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In Vitro Anticancer Potentiality and Molecular Modelling Study of Novel Cyclic Pentapeptides based on 1, 2-benzenedicarbonyl chloride

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In Loving Memory of Late Professor Doctor ""Mohamed Refaat Hussein Mahran""

Abstract

Cancer continues to be a leading cause of significant mortality globally. The conventional treatment methods currently in use are accompanied by notable side effects, and their efficacy falls short of achieving curative outcomes. Moreover, the considerable costs, technical demands, and cytotoxicity contribute to their limitations. Given their comparatively higher priorities, bioactive peptides with anti-cancer properties have emerged as treatment options within the therapeutic repertoire. This paper describes the synthesis and evaluation of a set of of Cyclo-(Nα-phthaloyl)-bis-[dipetide]-L- Lys -X, (X= ester, acid or hydrazide derivatives) with potential cytotoxic activity against four human cancer cell lines using MTT growth inhibition assay. Compound 8, in particular, demonstrated significant cytotoxicity against the human colon cancer cell line (CaCo-2). Furthermore, an additional exploration encompassed the molecular docking of the promising derivative 20 to examine its binding mode within the active site of the EGFR enzyme. The docking results indicate a favorable fit, attributed to various hydrogen bond interactions with protein residues, hinting at potential anticancer activity.

Keywords: Anti-cancer peptide, Bioactive peptide, cyclic pentapeptide, Nα-phthaloyl-bis-peptides, cytotoxicity.

1. Introduction

Chemotherapy is one of the most important treatment modalities for liver cancer, especially for those who are judged as being unsuitable for surgical resection, local ablative therapy, or trans arterial chemoembolization. However, the efficacy of chemotherapy is still unsatisfactory due to the long duration, side effects and the tendency to develop drug resistance. The development of novel anti- cancer drugs remains imperative. Cyclopeptides have been recognized as new chemical modalities in drug design due to their unique constrained structures, extensive biological activities, higher metabolic stability, cell permeability and bioavailability than linear peptides. A lot of cyclic peptides have been found with potential anti-proliferative activity against malignant cells, and many of them showed excellent anti-cancer activity [1]. Numerous cyclic peptides employed in clinical settings predominantly stem from natural sources. The inherent features that render cyclic peptides appealing as lead compounds for drug development and valuable tools for biochemical research have spurred diverse efforts by scientists to create biologically active cyclic peptide compounds [2]. Therapeutic cyclic peptides present a compelling alternative to proteins or antibodies, primarily due to their smaller size and exceptional ability to permeate cell membranes. These peptides showcase remarkable attributes, displaying high activity, specificity, and affinity while minimizing the potential for drug-drug interactions. Their versatility is evident in their diverse biological and chemical properties. An especially noteworthy advantage is their reduced accumulation in specific organs, a characteristic that contributes significantly to mitigating toxic side effects. This makes therapeutic cyclic peptides promising candidates for drug development, offering a balance between efficacy and safety in medical applications. [3]. Moreover, therapeutic peptide are easily modifiable [4] and exhibit lower immunogenicity compared to recombinant antibodies or proteins [5]. Peptides inherently interact with biological systems, making them potent therapeutics [6-8]. The development of anticancer agents has seen a rise in both natural and synthetic peptides [9,

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10], [11 -14]. The increasing number of peptides in clinical phases and advanced preclinical stages underscores their potential, making drug design and discovery in this domain a current research focus [15, [16 -21]. Peptide candidates exhibiting a spectrum of activities, including anticancer, anti-inflammatory, analgesic, and antimicrobial properties. [22 -49].

2. **Experimental** Details

2.1. Chemistry

IR Spectra were obtained using the Perkin Elmer FT-IR Spectrum BX apparatus. Melting points are uncorrected. Specific optical rotations were measured with a A. Krawss, Optronic, P8000 polarimeter. NMR Spectra were scanned in DMSO-d_6 on a Brucker NMR spectrophotometer at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Mass spectra were obtained using a GCMS-QP1000 EX spectrometer (70 e.V.) Elemental analyses were performed at the Microanalytical Center of Cairo University. (R_F) was determined using Thin Layer Chromatography (TLC) eluted with silica gel aluminum sheets, 60 F254 (E. Merck), it was eluted with (S_1; Butanol/water/acetic acid/ pyridine; 120/48/12/40) or ethyl acetate).

2.1.1. ***Synthesis of phthaloyl dichloride, (2),*** *was prepared according to the reported method [50].*

2.1.2. General procedure for synthesis of (N $^{\alpha}$ - phthaloyl)-)-bis-[dipeptides], (3-12), was prepared according to the reported method [51].

2.1.3. General Procedure for Synthesis of cyclo-(N$^{\alpha}$- Phthaloyl)-bis-[dipeptide] -L-Lys-OMe (Phthaloyl bridged cyclic Penta peptide methyl esters): (13-15).

ECF (2.9 ml, 30.1 mmol) was added to a stirred and cold solution (-20 ^{o}C) of Phthaloyl - *bis* - [dipeptide] (10-12), N $^{\alpha}$ - Phthaloyl - bis-[L-Ala – L-Phe - COOH], **10,** N $^{\alpha}$ - Phthaloyl - bis-[DL-NVa – DL-NVa -COOH], **11** and N $^{\alpha}$ - Phthaloyl - bis-[DL-NVa –L-Phe -COOH], **12**, (15.05 mmol) and TEA (3.3 ml, 30.1 mmol) in DCM (50 ml). The reaction mixture was stirred for additional 30 minutes, followed by the addition of an equivalent of free L-Lysine methyl ester (3.5 gm, 15.05 mmol, -20 ^{o}C) in DCM (50 ml). The reaction work up was continued according to the reported method [51] by the mixed anhydride method. The obtained solid was filtered off and precipitated from EtOH / pet. ether to afford the corresponding cyclic penta peptide methyl esters **(13-15),** Cyclo - (N $^{\alpha}$- Phthaloyl)- bis-[L-Ala – L-Phe]– L-Lys –OMe, **13,** Cyclo - (N $^{\alpha}$ – Phthaloyl) - bis-[DL-NVa – DL-NVa]– L – Lys –OMe, **14 and** Cyclo - (N $^{\alpha}$ – Phthaloyl) - bis-[DL-NVa –L-Phe]– L – Lys –OMe, **15**

Cyclo - (N$^{\alpha}$- Phthaloyl) - bis-[L-Ala –L-Phe]–L-Lys-OMe, 13

13. Yield: (90.9 %); m.p. 84–93 ^{0}C; [α] D = - 300 (C = 0.03).Rf x100 (the eluent) = 93.3 (S_1). *IR (cm^{-1}): (KBr): ν= (KBr): ν=* 3330 (NH stretching), 3070 (CH aromatic), 2945 (CH aliphatic), 1718 (C=O ester), 1648, 1536 and 1447 (C=O amide I, II and amide III, respectively). *^{1}H-NMR* (500 MHz, ppm, DMSO-d_6): δ= 8.58-8.32 (s, 6H, 6NH, D_2O exchangeable), 7.90-7.15 (m, 14H, aromatic), 5.03, 5.02 (t, 2H, NH**CH**CH_2Phe, α CH), 4.15 (t, 1H, CH_2**CH**NH, α CH, Lys), 3.99 (q, 2H, 2CH, NHCHCH$_3$, L-Ala, α CH), 3.62 (s, 6H, 2COOCH$_3$), 3.40 (d, 4H, 2CH_2, **CH_2**Phe, β CH_2),3.25 (t, 2H, NH**CH_2**CH_2, εCH_2, Lys), 2.30-2.00 (m , 6H, 3CH_2, NHCH$_2$**$CH_2CH_2CH_2$**CHNH, γ, δ, β CH_2, Lys), 1.60-1.11 (t, 6H, 2CH_3, NHCHCH$_3$, L-Ala, β CH_3). *MS (EI, 70 eV): m/z (%) =* **726.8 (M$^+$, 0.10%),** 727.8 (**M$^+$ +1,** 0.08%), 684.55 (0.16), 610 (2.01%), 541.70 (0.09%), 505.65 (0.59%), 403.75 (3.20%), 360.75 (8.39%), 277.80 (6.04%), 203.85 (4.88%), 130 (20.60 %), **90.95 (100%)**, 65 (10.03), 53 (1.31%). *Molecular formula (M.wt.),* $C_{39}H_{46}N_6O_8$(726.82): *calculated analysis;* C 64.45, H 6.38, N 11.56; *found analysis;* C 64.44, H 6.36, N 11.54.

Cyclo - (N$^{\alpha}$ – Phthaloyl) - bis-[DL-NVa – DL-NVa] – L – Lys –OMe; 14

14. Yield: (89 %); m.p. 73-75 ^{0}C; Rf x100 (the eluent) = 67.2 (S_1). *IR (cm^{-1}): (KBr): ν= (KBr): ν=* 3309 (NH stretching), 3064 (CH aromatic), 2957 (CH aliphatic), 1730 (C=O ester), 1650, 1536 and 1452 (C=O amide I, II and amide III, respectively). *^{1}H-NMR* (500 MHz, ppm, DMSO-d_6): δ= 9.00 (s, 6H, 6NH, D_2O exchangeable), 8.00-7.90 (m, 4H, aromatic), 4.65 (t, 4H, 4CH, NHCHCH$_2$, DL-NVa, α CH), 4.40 (t, 1H, CH_2**CH**NH, α CH, Lys), 3.85 (s, 3H, COOCH$_3$), 3.30 (m, 2H, NH**CH_2**CH_2, εCH_2, Lys), 2.55 - 1.80 (m , 6H, 3CH_2, NHCH$_2$**$CH_2CH_2CH_2$**CHNH, γ, δ, β CH_2, Lys), 1.50-1.35 (q, 8H, 4CH_2, DL-NVa, β CH_2), 1.20-1.10 (m, 2H, 2CH_2, DL-NVa, γ CH_2), 0.90-95 (s, 12H, 4CH_3, DL-NVa, δ CH_3). *MS (EI, 70 eV): m/z (%)* = **686.84 (M$^+$, 0.22%),**687.8 (**M$^+$ +1,** 0.28%), 601 (0.23%), 562 (0.35), 504.65 (0.10%), 403.75 (0.19%), 377.75 (0.22%), 301.80 (24.37%), 217.90 (0.22%), 185.90 (2.04%), 104.90 (7.17%), **72 (100%)**, 53 (2.91%). *Molecular formula (M.wt.),* $C_{35}H_{54}N_6O_8$ (686.84):*calculated analysis;* C 61.20, H 7.92 N 12.24; ;*found analysis;* C 61.19, 7.91, N 12.22.

Cyclo - (N$^{\alpha}$ – Phthaloyl) - bis-[DL-NVa –L-Phe]–L – Lys –OMe; 15

15. Yield: (84.7 %); m.p. 96-98 ^{0}C; Rf x100 (the eluent) = 83.33 (S_1). *IR (cm^{-1}): (KBr): ν=* 3303 (NH

stretching), 3060 (CH, aromatic), 2949 (CH, aliphatic), 1717 (C=O, ester), 1647, 1536 and 1436 (C=O amide I ,II and III, respectively). *^{1}H-NMR* (500 MHz, ppm, DMSO-d_6): δ= 9.00 (s, 4H, 4NH, D_2O exchangeable), 8.10-7.90 (m, 14H, aromatic), 4.70-4.55 (t, 2H, NH**CH**CH_2Phe, α CH), 4.40 (t, 4H, 4CH, NH**CH**CH_2, DL-NVa, α CH), 4.00 (s, 3H, COO**CH_3**), 3.50 (m, 2H, NH**CH_2**CH_2, εCH_2, Lys),3.25-3.15 (d, 4H, 2CH_2, **CH_2**Phe, β CH_2), 2.70 – 2.15 (m , 6H, 3CH_2, NHCH$_2$**$CH_2CH_2CH_2$**CHNH, γ, δ, β CH_2, Lys), 1.80-1.70 (q, 4H, 2CH_2, DL-NVa, β CH_2), 1.40 (m, 2H, 2CH_2, DL-NVa, γ CH_2), 0.90 (t, 6H, 2CH_3, DL-NVa, δ CH_3). *MS (EI, 70 eV): m/z (%)* = **782.92 (M^+, 0.10%),** 783.9 (**M^+ +1,** 0.04%), 720.6 (0.02%), 704.65 (0.05%), 655.70 (0.27%), 605.75 (1.07%), 514.75 (0.11%), 404.80 (1.30%), 300.85 (1.57%), 177.90 (0.20%), 120 (100%), 60.95 (0.29%), **55 (17.51%).** *Molecular formula (M.wt.),* $C_{43}H_{54}N_6O_8$ (782.92): *calculated analysis;* C 65.97, H 6.95 N 10.73; found *analysis;* C 65.96, H 6.91, N 10.71.

2.1.4. General Procedure for Synthesis of cyclo-(N^α- Phthaloyl)-bis-[dipeptide] -L-Lys-COOH (Phthaloyl bridged cyclic Penta peptide carboxylic acid): (16-18).

To a stirred and cold methanolic solution (-5 ^{o}C, 20 ml) of cyclic penta peptide methyl esters **(13-15),** Cyclo - (N ^{α}- Phthaloyl)- bis-[L-Ala – L-Phe]– L-Lys –OMe, **13,** Cyclo - (N ^{α} – Phthaloyl) - bis-[DL-NVa – DL-NVa]– L – Lys –OMe, **14 and** Cyclo - (N ^{α} – Phthaloyl) - bis-[DL-NVa –L-Phe]– L – Lys –OMe, **15** (1 mmol), sodium hydroxide solution (1N, 25 ml) was (dropwisely) added. The reaction mixture work up was continued according to the reported method [51] to give the corresponding cyclic Penta peptide acids: **(16-18),** Cyclo - (N^α- Phthaloyl)- bis-[L-Ala – L-Phe]– L- Lys-COOH, **16,** Cyclo - (N^α – Phthaloyl) - bis-[DL-NVa – DL-NVa]– L – Lys-COOH, **17 and** Cyclo - (N^α – Phthaloyl) - bis-[DL-NVa –L-Phe]– L – Lys-COOH, **18.**

Cyclo - (N^α- Phthaloyl) - bis-[L-Ala –L-Phe]–L-Lys-COOH, 16

16. Yield: (88.5%); m.p. 187-189 ^{0}C; [α] D = - 133 (C = 0.03).Rf x100 (the eluent) = 51.66 (S_1). *IR (cm^{-1}): (KBr): ν= (KBr): ν=* 3326 (NH stretching), 3064 (CH, aromatic), 2937 (CH, aliphatic), 1713 (C=O, acid), 1644 and 1538 (C=O amide I and II, respectively). *^{1}H-NMR* (500 MHz, ppm, DMSO-d_6): δ= 12.85 (s, 1 H, OH, D_2O exchangeable), 8.55-8.09 (s, 6H, 6NH, D_2O exchangeable), 7.58-7.27 (m, 14H, aromatic), 4.96 (t, 2H, NH**CH**CH_2Phe, α CH), 4.31 (t, 1H, CH_2**CH**NH, α CH, Lys), 3.45(q, 2H, 2CH, NHCHCH_3, L-Ala, α CH), 3.02 (d, 4H, 2CH_2, **CH_2**Phe, β CH_2),3.00 (t, 2H, NH**CH_2**CH_2, εCH_2, Lys), 2.00 – 1.80 (m , 6H, 3CH_2, NHCH$_2$**$CH_2CH_2CH_2$**CHNH, γ, δ, β CH_2, Lys), 1.24, 1.17 (t, 6H, 2CH_3, NHCHCH_3, L-Ala, β CH_3). *MS (EI, 70 eV): m/z (%)* = **740.80 (M^+, 0.03%),** 741.80 (M^+ +1**,** 0.02%), 641.55 (0.10), 600.7 (0.05%), 555.7 (0.05%), 503.75 (1.02%), 404.80 (2.41%), 300.9 (0.77%), 200.9 (0.99%), 155 (1.43%), 110.05 (2.53%), **91 (100%),** 59 (5.61), 50 (10.30%). *Molecular formula (M.wt.),* $C_{39}H_{44}N_6O_9$ (740.80): *calculated analysis;* C 63.23, H 5.99, N 11.34; *found analysis;* C 63.20, H 5.96, N 11.32.

Cyclo - (N^α – Phthaloyl) - bis-[DL-NVa – DL-NVa] – L – Lys-COOH; 17

17. Yield: (89.7 %); m.p. 188-190 ^{0}C; Rf x100 (the eluent) = 77.85 (S_1). *IR (cm^{-1}): (KBr): ν= (KBr): ν=* 3413 (NH stretching), 3033 (CH, aromatic), 2960 (CH, aliphatic), 1709 (C=O, acid), 1642 and 1539 (C=O amide I and II, respectively). *^{1}H-NMR* (500 MHz, ppm, DMSO-d_6): δ= 12.65 (s, 1 H, OH, D_2O exchangeable), 8.26 (s, 6H, 6NH, D_2O exchangeable), 7.66-7.53 (m, 4H, aromatic), 4.55 (t, 4H, 4CH, NHCHCH_2, DL-NVa, α CH), 4.31 (t, 1H, CH_2**CH**NH, α CH, Lys), 3.15 (m, 2H, NH**CH_2**CH_2, εCH_2, Lys), 2.40 - 1.95 (m , 6H, 3CH_2, NHCH$_2$**$CH_2CH_2CH_2$**CHNH, γ, δ, β CH_2, Lys), 1.65-1.45 (q, 8H, 4CH_2, DL-NVa, β CH_2), 1.35-1.25 (m, 2H, 2CH_2, DL-NVa, γ CH_2), 1.10-1.00 (s, 12H, 4CH_3, DL-NVa, δ CH_3). *MS (EI, 70 eV): m/z (%)* = **672.81 (M^+, 0.03%),** 673.8 (M^+ +1**,** 0.02%), 600 (0.04%), 550 (0.08%), 504 (0.05), 450.85 (0.27%), 403.90 (0.34%), 300.90 (8.94%), 265.95(0.53%), 205.90 (0.48%), 157 (1.00%), **72.05(100%)**, 59.05(4.47%), 50 (11.86%). *Molecular formula (M.wt.),* $C_{34}H_{52}N_6O_8$(672.81): *calculated analysis;* C 60.70, H 7.79, N 12.49; ;*found analysis;* C 60.68, H 7.77, N 12.47.

Cyclo - (N^α – Phthaloyl) - bis-[DL-NVa –L-Phe]– L – Lys-COOH; 18

18. Yield: (83.8 %); m.p. 112-114 ^{0}C; Rf x100 (the eluent) = 57.14 (S_1). *IR (cm^{-1}): (KBr): ν=* 3411 (NH stretching), 3063 (CH, aromatic), 2950 (CH, aliphatic), 1709 (C=O, acid), 1640 and 1541 (C=O amide I and II, respectively). *^{1}H-NMR* (500 MHz, ppm, DMSO-d_6): δ= 12.55 (s, 1 H, OH, D_2O exchangeable), 8.80 (s, 4H, 4NH, D_2O exchangeable), 7.90-7.80 (m, 14H, aromatic), 4.96 (t, 2H, NH**CH**CH_2Phe, α CH), 4.50 (t, 4H, 4CH, NHCHCH_2, DL-NVa, α CH), 4.00 (m, 2H, NH**CH_2**CH_2, εCH_2, Lys),3.12 (d, 4H, 2CH_2, **CH_2**Phe, β CH_2), 2.60 – 2.25 (m , 6H, 3CH_2, NHCH$_2$**$CH_2CH_2CH_2$**CHNH, γ, δ, β CH_2, Lys),2.00 (q, 4H, 2CH_2, DL-NVa, β CH_2), 1.30 (m, 2H, 2CH_2, DL-NVa, γ CH_2), 0.90 (t, 6H, 2CH_3, DL-NVa, δ CH_3). *MS (EI, 70 eV): m/z (%)* = **768.90 (M^+, 0.01%),** 617.0 (0.03%), 550.85 (0.11%), 500.75 (0.09%), 460 (0.58%), 403 (1.14%), 322 (0.08%). 285 (1.05%), 214 (0.61%), 172 (1.52%), 110 (2.15%), **91 (100%),** 60 (0.49%), 50 (4.52%). *Molecular formula (M.wt.),* $C_{42}H_{52}N_6O_8$(768.90): *calculated analysis;* C 65.61, H 6.82, N 10.93; found *analysis;* C 65.59, H 6.80, N 10.90.

2.1.5. General Procedure for Synthesis of cyclo-(N^{α}- Phthaloyl)-bis-[dipeptide] -L-Lys-$NHNH_2$ (Phthaloyl bridged cyclic Penta peptide hydrazide): (19-21).

To a stirred methanolic solution (50 ml) of cyclic penta peptide methyl esters (**13-15**), (1 mmol), anhydrous hydrazine hydrate (0.35 ml, 10 mmol) was added. The reaction mixture work up was continued according to the reported method [51] to give the corresponding cyclic Penta peptide hydrazides: **(19-21),** Cyclo - (N^{α}- Phthaloyl)- bis-[L-Ala – L-Phe]– L- Lys-$NHNH_2$, **19,** Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa – DL-NVa]– L- Lys-$NHNH_2$, **20 and** Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa –L-Phe]– L-Lys-$NHNH_2$, **21.**

Cyclo - (N^{α}- Phthaloyl) - bis-[L-Ala –L-Phe]–L-Lys-$NHNH_2$, 19

19. Yield: (60 %); m.p. Decompose at 268-270 ^{0}C; [α] D = - 150 (C = 0.03).Rf x100 (the eluent) = 65 (S_1). *IR (cm^{-1}): (KBr): ν= (KBr): ν=* 3374 (NH stretching), 2924 (CH, aromatic), 2855 (CH, aliphatic), 1640, 1462 and 1320 (C=O amide I, II and III, respectively). *^{1}H-NMR* (500 MHz, ppm, DMSO-d_6): δ= 9.85 (s, 1H, CO**NH**NH_2), 8.07-7.82 (s, 6H, 6NH, D_2O exchangeable), 7.27-7.15 (m, 14H, aromatic), 4.96 (t, 2H, NH**CH**CH_2Phe, α CH), 4.55 (t, 2H, NH**CH**CH_2Phe, α CH), 4.30 (s, 2H, CONH**NH_2**), 4.20 (t, 1H, CH_2**CH**NH, α CH, Lys), 3.50(q, 2H, 2CH, NHCHCH_3, L-Ala, α CH), 3.20 (d, 4H, 2CH_2, **CH_2**Phe, β CH_2),3.05 (t, 2H, NH**CH_2**CH_2, εCH_2, Lys), 2.15-1.95 (m , 6H, 3CH_2, NHCH_2**$CH_2CH_2CH_2$**CHNH, γ, δ, β CH_2, Lys), 1.40, 1.20 (t, 6H, 2CH_3, NHCHCH_3, L-Ala, β CH_3). *MS (EI, 70 eV): m/z (%)* = **726.8 (M^+, 0.03%),** 728 (**M^++1,** 0.02 %), 664 (0.05%), 601 (0.98%), 559 (0.16%), 510 (0.11%), 452 (0.27%), 403 (0.86%), 308 (0.59%), 200 (2.13%), 161 (4.53%), **120 (100%),** 85 (17.42%), 59 (12.37%). *Molecular formula (M.wt.),* $C_{38}H_{46}N_8O_7$ (726.82): *calculated analysis;* C 62.79, H 6.38, N 15.42; *found analysis;* C 62.78, H 6.36, N 15.40.

Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa – DL-NVa] – L- Lys-$NHNH_2$, 20

19. Yield: (70 %); m.p. Decompose at 230-232 ^{0}C; Rf x100 (the eluent) = 75.4 (S_1). *IR (cm^{-1}): (KBr): ν= (KBr): ν=* 3272 (NH stretching), 3154 (CH, aromatic), 2940 (CH, aliphatic), 1647, 1573 and 1448 (C=O amide I, II and III, respectively). *^{1}H-NMR* (500 MHz, ppm, DMSO-d_6): δ= 9.05 (s, 1H, CO**NH**NH_2), 8.09-8.06 (s, 6H, 6NH, D_2O exchangeable), 7.88-7.85 (m, 4H, aromatic), 4.30 (t, 4H, 4CH, NHCH_2, DL-NVa, α CH), 4.25(s, 2H, CONH**NH_2**), 4.15 (t, 1H, CH_2**CH**NH, α CH, Lys), 3.30 (m, 2H, NH**CH_2**CH_2, εCH_2, Lys), 2.10 - 1.85 (m , 6H, 3CH_2, NHCH_2**$CH_2CH_2CH_2$**CHNH, γ, δ, β CH_2, Lys), 1.40-1.20 (q, 8H, 4CH_2, DL-NVa, β CH_2), 1.15-1.00 (m, 2H, 2CH_2, DL-NVa, γ CH_2), 0.90-0.75 (s, 12H, 4CH_3, DL-NVa, δ CH_3). *MS (EI, 70 eV): m/z (%)* = **686.8 (M^+, 0.03%),**687.8 (**M^++ 1, 0.02**%), 611 (0.06%), 549 (1.65%), 503 (0.41), 438 (0.58%), 379 (0.56%), 306 (0.48%), 205 (0.43%), 159 (17.62%), 110(5.11%), 72 **(100%),** 59 (6.16%), 50 (3.43%). *Molecular formula (M.wt.),* $C_{34}H_{54}N_8O_7$(686.84): *calculated analysis;* C59.46, H 7.92, N 16.31; *found analysis;* C 59.44, H 7.90, N 16.30.

Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa –L-Phe]–L-Lys-$NHNH_2$, 21.

21. Yield: (77.5 %); m.p. Decompose at 280-282 ^{0}C; Rf x100 (the eluent) = 84.9 (S_1). *IR (cm^{-1}): (KBr): ν= (KBr): ν=* 3410 (NH stretching), 2930 (CH, aromatic), 2887 (CH, aliphatic), 1618, 1469 and 1319 (C=O amide I, II and III, respectively). *^{1}H-NMR* (500 MHz, ppm, DMSO-d_6): δ= 9.75 (s, 1H, CO**NH**NH_2), 8.10-8.05 (s, 6H, 6NH, D_2O exchangeable), 7.86-7.19 (m, 14H, aromatic), 4.90 (t, 2H, NH**CH**CH_2Phe, α CH), 4.45 (t, 4H, 4CH, NHC$H$$CH_2$, DL-NVa, α CH), 4.25 (s, 2H, CONH**NH_2**), 4.10 (m, 2H, NH**CH_2**CH_2, εCH_2, Lys), 3.05 (d, 4H, 2CH_2, **CH_2**Phe, β CH_2), 2.75 – 2.40 (m , 6H, 3CH_2, NHCH_2**$CH_2CH_2CH_2$**CHNH, γ, δ, β CH_2, Lys), 2.00 (q, 4H, 2CH_2, DL-NVa, β CH_2), 1.45 (m, 2H, 2CH_2, DL-NVa, γ CH_2), 1.00-0.85 (t, 6H, 2CH_3, DL-NVa, δ CH_3). *MS (EI, 70 eV): m/z (%)* = **782.8 (M^+, 0.02 %),** 705 (0.06%), 633 (0.79%), 569 (0.09%), 501 (0.35%), 403 (1.33%), 334 (1.12%). 292 (1.92%), 228 (3.01%), 201 (20.51%), 159 (32.8%), 120 (88.9%), **72 (100%),** 60 (3.91%), 50 (7.63). *Molecular formula (M.wt.),* $C_{42}H_{54}N_8O_7$ (782.93): *calculated analysis;* C64.43, H 6.95, N 14.31; *found analysis;* C 64.41, H 6.92, N 14.28 .

2.2. In-vitro cytotoxic activity against some selected human cancer cell lines

Human lung (A-549), colon (CaCo-2), prostate(PC-3) and breast (MCF) cancer cell were obtained from Karolinska Center, Department of Oncology and Pathology, Karolinska Institute and Hospital, Stockholm, Sweden. IC50 values were performed using SPSS computer program (SPSS for windows, statistical analysis software package /version 9/ 1989 SPSS Inc., Chicago, USA). The procedure was done in laminar air flow cabinet bio safety class II level. Culturing and sub culturing were carried out according to Thabrew [52]. Doxorubicin was used as a positive control. DMSO used as negative control. Cell Viability Assay was done according to (Selim) [53] as described by Mosmann [54]. The cells were seeded at concentration of 10x103 cells per well in case of MCF-7, 20x103 cells/well in case of HCT-116 cell lines using 96-well plates at 37 ^{o}C. After 48 hours' incubation, the medium was aspirated and 40 µl MTT salt (2.5 mg/ml) were added and further incubated for 4 hours. 200µl 10% sodium dodecyl sulphate (SDS) was added. The absorbance was measured at 595nm.

2.3. Molecular docking studies

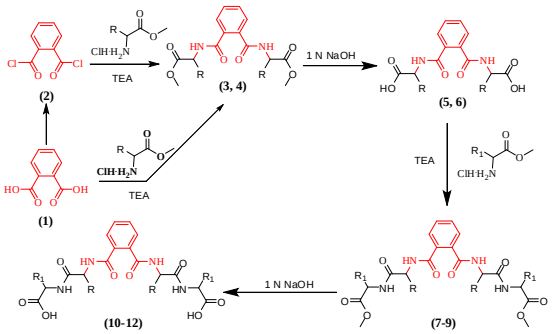
The molecular modeling of Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa – DL-NVa] – L- Lys-$NHNH_2$, **20** was carried out using Molecular Operating Environment (MOE, 10.2008) software. TheX-ray crystallographic structure of EGFR co crystallized with erlotinib as inhibitor (PDB ID: 1M17) [55] was downloaded from the protein data bank. The receptor was prepared for docking study using Protonate 3D protocol in MOE with default options followed by water molecules removal. The co-crystalized ligand was used to define the active site for docking. Docking setup was first validated by re-docking of the co crystallized ligand in the vicinity of the active site of the receptor. The validated setup was then used in predicting the ligand-receptor interactions at the active site for compound **20**.

3. Results and Discussion

3.1. Chemistry

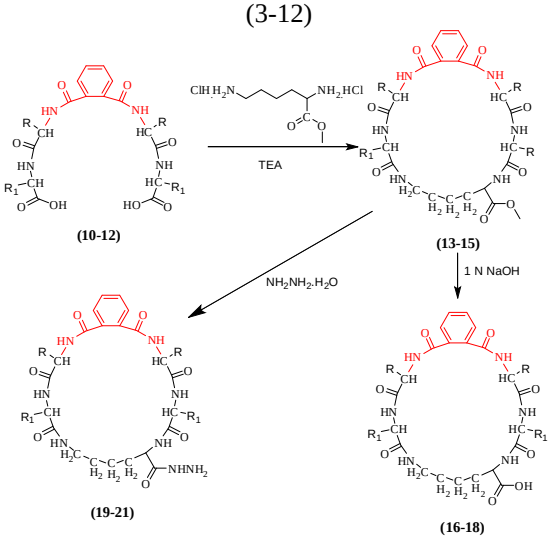
The coupling cyclization of the tetrapeptide precursors with L-Lysine-OMe to afford the cyclic pentapeptides could be considered as the critical synthetic step in this work. The synthesis cyclo-(N^{α}-phthaloyl)-bis-[dipeptide]-L-Lys-OMe (phthaloyl bridged cyclic pentapeptide methyl esters): (**13-15**) was based on phthaloyl-*bis*-[dipeptide] candidates, **(10-12),** which were converted to anhydride, formed with ethylchloroformate in the presence of TEA as tertiary base. The acid was then coupled with L-Lys-OMe in the presence of TEA. The IR spectra showed the presence of aromatic rings. The amide linkage confirmation was based on three characteristic bands observed at (1648, 1536, and 1447), (1650, 1536, and 1452), and (1647, 1536, and 1436) cm-1, corresponding to the regions of amide I, II, and III, respectively. Additionally, the presence of the ester group was supported by the observation of bands at 1718, 1730, and 1717 cm-1, respectively, ν (C=O) ester. In addition, the stretching vibration frequency of NH groups was observed at 3330, 3309 and 3303 cm^{-1} respectively. The EIMS of the obtained products **(13-15)** revealed the presence of molecular ion peaks at m/z: 726, 686 and 782, with base peak at m/z: 90, 72 and 55 (100%) respectively, which are consistent with their molecular weights. The ^{1}H-NMR spectra confirmed the existence of a singlet (3H) at δ (3.61-4.00) ppm for the (**CH_3** ester), a multiplet (4H) at δ (4.40-4.15) ppm for the (**CH**NH). Additionally, new characteristic signals appeared as a result of cyclization with L-Lysine ester: a multiplet (2H) at δ (3.32-3.5) ppm for the protons of the CH_2 adjacent to the NH group of lysine (NH**CH_2**), a multiplet (6H) at δ (1.80-270) ppm for the protons of the remaining CH_2 groups of lysine. The methyl groups of the L-Lys-OMe esters within the cyclic pentapeptides underwent conversion into carboxylic acid groups. The hydrolysis of pentapeptide methyl esters **(13-15)** with 1 N sodium hydroxide in methanol resulted in the formation of the corresponding compounds**:** *Cyclo - (N^{α} -Phthaloyl) - bis-[L-Ala – L-Phe]– L – Lys –COOH*, **16**, *Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa – DL-NVa]– L – Lys – COOH*, **17**, *Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa –L-Phe]– L – Lys –COOH***,** **18**. The IR spectra indicated the absence of the ν (C=O, ester) signal and, instead, revealed a band at 1713, 1709, and 1709 cm-1, corresponding to the presence of the ν(C=O, acid). The EIMS of the obtained products **(16-18)** revealed the presence of molecular ion peaks at m/z: 740, 672 and 768, respectively, with base peak at m/z: 91, 72 and 91 (100%), respectively, which are consistent with their molecular weights. The ^{1}H-NMR spectra revealed the disappearance of the singlet (3H) for the (CH_3 ester) and the appearance of a singlet (1H) at δ (12.48-12.85) ppm for carboxylic (OH) protons, which are exchangeable with D_2O.

The methyl groups of the L-Lys-OMe of the cyclic pentapeptides were converted to hydrazide functions. Hydrazinolysis of pentapeptide methyl ester derivatives **(13-15)** with hydrazine hydrate in methanol afforded the corresponding: Cyclo - (N^{α} – Phthaloyl) - bis-[L-Ala – L-Phe]– L – Lys - $NHNH_2$, **19**, Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa – DL-NVa]– L – Lys - $NHNH_2$, **20** and Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa –L-Phe]– L – Lys - $NHNH_2$, **21**. The IR spectra indicated the absence of ν (C=O, ester), and the amide linkage was well identified by its three characteristic bands in (1640, 1462 and 1320), (1647, 1573 and 144) and (1618, 1469 and 1319) cm^{-1} region respectively, (amide I, II and III respectively). In addition, the NH stretching vibrations of the amide and hydrazide groups appeared as a broad band centered at 3374, 3272 and 3410 cm^{-1} respectively. The EIMS of the obtained products (**19-21**) revealed the presence of molecular ion peaks at m/z: 726, 686 and 782, respectively with base peak at m/z: 120, 72 and 72, respectively (100%), which are consistent with their molecular weights. The ^{1}H-NMR spectra revealed the disappearance of the singlet (3H) for the (CH_3 ester) and the appearance of a broad singlet (2H) at δ (4.25-4.30) ppm for the amino protons (NH_2). The amide (NH) protons appeared as singlets (7H) at δ (8.18-7.82) ppm which are exchangeable with D_2O (scheme 2) for compounds (**13-21**).



(**3, 5**), R=CH_3; (**4, 6**), $CH_2CH_2CH_3$, (**7, 10**), R=CH_3, R_1=CH_2Phe; (**8, 11**), R= $CH_2CH_2CH_3$, R_1= $CH_2CH_2CH_3$; (**9, 12**), R= $CH_2CH_2CH_3$, R_1=CH_2Phe.

Scheme 1. Pathways for synthesizing compounds (3-12)



(**10, 13, 16, 19**), R=CH_3, R_1=CH_2Phe; (**11, 14, 17, 20**), R=CH_3, R_1= $CH_2CH_2CH_3$; (**12, 15, 18, 21**), R= CH_3, R_1=CH_2Phe.

Scheme 2. Pathways for synthesizing compounds (13-21)

2.2 Anti cancer activity of compounds 13-21 against different human cancer cell lines using MTT assay.

The anticancer activity of all synthesized peptides (13-21) was assessed against four cancer cell lines—human lung (A 549), colon (CaCo-2), prostate (PC-3), and breast (MCF-7) cancer cells—utilizing the MTT growth inhibition assay at a concentration of 100 µM [54]. Positive and negative controls, Doxorubicin, and DMSO, respectively, were employed. The results are detailed in Table 1. As indicated in Table 1, Cyclo - (N^{α}– Phthaloyl) - bis-[DL-NVa – DL-NVa] – L – Lys –COOH, **17**, displayed high cytotoxic activity against human lung (A 549), (growth inhibition 74.95%), while other compounds showed low cytotoxic activity. Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa – DL-NVa] – L – Lys - $NHNH_2$, **20**, revealed moderate anticancer activity towards prostate (PC-3) cell line, however, it displayed high cytotoxic activity towards colon (CaCo-2) cell line (growth inhibition 61.12 and 70.02% respectively), whereas other compounds displayed from low to moderate cytotoxic activity against them. Regarding to colon (MCF-7) cell line, all compounds showed low to moderate cytotoxic activity towards it (growth inhibition from 4.12 to 55.05%).

Table1: The percentage of the cytotoxicity of the promising compounds towards human cancer cell lines at 100 µM.

Compound NO	Compound Name	Growth Inhibition (%)			
		A-549	CaCo2	PC-3	MCF -7
13.	Cyclo - (N $^{\alpha}$- Phthaloyl)- bis-[L-Ala – L-Phe]– L- Lys –OMe	2.71	38.35	19.77	45.65
14.	Cyclo - (N $^{\alpha}$ – Phthaloyl) - bis-[DL-NVa – DL-NVa]– L – Lys –OMe	22.48	26.2	14.35	4.13
15.	Cyclo - (N $^{\alpha}$ – Phthaloyl) - bis-[DL-NVa – L-Phe]– L – Lys –OMe	30.45	18.65	9.23	6.16
16.	Cyclo - (N $^{\alpha}$ – Phthaloyl) - bis-[L-Ala – L-Phe]– L – Lys –COOH	8.55	66.4	48.35	55.05
17.	Cyclo - (N $^{\alpha}$ – Phthaloyl) - bis-[DL-NVa – DL-NVa]– L – Lys –COOH	74.95	29.5	13.6	52.5
18.	Cyclo - (N $^{\alpha}$ – Phthaloyl) - bis-[DL-NVa – L-Phe]– L – Lys –COOH	8.21	46.05	31.6	29.15
19.	Cyclo - (N $^{\alpha}$ – Phthaloyl) - bis-[L-Ala – L-Phe]– L – Lys - $NHNH_2$	5.75	20.33	16.85	23.65
20.	Cyclo - (N $^{\alpha}$ – Phthaloyl) - bis-[DL-NVa – DL-NVa]– L – Lys - $NHNH_2$	8.6	70.02	61.12	48.74
21.	Cyclo - (N $^{\alpha}$ – Phthaloyl) - bis-[DL-NVa – L-Phe]– L – Lys - $NHNH_2$	32.005	37.4	22.15	54.1
DMSO		0	0	0	0
Doxorubicin		100	100	100	100

IC_{50} was determined for compounds which achieved higher cytotoxic activity (> 70%). Doxorubicin served as the reference drug in this analysis, as presented in Table 2.

Table 2; IC_{50} of compound 20 which has the highest cytotoxic activity (> 70%) against CaCo -2 cell line.

Compd No.		IC50 (Mean ± SD) (mM) a
		CaCo -2 cell line
20	*Cyclo - (N $^{\alpha}$ – Phthaloyl) - bis-[DL-NVa – DL-NVa]– L – Lys - $NHNH_2$*	87.9 ±1.3
Doxorubicin		0.065 ± 1.00

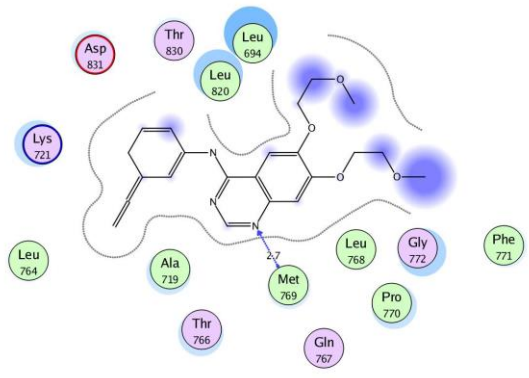
The IC_{50} values, along with their standard errors (±), were calculated using the SPSS statistical program.

3.2. Molecular docking studies

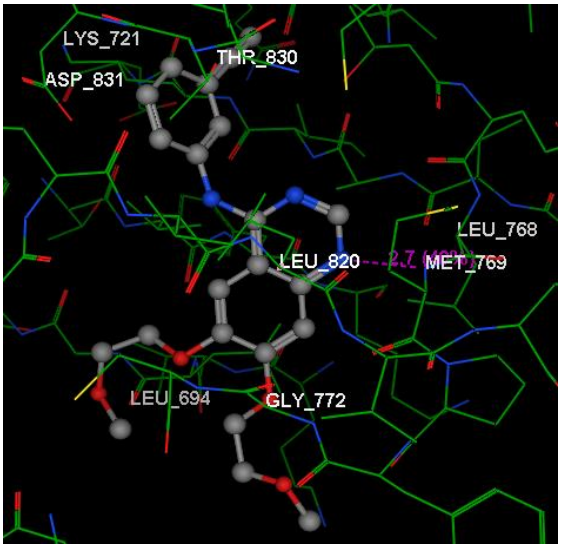
Depending on the promising cytotoxic activities ofthe newly synthesized Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa – DL-NVa] – L – Lys - $NHNH_2$, **20**, the docking study was applied using Molecular Operating Environment (MOE®) 2008.10 [56]. To illustrate their mechanism of action. Upon continuation of our research on isophthalamide based derivatives that revealed anticancer activities targeting the epidermal growth factor receptor (EGFR) [57], the docking study of Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa – DL-NVa] – L – Lys - $NHNH_2$, **20,** was achieved against EGFR to evaluate the binding affinity and the possible interactions. The X-ray crystallographic structure of EGFR bound with its ligand erlotinib was downloaded from protein data bank (pdb Id: 1M17) [57]. Redocking of the original ligand was established to evaluate RMSD value (1.02 A^{o}).

The original inhibitor (erlotinib) sharedthe binding with a hydrogen bond acceptor between its quinazoline nitrogen atom and the backbone of Met769 (distance: 2.70 Å) [56]. Figure 1.

The docking of the phthaloyl bridged cyclic pentapeptide hydrazide; Cyclo - (N^{α} – Phthaloyl) - bis-[DL-NVa – DL-NVa] – L – Lys - $NHNH_2$, **20** was inserted in figure 2. The benzamide part played an essential rule in binding via formation of two arene-cation interactions with Lys730 and Lys851 and fixation of Glu734 within the centre of the cyclic shape via hydrogen bonding with NH group. On the other hand, the terminal amino group formed H-bond donor and acceptor with the backbone of Asp831 and Gly833 (distance: 2.54 and 2.57 Å, respectively).

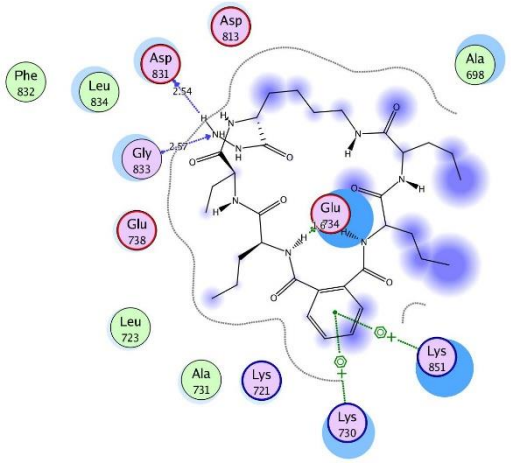


A

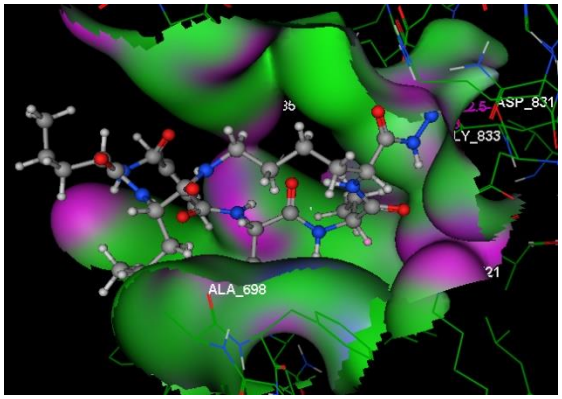


B

Figure 1. (A & B) the 2D and 3D visualization docking of erlotinib into the bending site of EGFR, PDB: 1M17. Hydrogen bonds are illustrated as dotted lines with arrows, while carbon (C) atoms are shaded in gray, nitrogen (N) in blue, and oxygen (O) in red.



A



B

Figure 2: (A & B) the 2D and 3D visualization docking of compound 20 into the binding site of EGFR, PDB: 1M17. green color signifies the hydrophobic area, pink indicates the high polar area, and blue designates the mild polar area. Hydrogen bonds are depicted as dotted lines with arrows, with carbon (C) atoms colored gray, nitrogen (N) in blue, and oxygen (O) in red.

4. Conclusion

The primary objective of this study was to synthesize novel cyclic pentapeptides using phthaloyl dichloride (1, 2-benzenedicarbonyl chloride). The synthesized compounds, characterized by diverse spectral data, conform to the general structures of Cyclo-(Nα-phthaloyl)-bis-[dipeptide]-L-Lys-X, where X represents ester, acid, or hydrazide. The cytotoxic activities of these novel peptide candidates were evaluated against four human carcinoma cell lines (CaCo-2, A-549, MCF-7, and PC-3) using MTT assay at a concentration of 100 μM. Several compounds exhibited significant cytotoxic activity, particularly against CaCo-2 cell lines. Molecular docking studies were conducted for the highly promising derivative 20 within the EGFR binding site to elucidate its mechanism of action. The conclusion suggests the necessity for further comprehensive biological investigations, particularly conventional anticancer studies using experimental animal models.

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