 h, room temperature overnight, ≈65 %

bacteria mostly Gram-negative); cefotaxime (used against most Gram-negative enteric bacteria); ofloxacin (inhibiting DNA-gyrase, preventing the bacteria from reproducing); tetracyclines (effects through the inhibition of protein synthesis); nystatin (binding to sterols in the fungal cellular membrane, causing leakage of the cellular contents); ketoconazole (inhibiting the formation of the fungal cell wall) and clotrimazole (causing the fungal cells leakage).

Experimental

General

All chemicals including isophthaloyl chloride and solvents were reagent grade and were used as purchased without further purification except dichloromethane (CaH_2 under Ar) and toluene (Na/benzophenone under Ar). Melting points were determined using an Electro-thermal 9100 melting-point apparatus. Analytical data were obtained with a Thermo Finnigan Flash EA 1112 analyzer. Mass (ESI) analyses were carried out in positive ion modes using a Zivac® Tandem Gold Triple quadruple MS Spectrometer. FT-IR spectra were recorded on a Bruker Alpha-P (mid 4,000–400 cm^{-1}) as solid and far IR spectra (700–180 cm^{-1}) were recorded (as polyethylene pellets) on a Jasco FT/IR-600 Plus Spectrometer. Routine ^1H (400 MHz) and ^{13}C (100 MHz) spectra were recorded in DMSO-d_6 or $CDCl_3$ at ambient temperature on a Bruker Ultrashield Plus 400 MHz instrument. Chemical shifts (δ) are expressed in units of parts per million relative to TMS. The analytical data, Mass, FT-IR, NMR and physical properties are summarized for each experiment.

Synthesis of pyridine-2,6-dicarbonyl dichloride

This was adapted from the procedure [25] using 2,6-pyridine dicarboxylic acid (1.25 g, 7.5 mmol) and thionyl chloride (12 mL, 16.5 mmol) in methylene chloride (25 mL) (1.31 g, 86 %). Selected IR bands (cm^{-1}): 1,750 ν(CO).

Pyridine-2,6-dicarboxylic acid diethyl ester

According to procedure [27] pyridine-2,6-dicarboxylic acid (11.7 g, 70 mmol) and ethanol (100 mL), benzene (10 mL) and concentrated sulfuric acid (3 mL) was refluxed using a Dean-Stark receiver trap for 12 h. White crystalline solid was obtained (13.6 g, 87 %). M.P. 41–42 °C. (Ref. [27] 45–46 °C). ^1H NMR ($CDCl_3$), δ_H ppm: 1.47 (t, $2CH_3$, J = 7.25 Hz), 4.50 (q, $2CH_2$, J = 7.16 Hz), 8.01 (t, 2H, J = 7.75 Hz), 8.29 (d, H, J = 7.92 Hz).

2,6-Bis(hydroxymethyl)pyridine

According to procedure [28], pyridine-2,6-dicarboxylic acid diethyl ester (5.58 g, 25 mmol) and $NaBH_4$ (4.16 g, 110 mmol) in THF (50 mL) were used and a white solid

was obtained (2.96 g, 85 %). M.P. 112–114 °C. (Ref. [28] 115–120 °C) ATR (solid, cm^{-1}): 3,346 *ν*(O–H), 3,099 *ν*(C≡H), 1,593 *ν*(C≡C). ^{1}H NMR ($CDCl_3$), δ_H ppm: 4.79 (s, 4H), 7.20–7.79 (m, 3H).

2,6-[(Tosyloxy)methyl]pyridine

2,6-Bis(hydroxymethyl)pyridine (1.39 g, 10 mmol) in CH_2Cl_2 (35 mL), KOH (40 % aq, 35 mL) and *p*-toluenesulfonyl chloride (3.81 g, 20 mmol) were reacted according to procedure [26], affording white solid (3.89 g, 87 %). M.P. 119–121 °C. (Ref. [26] 121–122 °C) ATR (solid, cm^{-1}): ^{1}H NMR ($CDCl_3$), δ_H ppm: 2.46 (s, 6H, CH_3), 5.08 (s, 4H, CH_2), 7.34–7.83 (m, 11H).

2,6-Bis(2-aminophenylsulfanyl methyl) pyridine

This was prepared according to the procedure [29]. Reaction of 2-aminothiophenol (1.25 g, 10 mmol) in absolute EtOH (8 mL) containing Na (0.23 g, 10 mmol) and 2,6-[(tosyloxy)methyl]pyridine (2.23 g, 5 mmol) gave white solid product (1.47 g, 84 %). M. P. 80–82 °C (Ref. [29] 80–82 °C). ATR (solid, cm^{-1}): 3,410, 3,310 *ν*(N–H); 1,605 *δ*(N–C). ^{1}H NMR ($CDCl_3$), δ_H ppm: 4.00 (s, 4H, CH_2), 5.38 (s, 4H, NH), 6.40–7.58 (m, 11H). ^{13}C NMR ($CDCl_3$), δ_C ppm: 41.48 (CH_2), 115.09, 117.12, 118.42, 121.66, 130.37, 136.76, 136.82, 148.97, 158.01.

General procedure for [1+1] condensed cyclopeptides synthesis

A 250 mL two-necked, round-bottomed flask fitted with two dropping funnels was charged with dry CH_2Cl_2 (50 mL) and dry pyridine (3 mL) at 0 °C. A solution of diacid chloride (1 mmol) in dry methylene chloride (50 mL) and a solution of diamine (1 mmol) in dry methylene chloride (50 mL) were simultaneously added dropwise to a well stirred solution at the same rate under dry argon atmosphere for 5 h, while maintaining the temperature at 0 °C. After the addition was complete, the reaction mixture was left stirring at room temperature overnight. The solvent was evaporated on a rotary apparatus under vacuum and the residue was added very slowly into 300 mL of ice-cold distilled water with vigorous stirring. The white solid products were collected, washed several times with cold water, than with cold acetone and dried under vacuum.

*1,9(1,2)-Dibenzena-2,8-diaza-3,7-dioxo-10,14-dithia-12(2,6)-pyridinacyclotetradecaphane (**L_1**)*

3 mL Pyridine in CH_2Cl_2 (50 mL), glutaryl chloride (168 mg, 1 mmol) in CH_2Cl_2 (50 mL) and 2,6-bis(2-aminothiophenoxymethyl)pyridinde (352 mg, 1 mmol) in CH_2Cl_2 (50 mL) were reacted and white solid product was obtained (320 mg, 72 %). M. P. 220 °C. Found (calculated) $C_{24}H_{23}N_3O_2S_2$: C, 63.98 (64.12); H, 5.21 (5.16); N, 9.21 (9.35); S, 14.06 (14.26). FT-IR (solid, cm^{-1}): 3,298, 3,232 *ν*(N–H), 3,060 *ν*(C≡H), 1,692 *ν*(C=O), 1,650 *δ*(N–H), 1,580 *ν*(C≡C), 1,520 *δ*(N–H), 746 *δ*(C≡H). Far-IR (cm^{-1}): 562, 491, 447, 432, 387, 334, 320, 214. ^{1}H NMR (DMSO-d_6), δ_H ppm: 1.92 (quintet, CH_2, J = 7.1 Hz), 2.43 (t, $2CH_2$, J = 7.0 Hz), 4.21 (s. $2CH_2$), 7–7.66 (m, 11H), 9.12 (s, 2NH). ^{13}C NMR (DMSO-d_6), δ_C ppm: 21.26 (CH_2), 35.53 ($2COCH_2$), 40.45 ($2SCH_2$), 121.64, 124.42, 125.70, 127.41, 129.44, 131.46, 137.11, 137.54, 156.48, 170.73 (2CO).

*1,10(1,2)-Dibenzena-2,9-diaza-3,8-dioxo-11,15-dithia-13(2,6)-pyridinacyclopentadecaphane (**L_2**)*

3 mL Pyridine in CH_2Cl_2 (50 mL), adipoyl chloride (183 mg, 1 mmol) in CH_2Cl_2 (50 mL) and 2,6-bis(2-aminothiophenoxymethyl)pyridine (353 mg, 1 mmol) in CH_2Cl_2 (50 mL) were reacted and white solid product was obtained (270 mg, 60 %). Dec. 210 °C. Found (calculated) $C_{25}H_{25}N_3O_2S_2$: C, 64.45 (64.77); H, 5.28 (5.44); N, 8.97 (9.06); S, 13.76 (13.83). MS (ESI) *m/z* calculated for $C_{25}H_{25}N_3O_2S_2$, 463.61; found: 464.40 [M + H] (75 %). FT-IR (solid, cm^{-1}): 3,324, 3,255 *ν*(N–H), 3,050 *ν*(C≡H), 2,950 ν(C–H), 1,689 ν(C=O), 1,658 *δ*(N–H), 1,576 *ν*(C≡C), 1,506 *δ*(N–H), 755 *δ*(C≡H). Far-IR (cm^{-1}): 558, 537, 465, 449, 419, 371, 308, 282, 234, 189. ^{1}H NMR ($CDCl_3$), δ_H ppm: 1.93 (t, $2CH_2$), 2.54 (t, $2COCH_2$), 4.04 (s, $2SCH_2$), 7.0–8.40 (m, 11H), 8.71 (s, 2NH). ^{13}C NMR ($CDCl_3$), δ_C ppm: 29.11 ($2CH_2$), 38.65 ($2COCH_2$), 43.31 ($2SCH_2$), 120.86, 122.26, 124.62, 130.76, 130.98, 136.16, 137.78, 140.12, 157.18, 169.67 (2C=O).

*1,9(1,2)-Dibenzena-2,8-diaza-3,7-dioxo-5,10,14-trithia-12(2,6)-pyridinacyclotetradecaphane (**L_3**)*

Pyridine (3 mL) in CH_2Cl_2 (50 mL), 2,2-thio diacetyl chloride (187 mg, 1 mmol) in CH_2Cl_2 (50 mL) and 2,6-bis(2-aminothiophenoxymethyl)pyridine (353 mg, 1 mmol) in CH_2Cl_2 (50 mL) were reacted and white solid product was obtained (290 g, 62 %). Dec. 232 °C. Found (calculated) $C_{23}H_{21}N_3O_2S_3$: C, 58.93 (59.07); H, 4.61 (4.53); N, 8.75 (8.99); S, 20.38 (20.57). MS (ESI) *m/z* calculated for $C_{23}H_{21}N_3O_2S_3$, 467.63; found: 468.33 [M + H] (81 %). FT-IR (solid, cm^{-1}): 3,274 ν(N–H), 3,053 ν(C≡H), 2,968 ν(C–H), 1,659 ν(C=O), 1,576 ν(C≡C), 1,505 *δ*(N–H), 757 *δ*(C≡H). Far–IR (cm^{-1}): 570, 525, 497, 455, 405, 310, 277, 200. ^{1}H NMR ($CDCl_3$), δ_H ppm: 3.63 (s, $2CH_2$), 4.09 (s, $2CH_2$), 6.90–8.25 (m, 11H), 9.28 (s, 2NH). ^{13}C NMR ($CDCl_3$), δ_C ppm: 43.93 ($2CH_2$), 44.5 ($2CH_2$), 120.28, 122.01, 122.88, 124.93, 130.77, 136.5, 137.68, 140.07, 157.01, 166.4 (2C=O).

*1,10(1,2)-Dibenzena-2,9-diaza-3,8-dioxo-5,6,11,15-tetrathia-13(2,6)-pyridinacyclopentadecaphane (**L_4**)*

Pyridine (3 mL) in CH_2Cl_2 (50 mL), 2,2-disulfanediyldiacetyl chloride (219 mg, 1 mmol) in CH_2Cl_2 (50 mL) and 2,6-bis(2-aminothiophenoxymethyl)pyridine (353 mg, 1 mmol) in CH_2Cl_2 (50 mL) were reacted and white solid product was obtained (290 mg, 58 %). Dec. 166 °C. Found (calculated) $C_{23}H_{21}N_3O_2S_4$: C, 55.17 (55.28); H, 4.35 (4.24); N, 8.29 (8.41); S, 25.49 (25.67). MS (ESI) *m/z* calculated for $C_{23}H_{21}N_3O_2S_4$, 499.69; found: 500.33 [M + H] (56 %). FT-IR (solid, cm^{-1}): 3,257 ν(N–H), 3,060 ν(C⎓H), 2,929 ν(C–H), 1,664 ν(C=O), 1,577 ν(C⎓C), 1,506 δ(N–H), 751 δ(C⎓H). Far-IR (cm^{-1}): 580, 531 ν(S–S), 500, 465, 453, 389, 336, 298, 276, 258. ^{1}H NMR ($CDCl_3$), δ_H ppm: 3.7 (s, $2SCH_2CO$), 4.08 (s, $2SCH_2$), 7.10–8.47 (m, 11H), 9.5 (s, 2NH).

*1,11(1,2)-Dibenzena-2,10-diaza-3,9-dioxo-6,12,16-trithia-14(2,6)-pyridinacyclohexadecaphane (**L_5**)*

Pyridine (3 mL) in CH_2Cl_2 (50 mL), 3,3-thiodipropanoyl chloride (214 mg, 1 mmol) in CH_2Cl_2 (50 mL) and 2,6-bis(2-aminothiophenoxymethyl)pyridine (353 mg, 1 mmol) in CH_2Cl_2 (50 mL) were reacted and white solid product was obtained (0.310 g, 63 %). Dec. 216 °C. Found (calculated) $C_{25}H_{25}N_3O_2S_3$: C, 60.72 (60.58); H, 5.19 (5.08); N, 8.37 (8.48); S, 19.36 (19.41). MS (ESI) *m/z* calculated for $C_{25}H_{25}N_3O_2S_3$, 495.68; found: 496.33 [M + H] (100 %). FT-IR (solid, cm^{-1}): 3,358, 3,256 ν(N–H), 3,057 ν(C⎓H), 2,954 ν(C–H), 1,684 ν(C=O), 1,658 δ(N–H), 1,575 ν(C⎓C), 1,508 δ(N–H), 751 δ(C⎓H). Far-IR (cm^{-1}): 577, 552, 461, 454, 380, 302, 261, 190. ^{1}H NMR ($CDCl_3$), δ_H ppm: 2.71 (t, $2COCH_2$, J = 5.82 Hz), 3 (t, $2SCH_2$, J = 5.80 Hz), 4.10 (s, $2SCH_2$), 7.0–8.41 (m, 11H), 8.73 (s, 2NH). ^{13}C NMR ($CDCl_3$), δ_C ppm: 29.92 ($2SCH_2$), 38.47 ($2COCH_2$), 43.31 ($2SCH_2$), 121.41, 122.14, 123.55, 125.25, 130.49, 135.88, 137.60, 139.57, 156.97, 166.06 (2C=O).

Pharmacology

Microorganisms

The antimicrobial activities were evaluated against Gram-positive (*Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 7064, *Micrococcus luteus* La 2971, *Mycobacterium smegmatis* CCM 2067, *Listeria monocytogenes* ATCC 15313) and Gram-negative (*Escherichia coli* ATCC 11230, *Proteus vulgaris* ATCC 8427, *Klebsiella pneumoniae* UC57, *Pseudomonas aeruginosa* ATCC 27853) bacteria and yeast cultures (*Candida albicans* ATCC 10231, *Kluyveromyces fragilis* NRRL 2415, *Rhodotorula rubra* DSM 70403, *Debaryomyces hansenii* DSM 70238 and *Hanseniaspora guilliermondii* DSM 3432) using both disc diffusion and dilution methods.

Antimicrobial screening

Disc diffusion method

The antimicrobial activity of the compounds was evaluated using a slightly modified disc diffusion method [30, 31]. The compounds were dissolved in DMSO (2 mg mL^{-1}). Sterilized antibiotic discs (6 mm, Schleicher & Schull No. 2668, Germany) were each impregnated with 20 µL of solution. To ensure that the solvent had no effect on bacterial growth, a control test was performed with test medium supplemented with DMSO in the same procedures used in the experiments. All the bacteria were incubated at 30 ± 0.1 °C for 24 h by inoculation into nutrient broth (Difco) and the yeasts were incubated in malt extract broth (Difco) for 48 h. An inoculum containing 10^6 bacterial cells or 10^8 yeast cells/mL was spread on Mueller-Minton agar (Oxoid) plates (1 mL inoculum/plate). The discs injected with solutions were placed on the inoculated agar by pressing slightly and incubated at 35 °C (24 h) for the bacteria and at 25 °C (72 h) for the yeasts. On each plate an appropriate reference antibiotic disc was applied depending on the test microorganisms. In each case triplicate tests were performed and the average was taken as the final reading.

Dilution method

Screening for antibacterial and antifungal activities was carried out by preparing a broth micro dilution, following the procedure outlined in Manual of Clinical Microbiology [32]. All the bacteria were incubated and activated into nutrient broth at 30 °C for 24 h and the yeasts were incubated in malt extract broth for 48 h. The compounds were dissolved in DMSO (2 mg mL^{-1}) and then diluted using caution adjusted Mueller-Hinton broth (Oxoid). Two fold serial concentrations of the compounds were employed to determine the (MIC) ranging from 200 to 1.56 µg mL^{-1}. Cultures were grown at 37 °C (20 h) and the final inoculation was approximately 10^6 cfu mL^{-1}. Test cultures were incubated at 37 °C (24 h). The lowest concentrations of antimicrobial agents that result in complete inhibition of microorganisms were represented as (MIC) µg mL^{-1}. In each case triplicate tests were performed and the results are expressed as means.

Ion transport

Macrocyclic peptide (**L_5**) (60 mg, 0.12 mmol) was dissolved in dichloromethane (60 mL) and transferred in a

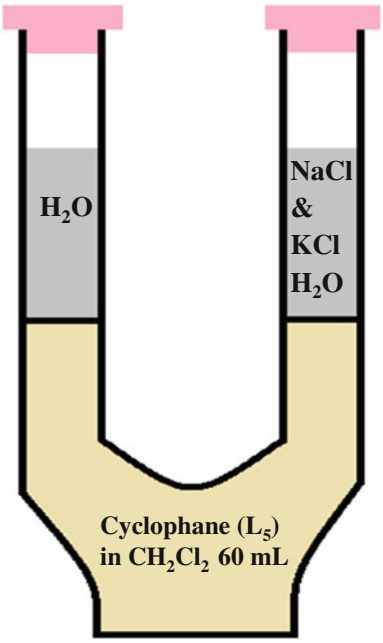


Fig. 2 Apparatus used for ion transport study

glass vessel as shown in Fig. 2. One arm of the U tube is filled with distilled water (6 mL) in which NaCl (1.17 g, 20 mmol) was dissolved and the other arm is filled with triply distilled water. In another identical glass vessel similar procedure was carried out using KCl (1.49 g, 20 mmol) instead of NaCl. The dichloromethane layer was stirred gently for 72 h for each vessel. The arm that was filled with triply distilled water showed the presence of K^+ ion (500 mg) and Na^+ ion level was below the measuring limit. Thus the experiment proved that K^+ ions were transported by cyclophane peptide (**L_5**) from one arm to the other. This indicates that the size of K^+ ion (ionic radius: 1.33 Å) and the cavity size of the cyclophane amide (**L_5**) are complementary to each other, whereas the size of Na^+ ion (ionic radius: 0.95 Å) does not match with cavity size. Hence cyclophane amide (**L_5**) could be used as a potential ion filtering system for retaining the biologically important K^+ ion and eliminating Na^+ ion or possible continuous purification system for these ions. A blank experiment was also performed in dichloromethane without cyclophane amide and no such ion mobility was observed.

Results and discussions

Mass spectra

The mass spectra of the cyclic peptides give important structural information about the nature of the condensation (1+1), the ring size and molecular conformation of the compounds. The mass spectra show a molecular positive ion peaks $[M + H]^+$ for each individual cyclic compound, suggesting the monomeric nature of the compounds and confirming the proposed formula. The mass spectra of these compounds also show a series of peaks corresponding to various fragments. Their intensity may give an idea of the stability of the fragments.

Vibrational spectra

In most cases secondary amides usually have their NH and C=O groups *trans* to each other. *cis*-Configured secondary amides are found practically only in forms of lactams, where the *trans*-configuration is difficult because of the high ring tension. However, the bands lie essentially in the same regions as for the *trans*-configured derivatives. The vibrational spectra for the cyclic compounds can be discussed in terms of two characteristic wave regions: 3,370–2,900 cm^{-1} corresponding to the ν_{as}(N–H), ν_s(N–H), ν(C⎓H) aromatic, ν(C–H) aliphatic characteristic modes, and 1,750–1,500 cm^{-1} belonging to ν(CO), δ(N–H), ν(C⎓C) vibration modes. The bands corresponding to free aniline $–NH_2$ ν(N–H) (3,410 cm^{-1}) and acid chloride COCl *ν*(CO) (1,750 cm^{-1}) groups are not observed in the IR spectra of the products, which suggests complete condensation of the reactions and possible formation of amide linkages. The medium bands observed in the regions 3,370–3,320 and 3,280–3,230 cm^{-1} are assignable to ν_{as}(N–H) and ν_s(N–H) amide vibration modes, respectively. These modes of vibration are not dependent on the backbone conformation but are very sensitive to the strength of a hydrogen bonding, which may cause broadening and as well as causing multiplicity in certain cases [33]. The peptide bond formation could also be confirmed due to the observance of two major bands in the frequencies 1,690–1,650 (amide I) and 1,520–1,500 cm^{-1} (amide II). The amide I band is the most intensive absorption in the IR spectrum and it is almost fully governed by the stretching vibration of the C=O mode with a very small contribution of the C–N groups. Amide II is more complex than amide I, it's signal derives mainly from in-plane N–H bending vibration with a certain contribution of ν(C–N) groups [33, 34]. Stretching vibration C=O amide is considerably lower than corresponding acyl ν(CO) vibration frequencies. The characteristic ν(CH) modes of aromatic and aliphatic groups are observed in the wave region 3,100–3,000 and 2,980–2,920 cm^{-1} respectively (see "Experimental" section).

Magnetic resonance spectra

The ^{1}H NMR spectra of the cyclic compounds do not give any signal corresponding to aniline $–NH_2$ at 5.38 ppm, instead sharp bands appear in the regions 8.70–9.50 ppm

corresponding to amide (2CONH) proton atoms. As expected no significant changes are observed for the methylene proton chemical shifts compared with the starting material diamine. They appear around 4 ppm as sharp singlets (4H, $2SCH_2$) for each of the cyclic compound.

The peaks corresponding to acid chlorides COCl ν(C=O) ($\sim$173 ppm) groups are not observed in the ^{13}C NMR spectra of the cyclic products, instead the signals which appeared for each product in the regions 166–171 ppm are attributed to a (CONH) group, suggesting complete condensation of the reactions, which was also predicted from IR spectra.

Microbial activity

The results concerning in vitro antimicrobial activities of the macrocycles together with inhibition zone (mm) and (MIC) values of compared antibiotic and antifungal reagents are listed in Tables 1 and 2. The overall macrocycles can interact through van der Waals and hydrophobic interactions, while the individual hetero atoms present in the structure particularly nitrogen sulfur atoms, could interact by strong hydrogen bonding. As far as hydrogen bonding is concerned, there is an important electronegative aspect regarding to nitrogen atoms. The nitrogen atom is the most common atom involved as hydrogen bond acceptor in biological systems. Moderate biological activity shown by the compounds may be related to their hydrogen bonding capability due to the presence of several hetero atoms capable of hydrogen bonding with biomolecules. Comparing the inhibition zone values, in most cases the compounds baring sulfur atoms such as CSC (**L_3**) and CS_2C (**L_4**) containing units seems to show slightly higher biological activity than the units such as C_3 (**L_1**), C_4 (**L_2**), which has no sulfur atoms. As a result the inhibition activity seems to be governed to a certain degree by the percentage amount of nitrogen presence in these compounds, because the (**L_4**) macrocycle contains higher numbers of nitrogen atoms compared with the other four compounds. In a similar pattern, the least nitrogen containing compound **L_1** in most cases shows the lowest antibiotic activity. This pattern repeats itself for both inhibition zone and MIC values (Tables 1, 2).

Of all the tested compounds (**L_3**) and (**L_4**) showed moderate activity against both the Gram-positive pathogen *B. cereus* (**L_3** and **L_4** = 6.25 μg mL^{-1}) and on Gram-negative bacteria *P. vulgaris* both **L_3** and **L_4** showed strong activity 6.25 μg mL^{-1} compared with Gentamycin (6.25 μg mL^{-1} (Table 2). In the case of inhibition zone values these two compounds also showed (in most cases) slightly higher activity than the other compounds. Showing moderate antibacterial activity on both Gram-positive and Gram-negative bacteria, these compounds could be considered to possess a broad-spectrum potency.

The inhibition zone and (MIC) values indicate that almost all the compounds exhibited a moderate antifungal activity against most of the yeast cultures. Once more the compound (**L_4**) shows slightly higher activity (zone 20 mm and MIC 6.25 μg mL^{-1}) against *K. fragilis* in comparison

Table 1 In vitro antimicrobial activity (mm zone) of (**L_1–L_5**) and standard reagents

Microorganisms	L_1	L_2	L_3	L_4	L_5	PE	AM	CT	OF	TE	NY	KE	CL
Staphylococcus aureus$^+$	12	15	16	18	15	14	15	14	22	26	–	–	–
Bacillus cereus$^+$	14	15	16	17	14	12	14	14	36	22	–	–	–
Micrococcus luteus$^+$	12	13	12	10	11	34	30	34	28	20	–	–	–
Mycobacterium smegmatis$^+$	14	17	17	18	18	17	20	14	28	30	–	–	–
Listeria monocytogenes$^+$	14	14	18	18	14	12	14	14	30	28	–	–	–
Escherichia coli$^-$	15	14	16	17	14	16	14	12	30	25	–	–	–
Proteus vulgaris$^-$	15	16	14	15	15	12	18	20	32	24	–	–	–
Klebsiella pneumoniae$^-$	14	16	14	15	16	18	15	16	26	30	–	–	–
Pseudomonas aeruginose$^-$	15	14	11	14	12	10	10	48	40	38	–	–	–
Kluyveromyces fragilis	13	15	18	20	14	–	–	–	–	–	18	16	18
Rhodotorula rubra	12	13	17	15	16	–	–	–	–	–	16	22	16
Candida albicans	17	18	15	17	15	–	–	–	–	–	20	22	16
Hanseniaspora guilliermo	14	15	19	21	18	–	–	–	–	–	23	22	24
Debaryomyces hansenii	14	16	18	20	18	–	–	–	–	–	16	16	18

In each case triplicate tests were performed and the average was taken as the final reading

+ Gram positive, − Gram negative, *PE* penicillin G (10 units), *AM* ampicillin 10 μg, *CT* cefotaxime 30 μg, *OF* ofloxacin 5 μg, *TE* tetracycline 30 μg, *NY* nystatin 100 μg, *KE* ketaconazole 20 μg, *CL* clotrimazole 10 μg

Table 2 In vitro antimicrobial activity (MIC, μg mL^{-1}) of **(L_1–L_5)** and standard reagents

Microorganisms/compounds	L_1	L_2	L_3	L_4	L_5	GEN	NY
Staphylococcus aureus^{+}	25	12.5	25	12.5	12.5	25	–
Bacillus cereus^{+}	12.5	12.5	6.25	6.25	12.5	6.25	–
Micrococcus luteus^{+}	50	25	25	50	50	50	–
Mycobacterium smegmatis^{+}	25	12.5	25	12.5	12.5	12.5	–
Listeria monocytogenes^{+}	25	25	12.5	6.25	25	12.5	–
Escherichia coli^{-}	12.5	12.5	12.5	6.25	12.5	6.25	–
Proteus vulgaris^{-}	25	12.5	6.25	6.25	25	6.25	–
Klebsiella pneumoniae^{-}	12.5	12.5	12.5	12.5	12.5	6.25	–
Pseudomonas aeruginose^{-}	50	25	50	25	50	6.25	–
Kluyveromyces fragilis	12.5	12.5	6.25	6.25	25	–	6.25
Rhodotorula rubra	50	25	12.5	12.5	12.5	–	3.125
Candida albicans	12.5	6.25	12.5	12.5	12.5	–	3.125
Hanseniaspora guilliermondii	12.5	12.5	6.25	3.125	6.25	–	3.125
Debaryomyces hansenii	25	12.5	6.25	3.125	6.25	–	12.5

In each case triplicate tests were performed and the average was taken as the final reading

+ Gram positive, − Gram negative, *GEN* gentamycin, *NY* nystatin

with Nystatin (zone 18 mm and MIC 6.25 μg mL^{-1}). Nystatin acts by binding to ergosterol in the cell membrane of susceptible species resulting in a change in membrane permeability and the subsequent leakage of intracellular components. Considering the presence of peptide bonds in the molecules, a similar mechanism may also be considered for these compounds.

Ion transport

The physical and chemical properties of Na^+ and K^+ ions are quite similar. Both are closed shell, hard ions, they have identical coordination geometries and display similar exchange kinetics for bound water molecules. Despite these similarities, K^+ ion channels, a class of transmembrane proteins responsible for a host of biological events, can discriminate with unprecedented selectivity for K^+ over Na^+ by a factor of 10^3–10^4. Macrocycles with open pores and varying size of the cavity, particularly cyclophane peptides having amphipathic properties are useful as transport vehicles for biologically important ions [17].

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