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Synthesis and investigation of a new cyclo (N^{α}-dipicolinoyl) pentapeptide of a breast and CNS cytotoxic activity and an ionophoric specificity

M. Abo-Ghalia and **A. Amr**

National Research Center, Dokki, Cairo, Egypt

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Summary. A new acylated cyclopentapeptide namely, Cyclo-(N^{α}-dipicolinoyl)-*bis*-[L-Leu-DL-Nval]-L-Lys OMe (**5**) was suggested and synthesized. The structural conception of **5** was rationalized by analogy to the structural features of some known cyclodepsipeptides exemplified by the antibiotic and DNA intercalator actinomycin D (NSC: 3053), the ionophore and anti-HIV enniatin B (NSC: 692895) and the ionophore and antibiotic valinomycin (NSC: 630175).

The cyclopeptide **5** was chemically synthesized, starting from its linear tetrapeptide ester precursor **2** by coupling L-lysine methyl ester to the prepared tetrapeptide acid **3** or hydrazide **4** via the mixed anhydride or azide method, respectively.

A cytotoxic activity (cell killing) in both breast (NCF7) and CNS (SF-268) cell lines NCI, USA) was realized for **5**, while less active cytotoxic profile was determined for **2**.

Moreover, we have recently reported general ionophoric and sensor characteristics particularly, for Pb (II) ions for both **5** and **2**. Correlation between the cytotoxic activity and the ionophoric potency is a matter of future investigations.

Keywords: Cyclopeptides – 2,6-Pyridinedicarboxlic acid – Peptide coupling methods – Cytotoxic agents – Ionophores

1. Introduction

Since the mid of the past century, cyclic peptides represent a fascinating area of bioorganic and medicinal chemistry investigations (Schröder et al., 1966; Goodman et al., 2000). Actinomycins, enniatins and valinomycins are three illustrative examples of updated cyclodepsipeptide literature reports.

Analogously, some front line anticancers are mechanistically confirmed to interact with DNA as their primary therapeutic targets. Several DNA intercalators are, consequently aimed at cancer chemotherapy (Waring, 1981; Sriram et al., 1996). The cyclodepsipeptide antibiotic actionomycin D (Dactinomycin, $C_{62}H_{86}N_{12}O_{16}$, [1255.5], CAS: 50-76-0, NSC: 3053, *Streptomyces antibioticus)*, is an anticancer drug that exhibits both an intercalation and DNA groove binding properties. Similarly, the cyclodepsipeptids enniatins, particularly, the antibiotic and anti-HIV, enniatin B ($C_{33}H_{57}N_3O_9$, NSC: 692895, HIV: EC50, 16.0 μM, HIV: IC50, 0.78 μM, *Fusarium spp*) has diverse biological and ionophoric activities with high specificity for sodium and potassium ions (Lifson et al., 1984).

Moreover, the cyclo-dodecadepsipetide antibiotic and antitumor valinomycin (*Streptomyce fulvissimus* $C_{54}H_{90}N_6O_{18,}$ CAS: 2001-95-8, NSC: 630175) which is a 36-membered ring, of carboxylic acids and amino acids, is known as a potassium ion specific ionophore. The high affinity for potassium infers for the compound its activity as an antibiotic, an insecticide, a nematodicide, and particularly in assembling K^+ specific electrodes (Neupert-Laves et al., 1975; Collison et al., 1989; Boitano and Omoto, 1991).

Correspondingly, this work was principally suggested as a continuation to our interest in the chemistry and biological activity of pyridoyl amino acid and peptide conjugates (Abo-Ghalia et al., 1979, 1980, 2003; Attia et al., 1995, Abo-Ghalia and Soliman, 1996, Abo-Ghalia and Amr, 2002). It equally presents an extrapolation to our previous approaches to synthesize some enniatin mimics (Attia et al., 1994, 2000; Amr et al., 1999).

In a similar context, Talama et al. (1985) reported the synthesis of chiral-bridged *bis*-coupled amino acid dihydropyidines starting from 3,5-pyridine dicarboxylic acid (dinicotinic acid) and a variety of L-amino acids. These

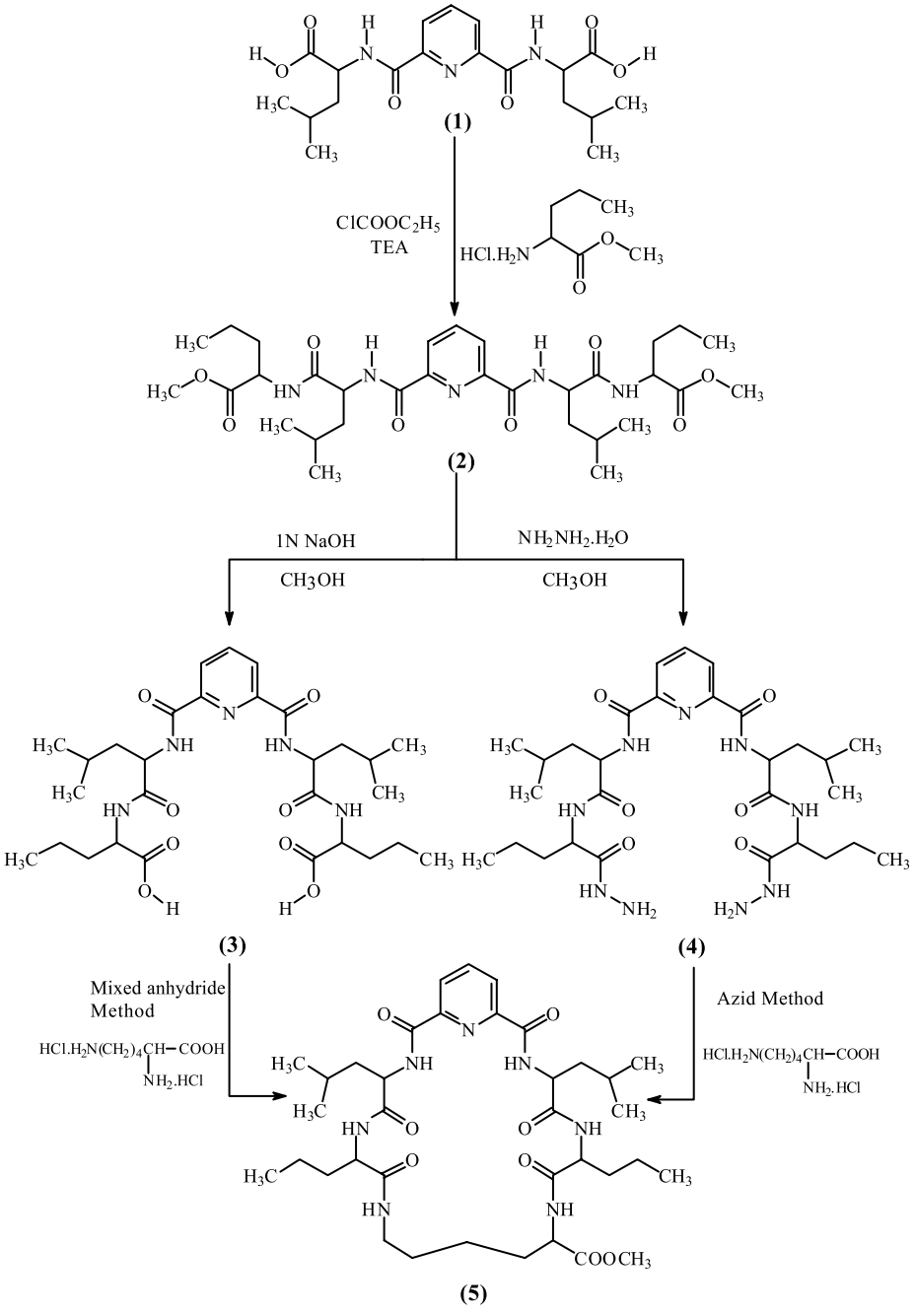
enniatin mimics were also valuable for their chemical activity comparable to enzyme cofactors. Synthesis of some new potential *bis*-intercallators based on chiral pyridine-2, 6-dicarboxamides was equally reported (Amr et al., 1999).

Herein, taking in to consideration the depsicyclopeptide structural features of actinomycins, enniatins and valinomycin and their corresponding cytotoxic, antibiotic and/or ionophoric potencies, the structure of a simplified dipicolinc acid based cyclopeptide **5** was suggested for the investigation of its diverse characteristics namely, its cytotoxic properties.

2. Materials and methods

2.1. Chemistry

Syntheses were performed on 1–5 mmole scale of the starting reactants and generally in ~5 ml solvent/mmole. Melting points were recorded on



Scheme 1. Synthesis of cyclo-(N^{α}-dipicolinoyl)-*bis*-[L-Leu-DL-Nval]-L-Lys OMe, (**5**)

an "Electrothermal IA 9000 digital melting point apparatus (England). Analytical data were obtained from the microanalytical unit, National Research Center, Cairo, Egypt. The IR spectra (KBr) were recorded on a FT IR-8201 PC spectrophotometer (Shimadzu, Japan). ^{1}H- and ^{13}C-NMR spectra were realized via (Jeol-GLM 270 MHz spectrometer (Japan). Chemical shifts were recorded in δ-scale (ppm) relative to TMS as a standard. Mass spectra were run at 70 ev with Finning SSQ GC/MS Spectrometer using the electron ionization technique (EI). The reactions were followed by analytical TLC (Silica gel, aluminum sheets 60 F254 (Merck, Germany) using a methanol/chloroform solvent systems. Evaporation of the solvents was achieved at room temperature and under reduced pressure (rotatory evaporator). Compound **1** was prepared according to our published procedure (Amr et al., 1999).

2.1.1. N$^{\alpha}$-dipicolinoyl-*bis*-[L-Leu-DL-Nval-OMe], (**2**)

To a cold and stirred dry methylene chloride solution (−20°C) of diacid **1** (1 equivalent, Amr et al., 1999), ethyl chloroformate (2 equivalents) and triethylamine (2 equivalents) were successively added. Ten minutes later, a cold methylene chloride solution (−20°C) of DL-norvaline methyl ester (2 equivalents) was added. Stirring of the cold reaction mixture (−20°C) was continued for three hours and at room temperature for additional twelve hours. The solution was then washed with water, 1N HCl, 1N sodium bicarbonate and finally with water (∼2 × 3 ml). The dried solution (anhydrous $CaCl_2$) was evaporated and the obtained oily residue was solidified by dry ether trituration, filtered off, dried under vacuum and crystallized from methanol/ether to afford **2**. Yield 78%, M.p. 113–115°C, Found/Calculated for $C_{31}H_{49}N_5O_8$ (619.7): C, 59.95/60.08, H, 7.90/7.97, N, 11.22/11.30. IR (cm^{-1}): 3396 (ν NH), 1746 (ν CO, ester) and 1650 (CO, amide).

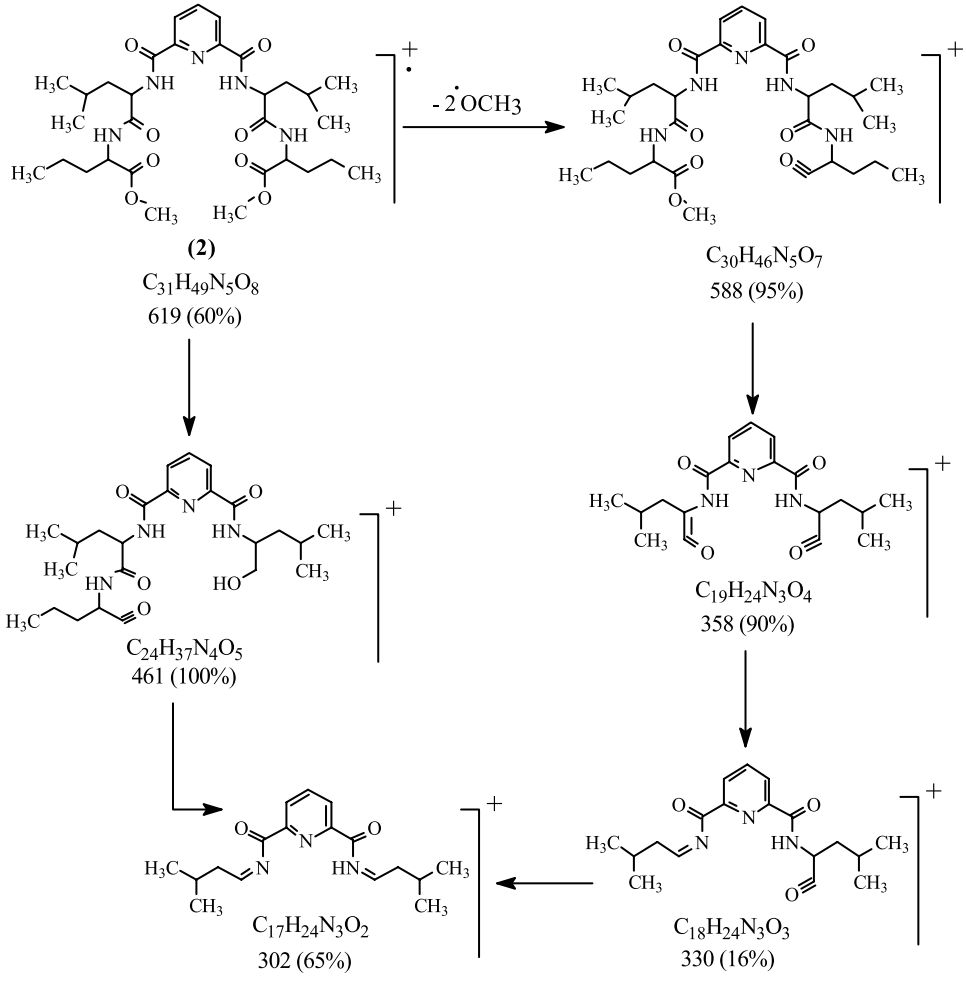
^{1}H NMR (DMSO-d_6): 8.57 and 8.53 (2s, 4H, 4 × NH, exchangeable with D_2O), 8.40–8.20 (m, 3H, pyridyl-H), 4.65–4.60 (t, 2H, 2 × CHN), 4.30–4.25 (t, 2H, 2 × CHN), 3.60 (s, 6H, 2 × OCH_3), 2.45 (m, 2H, 2 × CH), 1.85–1.80 (m, 4H, 2 × CH_2), 1.70–1.65 (m, 4H, 2 × CH_2), 1.40–1.30 (m, 4H, 2 × CH_2), 0.95–0.85 (m, 18H, 6 × CH_3).

^{13}C NMR (DMSO-d_6): 173.03, 172.44 (2C, 2 × CO-ester), 163.73, 163.66 (4C, 4 × CO-amide), 149.43 (2C, the pyridine positions-2,6), 140.08 (1C, the pyridine position-4), 125.39 (2C, the pyridine positions-3,5), 66.89 (2C, 2 × OCH_3-ester), 52.57, 52.35 (4C, 4CH-NH), 41.88, 41.48 (2C, 2 × CH_2), 33.79, 33.61 (2C, 2 × CH), 25.36, 25.26 (2C, 2 × CH_2), 23.76 (2C, 2 × CH_2), 22.66, 22.58, 19.44, 19.35 (4C, 4 × CH_3), 14.24, 14.14 (2C, 2 × CH_3).

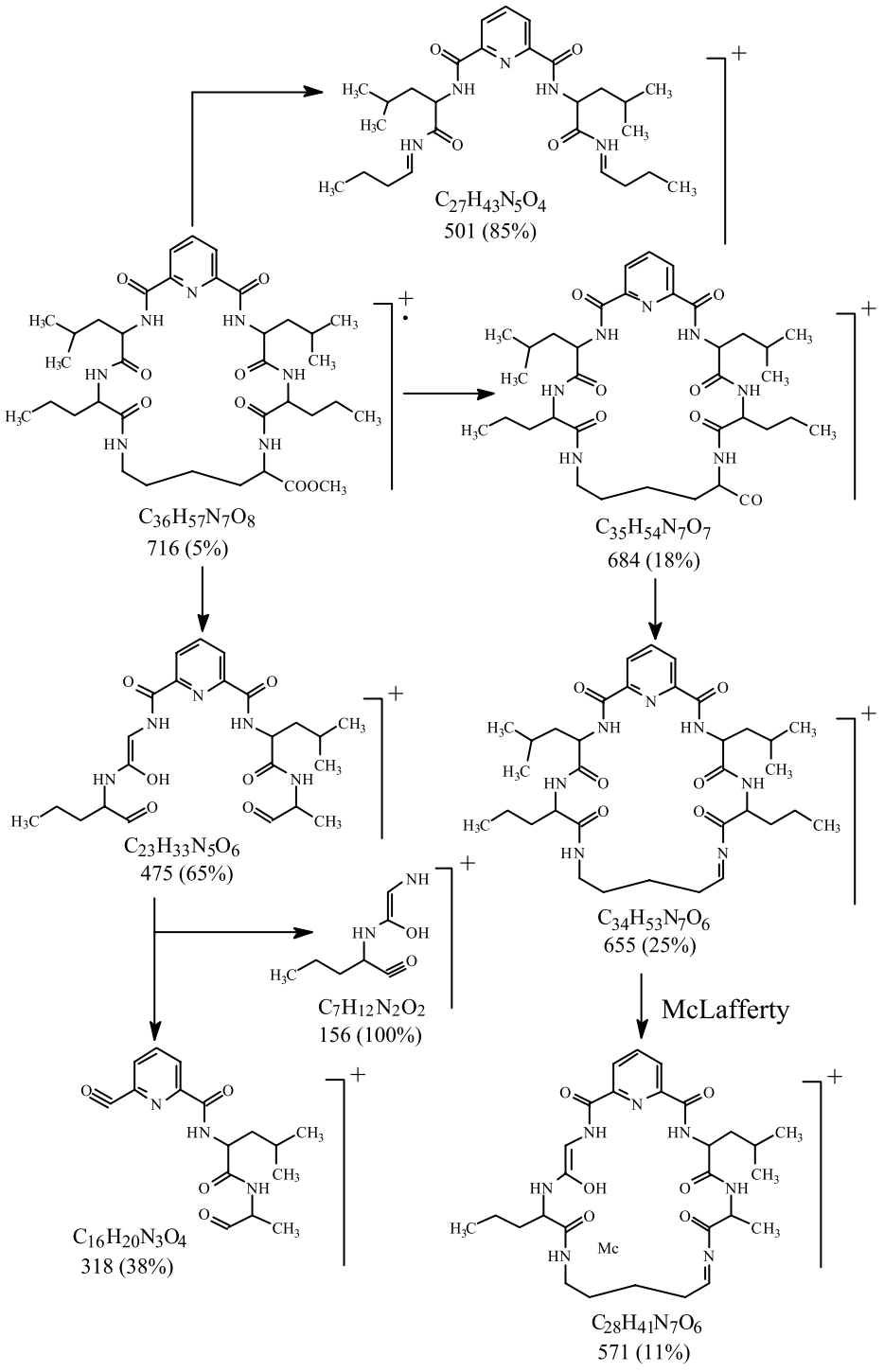
M.S.: 619 (M^+, 60%) and 461 ($C_{24}H_{37}N_4O_5$, 100%), (cf. a suggested fragmentation pattern, Scheme 2).

2.1.2. N$^{\alpha}$-dipicolinoyl-*bis*-[L-Leu-DL-Nval], (**3**)

Sodium hydroxide (1N, 3 equivalents) was dropwisely added to a cold and stirred methanolic solution (1 equivalent, −5°C) of **2**. Stirring was continued at that temperature for two hours and then for twelve hours at



Scheme 2. Suggested fragmentation pattern of N$^{\alpha}$-dipicolinoyl-*bis*-[L-Leu-DL-Nval] OMe, (**2**)



Scheme 3. Suggested fragmentation of cyclo-(N^{α}-dipicolinoyl)-*bis*-[L-Leu-DL-Nval]-L-Lys OMe, (**5**)

room temperature followed by evaporation of the solvent. The cold reaction mixture was acidified (0.1N HCl) to pH∼3, and the obtained solid was filtered off, washed with cold water and crystallized from ethanol/ether mixture to afford **3**. Yield 68%, m.p. 135–137°C, Found/Calculated for $C_{29}H_{45}N_5O_8$ (591.7): C, 58.75/58.87, H, 7.58/7.67, N, 11.63/11.83. IR (Cm^{-1}): 3655 (ν OH), 3325 (ν NH), 1725 (ν CO, acid) and 1656 (amide I).

^{1}H NMR (DMSO-d_6): 12.50 (s, 2H, 2 × OH, exchangeable with D_2O), 8.90 and 8.45 (2s, 4H, 4 × NH, exchangeable with D_2O), 8.30–8.20 (m, 3H, pyridyl-H), 4.70–4.65 (t, 2H, 2 × CHN), 4.25–4.20 (t, 2H, 2 × CHN),

2.40 (m, 2H, 2 × CH), 1.80–1.75 (m, 4H, 2 × CH_2), 1.70–1.60 (m, 4H, 2 × CH_2), 1.35–1.30 (m, 4H, 2 × CH_2), 0.95–0.85 (m, 18H, 6 × CH_3).

M.S.: 591(M^+, 20%) and 302 ($C_{17}H_{24}N_3O_2$, 100%).

2.1.3. N^{α}-dipicolinoyl-*bis*-[L-Leu-DL-Nval] hydrazide], (**4**)

Hydrazine hydrate (90%, 10 equivalents) was added to a methanolic solution of **2** (1 equivalent). The reaction mixture was refluxed for five hours after which the solvent was evaporated. The obtained residue was triturated with ether, filtered off and crystallized from methanol/ether to afford **4**. Yield 80%, m.p. 130–132°C, Found/Calculated for $C_{29}H_{49}N_9O_6$ (619.76) C, 56.16 /56.20, H, 7.94/7.97, N, 20.28/20.34. IR (Cm^{-1}): 3350 (ν NH), 1745 (ν CO), ester and at 1656 (CO), amide.

^{1}H NMR (DMSO-d_6): 9.10, 8.35, 8.0 (3s, 6H, 6 × NH, exchangeable with D_2O), 8.25–8.15 (m, 3H, pyridyl-H), 4.60–4.55 (m, 2H, 2 × CHN), 4.25–4.20 (m, 2H, 2 × CHN), 3.45 (bs, 4H, 2 × NH_2, exchangeable with D_2O), 2.40 (m, 2H, 2 × CH), 1.80–1.75 (m, 4H, 2 × CH_2), 1.65–1.50 (m, 4H, 2 × CH_2), 1.30–1.20 (m, 4H, 2 × CH_2), 0.90–0.80 (m, 18H, 6 × CH_3).

M.S.: 620 ($M^+ + 1$, 10%) and 302 ($C_{17}H_{24}N_3O_2$, 100%).

2.1.4. Cyclo-(N^{α}-dipicolinoyl)-*bis*-[L-Leu-DL-Nval]-L-Lys OMe, (**5**)

A Mixed anhydride method. Triethylamine (2 equivalents) was added to a cold and stirred dichloromethane (−20°C) suspension of diacid **3** (1 equivalent), and ethyl chloroformate (2 equivalents). Stirring was continued for twenty minutes after which L-lysine methyl ester (1 equivalent) was added. The reaction mixture was stirred at (−20°C) for three hours and then for twelve hours at room temperature. The reaction mixture was washed with water, 1 N hydrochloric acid, 1 N sodium bicarbonate and water then dried over anhydrous calcium chloride. The solvent was evaporated and the crude product was purified by preparative thin layer chromatography using methanol/benzene mixture (1/9 by volume) as an eluent to give the corresponding cyclic peptide ester **5**. Yield 65%. Found/Calculated for $C_{36}H_{57}N_7O_8$ (715.89): C, 60.10/60.40, H, 7.88/8.02, N, 13.60/13.70. IR 3350 (ν NH), 1745 (ν CO ester) and at 1656 (CO amide I).

^{1}HNMR (DMSO-d_6): 9.01, 8.65, 7.85 (3s, 6H, 6 × NH, exchangeable with D_2O), 8.20–8.15 (m, 3H, pyridyl-H), 4.65–4.60 (m, 2H, 2 × CHN), 4.50–4.40 (m, 2H, 2 × CHN), 4.10–3.95 (m, 2H, 2 × CH), 3.60 (s, 3H, OCH_3), 3.30–3.20 (m, 4H, 2 × CH_2), 2.40 (m, 2H, 2 × CH), 1.95–1.80 (m, 4H, 2 × CH_2), 1.70–1.65 (m, 2H, CH_2), 1.65–1.50 (m, 4H, 2 × CH_2), 1.35–1.20 (m, 4H, 2 × CH_2), 1.15–1.10 (m, 2H, CH_2), 0.90–0.80 (m, 18H, 6 × CH_3).

^{13}C NMR (DMSO-d_6): 176.89 (1C, CO-ester), 174.12, 173.64, 173.04, 172.65, 172.15, 163.81 (6C, 6 × CO-amide), 149.57 (2C, the pyridine positions-2,6), 139.37 (1C, the pyridine position-4), 125.72, 125.23 (2C, the pyridine positions-3,5), 60.50, 60.09 (2C, 2 × CH), 54.48 (1C, OCH_3-ester), 52.80, 52.36 (4C, 4CH–NH), 42.01(2C, CH_2–NH), 41.51, 41.32 (2C, 2 × CH_2), 32.26 (2C, 2 × CH), 31.08 (1C, CH_2), 29.22 (1C, CH_2), 25.25 (2C, 2 × CH_2), 23.76, 23.57 (2C, 2 × CH_2), 22.98 (1C, CH_2), 22.62, 22.45 (4C, 4 × CH_3), 15.33, 14.75 (2C, 2 × CH_3).

M.S.: 716 (M^+, 5%) and 156 ($C_7H_{12}N_2O_2$, 100%), [cf. a suggested fragmentation pattern, Scheme 3).

B Azide method. An aqueous solution of sodium nitrite (10%, 2 equivalents) was added to a cold (−5°C) and stirred solution of the dihydrazide **4** (one equivalent) in 5N HCl (3 ml/mmole) and acetic acid (3 ml/mmole). Stirring was continued for thirty minutes after which the reaction mixture was extracted with ether, washed with water, $NaHCO_3$, and water then dried over anhydrous sodium sulfate. The cold ethereal solution (−5°C) was then added to a cold (−5°C) dichloromethane solution of L-lysine methyl ester (1 equivalent, 10 ml/mmole). Stirring was continued for 5 hours and at room temperature for two hours. The reaction mixture was washed with 1N HCl, water and then dried (anhydrous sodium sulfate). Evaporation of the solvent afforded crude **5**, which upon preparative TLC was found identical with that, obtained via the mixed anhydride method. Yield ∼35%.

2.2. Cytotoxic activity

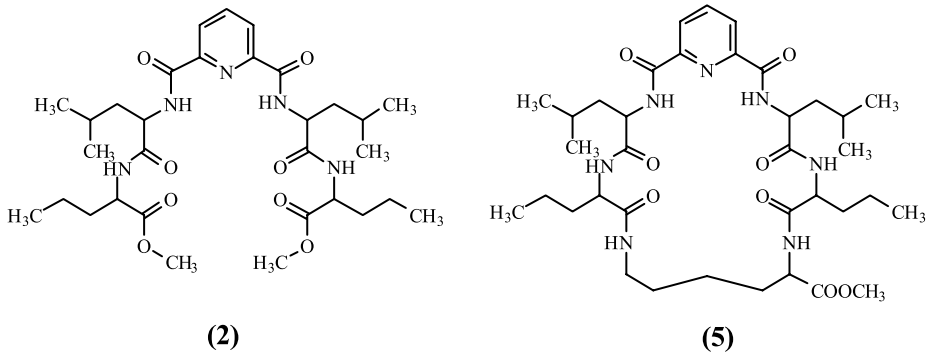
The activity was carried out by the National Cancer Institute (NCI, Maryland, USA). The testing protocol involved three cell lines, namely, NCI-H460, (Lung), MCF7 (Breast) and SF-268 (CNS). Each cell line was inoculated and preincubated on a microtiter plate. Test agents were then added at a single concentration (0.0001 M) and the culture incubated for 48 hours. End point determinations were made with Suforhodamine B, a protein binding dye. Results for each test agent were reported as the percent of growth of the treated cells when compared to the untreated control cells. Compounds which reduced the growth of a cell lines to 32% or less (negative numbers indicate cell killing) were considered promising for further evaluation in the full panel of 60 cell lines. Table 1 illustrates the obtained results.

Table 1. Cytotoxic potency of the cyclic peptide **5** and its linear tetrapeptide precursor **2**

Compound	NCI-H460 (Lung)	MCF7 (Breast)	SF-268 (CNS)
2	100	78	86
5	54	−31 (Active)	−56 (Active)

3. Results and discussions

Searching for new potent cytotoxic agents, the suggestion, synthesis, and investigation of new candidates represented by the peptides **2** and **5** was herein undertaken (Formula 1).



(2) **(5)**

Formula 1

Synthetically, either the mixed anhydride or azide methods of coupling were successfully applied as the method of choice for assembling the peptide bond with special preference to the mixed anhydride methodology. The acid chloride method however, was found more convenient for acylating L-leucine with dipicolinc acid. The selection of norvaline in its racemized (R&S) form was intended, in this preliminary study, to permit the future investigation of the activity of each of the R and S enantiomer consequently permitting the structure/activity relationships.

Scheme 1 resumes the followed synthetic protocols. Scheme 2 and 3 present suggested mass spectral fragmentation patterns for compounds **2** and **5**, respectively.

In the present work, while the preliminary cytotoxic screening (Table 1) indicated a considerable cell killing potency for cyclic peptide **5**, in both breast (NCF7) and CNS (SF-268) cell lines, no comparable activity was observed for its linear tetrapeptide precursor **2**.

Structural analogy with the most mechanistically studied cytotoxic agents could eventually suggest for the cyclic peptide **5**, intercalating characteristics. Further physico-chemical studies, however, are required to validate such hypothesis.

Prospectively, while replacing norvaline by phenylalanine or tyrosine could propose an insight to a *bis*-intercalating possibility, replacing lysine with shorter or longer amino acids or aliphatic diamine bridges could additionally reveal the intercalating structural adaptability of future cyclic peptide candidates. This work is currently in progress in this laboratory.

It seems worthwhile to mention that, guided by the reported ionophoric properties of some biologically active cyclic peptides namely enniatin B (Lifson et al., 1984, NSC: 692895) and. valinomycin (Neupert-Laves et al., 1975; Collison et al., 1989; Boitano and Omoto, 1991, NSC: 630175), we have, in the mean time, investigated and reported the ionophoric behavior both of **5** and **2** (Hassan et al., 2003). The obtained results indicated that both **2** and **5** exhibited a general ionophoric potency for divalent cations. High selectivity however, for Pb (II) ions over alkali, alkaline earth and several transition metal ions was determined (Hassan et al., 2003).

4. Conclusion

The dipicolinic acid acylated cyclopeptide **5** exhibited a promising preliminary cytotoxic activity. Referring to its the reported ionophoric potency, the peptide seems as an interesting candidate for further profound investigations. Correlation between the cytotoxic activity and the ionophoric potency could be a matter of future investigations.

Acknowledgement

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Authors' address: Dr. M. H. Abo-Ghalia, Ass. Research Professor, Dep. Chem. Tanning Mat. & Proteins, National Research Center, El-Tahrir St., 12622-Dokki-Cairo, Egypt,
E-mail: mhaboghalia@yahoo.com