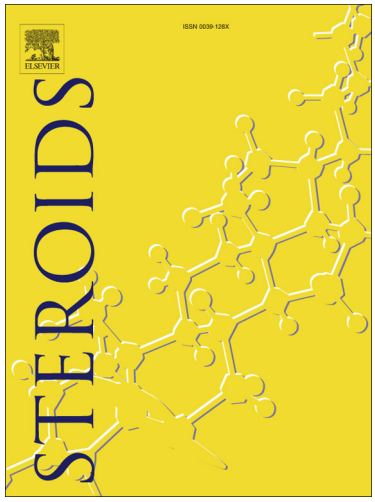

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Synthesis, Characterization and Biological evaluation of Bile acid-aromatic/heteroaromatic amides linked *via* amino acids as anti-cancer agents

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Abstract

A series of bile acid (Cholic acid and Deoxycholic acid) aryl/heteroaryl amides linked *via* α-amino acid were synthesized and tested against 3 human cancer cell-lines (HT29, MDAMB231, U87) and 1 human normal cell line (HEK293T). Some of the conjugates showed promising results to be new anticancer agents with good *in vitro* results. More specifically, Cholic acid derivatives **6a** (1.35 μM), **6c** (1.41 μM) and **6m** (4.52 μM) possessing phenyl, benzothiazole and 4-methylphenyl groups showed fairly good activity against the breast cancer cell line with respect to Cisplatin (7.21 μM) and comparable with respect to Doxorubicin (1 μM), while **6e** (2.49 μM), **6i** (2.46 μM) and **6m** (1.62 μM) showed better activity against glioblastoma cancer cell line with respect to both Cisplatin (2.60 μM) and Doxorubicin (3.78 μM) drugs used as standards. Greater than 65% of the compounds were found to be safer on human normal cell line.

Keywords: cancer; cholic acid; coupling; cytotoxicity; amino acid; heteroaryl amines

1. Introduction

Primary bile acids such as Cholic acid (CA) and Chenodeoxycholic acid (CDCA) are endogenous steroids that are excreted into the bile canaliculus and the digestive tract after cholesterol catabolism [1]. Bacterial metabolism of tauryl and glycyl conjugates of CA and CDCA in distal small intestine and colon produces secondary bile acids such as Deoxycholic acid (DCA), Ursodeoxycholic acid (UDCA), and Lithocholic acid (LCA) [2]. Slight differences in the chemical structure of these bile acids make them behave distinctly in the

biological environment. Earlier reports on hydrophobic bile acids indicated their cytotoxic behaviour towards normal tissues [3], while less hydrophobic bile acid such as UDCA protect normal cells against apoptosis induced by direct prevention of mitochondrial membrane perturbation [4]. UDCA and its derivatives have been extensively studied as potent scaffolds in medicinal chemistry [5]. *In vitro* and animal studies have suggested the chemopreventive behaviour of UDCA towards colorectal cancer [6]. UDCA has been used for the prevention of gastrointestinal disorders in patients having various cancers such as stomach, colon, lung, breast, and liver cancer [7]. UDCA itself has been evaluated as a drug in clinical phase III trial therapy to prevent colorectal adenoma recurrence [8]. Recently, Qui and co-workers reported anticancer effect of UDCA in human oral squamous carcinoma HSC-3 cells through the caspases [9]. UDCA and its metal complexes decreased the viability and proliferation of cultured human and animal tumour cells in a time- and concentration-dependent manner [10]. In addition, UDCA expresses an inhibitory effect on the induction of P-glycoprotein expression and reactive oxygen species by Doxorubicin in HepG2 human hepatoma cells [11].

In 2001, Powell and co-workers reported a direct correlation between bile acids hydrophobicity and induction of apoptosis and/or growth arrest in HCT116 cells, however no apparent link was observed between a particular structural modification and its biological activity [12]. Nonetheless, reports in the last two decades suggested the induction of apoptosis by hydrophobic bile acid derivatives in many human cancer cells [13], such as prostate cancer cells [14], leukemic T cells [15], heaptocellular carcinoma cells [16], colon cancer cells [17], breast carcinoma cells [18], osteosarcoma cells [19], and cervical carcinoma cells [20]. However, low cytotoxicity (IC_{50}> 100 μM) and tumour promoter ability of certain bile acid derivatives has compelled medicinal chemist to design more efficient bile acid derivatives with potent anti-proliferative and apoptotic properties. Thus, physiological and carcinogenesis studies of bile acid derivatives have experienced a significant progress in the last decade.

Many bile acid-amino acid conjugates have been studied for their anti-cancer properties. Among these, compounds HS-1183, HS-1199 and HS-1200 (Figure 1) showed promising results in inducing apoptosis with an IC_{50} values from 25 to 50 μM for SiHa human cervical carcinoma cells, 30 to 45 μM for MDA-MB-231 cells and 30 to 150 μM for MCF-7. Reports also suggests unnatural long chain amino acid conjugates of bile acid to possess strong anti-

cancer activity against several tumour cell lines including human breast adenocarcinoma (ER-, MDA-MB-231), breast adenocarcinoma (ER+, MCF-7), cervix epiteloid carcinoma, (HeLa S-3) and prostate cancer (PC-3) [21]. Khiel and co-workers reported piperazinyl linked aryl and heteroaryl substituted bile acid derivatives with apoptosis-inducing activity on multiple myeloma cancer cell line KMS-11[22]. Bile acid–polyaminocarboxylate conjugates containing NE3TA, a potential iron chelator displayed significant cytotoxicity in both HeLa and HT29 colon cancer cells [23]. Other reports on the synthesis and structural studies of bile acid-4-aminopyridine conjugates [24], *N*-(2-aminoethyl)amido linked bile-acid–heteroaryl conjugates [25], bile acid-arene conjugates [26] and bile acid-glutamyl-pyridines [27] have been described, however no anticancer-activity evaluation studies were reported.

In view of the preceding discussion, we herein describe the design (Figure 1) and synthesis of new bile acid-aromatic/heteroaromatic amides linked *via* amino acids. *In-vitro* anti-cancer studies were accomplished on human breast adenocarcinoma cell line (MDA-MB-231), human colorectal adenocarcinoma cell line (HT29) and human glioblastoma cell line (U87).

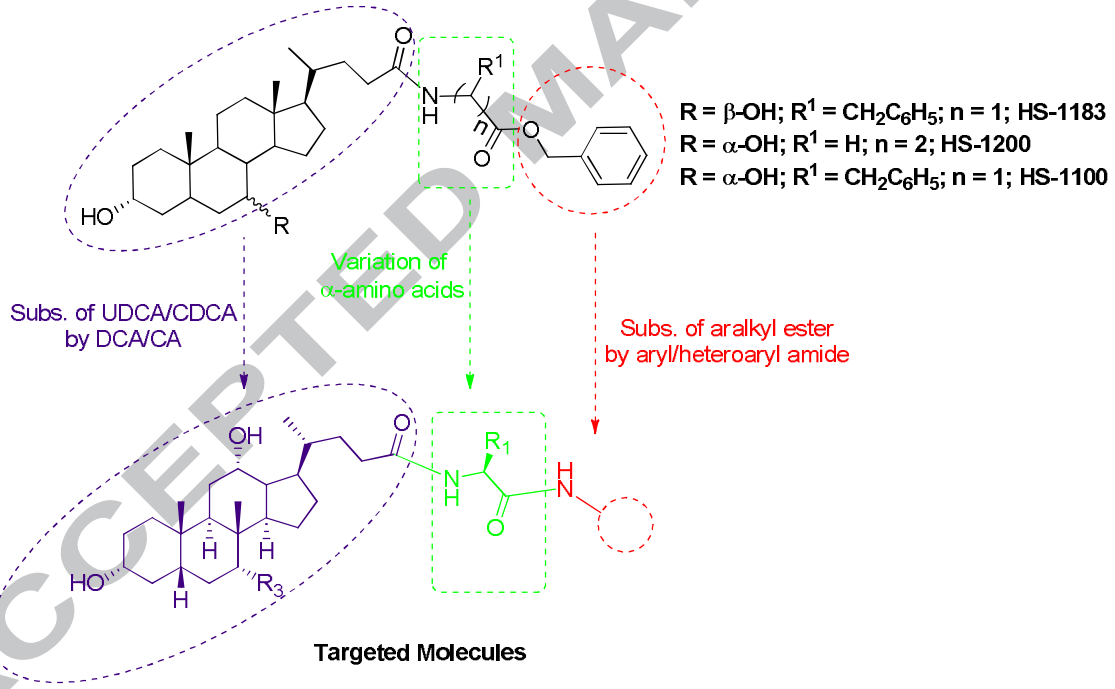


Figure 1. Design of targeted molecules with reference to known anti-cancer bile acid-amino acid conjugates

2. Experimental

2.1 General

All the chemicals were purchased from Sigma-Aldich, Alfa Aesar, and Spectrochem India Pvt. Ltd and used without further purification. The solvents used were purchased from Merck

(India) and were distilled and dried before use. Nuclear magnetic resonance spectra were recorded on Bruker 400 spectrometer. The ^{1}H NMR experiments were reported in δ units, parts per million (ppm), and were measured relative to residual chloroform (7.26 ppm) or DMSO (2.5 ppm) in the deuterated solvent. The ^{13}C NMR spectra were reported in ppm relative to deuterochloroform (77.0 ppm) or DMSO-d_6 (39.5 ppm). All coupling constants J were reported in Hz. The following abbreviations were used to describe peak splitting patterns when appropriate: s = singlet, d = doublet, t = triplet, dd = doublet of doublet, m = multiplet and br s = broad singlet. Melting points were determined on a capillary point apparatus equipped with a digital thermometer and are uncorrected. Reactions were monitored by using thin layer chromatography (TLC) on 0.2 mm silica gel F254 plates (Merck). The chemical structures of final products were confirmed by a high-resolution ESI/APCI- hybrid quadrupole time-of-flight mass spectrometer. High resolution mass spectrometry (HRMS) was performed with a waters synapt G2 HDMS instrument using time-of-flight (TOF-MS) with ESI/APCI- hybrid quadrupole. Optical rotations were recorded using a Perkin Elmer 343 series polarimeter in methanol.

2.2 General Synthesis of Boc protected aminoacyl aromatic/heteroaromatic amides

To the stirred solution of **1** (1 mmol) in DMF (20 mL), triethyl amine (2.5 mmol) was added at 0 ^{o}C and subsequently EDC.HCl (1.5 mmol) and HOBt (1 mmol) was added. The reaction mixture was stirred for 15 min. at 0 ^{o}C, after that aryl/heteroaryl amines (**2**) (1.1 mmol) was added and the reaction was stirred at room temperature for 6-8 h. The completion of the reaction was monitored by TLC. After the completion of the reaction, crushed ice was added. The resulted precipitate was filtered, washed with cold water and recrystallized with ethyl acetate/hexanes to yield pure **3a-g**.

*2.2.1 tert-Butyl ((2S,3R)-3-methyl-1-oxo-1-(phenylamino)pentan-2-yl)carbamate (**3a**)*

Off-white solid; yield: 81%, mp: 130-132 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (br s, 1H, NH_{Ar}), 7.47 (d, J = 8.0 Hz, 2H, H-3$_{Ar}$ & H-5$_{Ar}$), 7.21 (t, J = 7.0 Hz, 2H, H-2$_{Ar}$ & H-6$_{Ar}$), 7.03 (t, J = 7.2 Hz, 1H, H-4$_{Ar}$), 5.39 (d, J = 8.6 Hz, 1H, $NH_{isoleucine}$), 4.13 (t, J= 8.0 Hz, 1H, $CH_{isoleucine}$), 1.90-1.99 (m, 1H), 1.42 (s, 9H, 3xMe_{Boc}), 1.30 – 1.07 (m, 2H), 0.99 (d, J = 6.8 Hz, 3H), 0.90 (t, J = 7.4 Hz, 3H).^{13}C NMR (101 MHz, $CDCl_3$) δ 170.54 ($C{=}O_{amide}$), 156.30 ($C{=}O_{Boc}$), 137.63 (C-1$_{Ar}$), 128.79 (C-3$_{Ar}$ & C-5$_{Ar}$), 124.20 (C-4$_{Ar}$), 119.95 (C-2$_{Ar}$ & C-6$_{Ar}$), 80.16, 60.07 (NH-**C**H$_{isoleucine}$), 37.00, 28.30, 24.89, 15.57, 11.08. IR (KBr, υ, cm^{-1}) 3333,

2970, 2932, 1668, 1605, 1528, 1173, 748. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{17}H_{27}N_2O_3$: 307.2021 $[M + H]^+$, found : 307.2009.

*2.2.2 tert-Butyl ((2S,3R)-1-(benzo[d]thiazol-2-ylamino)-3-methyl-1-oxopentan-2-yl)carbamate (**3b**)*

White solid; yield: 68%, mp: 180-182 °C, ^{1}H NMR (400 MHz, $CDCl_3$+DMSO-d_6) δ 12.11 (s, 1H, NH_{Ar}), 7.75 (d, J = 7.8 Hz, 1H, $H_{benzothiazolyl}$), 7.63 (d, J = 8.0 Hz, 1H, $H_{benzothiazolyl}$), 7.31 (ddd, J = 8.2, 7.3, 1.2 Hz, 1H, $H_{benzothiazolyl}$), 7.19 (td, J = 7.8, 1.1 Hz, 1H, $H_{benzothiazolyl}$), 6.47 (d, J = 9.0 Hz, 1H, $NH_{isoleucine}$), 4.17 (t, J = 8.2 Hz, 1H, $CH_{isoleucine}$), 1.81 – 1.72 (m, 1H), 1.34 (s, 9H, 3xMe_{Boc}), 1.21 – 0.99 (m, 2H), 0.84 (d, J = 6.8 Hz, 3H), 0.80 (t, J = 7.4 Hz, 3H).^{13}C NMR (101 MHz, $CDCl_3$ + DMSO-d_6) δ 172.04 ($C{=}O_{amide}$), 157.95 ($C{=}O_{Boc}$), 155.63 (C-2_{Ar}), 148.90, 131.98, 126.00, 123.60, 121.34, 120.84, 78.9, 59.39 (NH-**C**$H_{isoleucine}$), 37.19, 28.48, 24.83, 15.59, 11.15. IR (KBr, υ, cm^{-1}) 3240, 2970, 1705, 1666, 1536, 1173, 764. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{18}H_{26}N_3O_3S$: 364.1694 $[M + H]^+$, found : 364.1676.

*2.2.3 (S)-tert-Butyl (1-((1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)amino)-1-oxopropan-2-yl)carbamate (**3c**)*

Orange solid; yield: 76%, mp: 187-190 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.98 (s, 1H, NH_{Ar}), 7.43 (t, J = 7.8 Hz, 2H, $H_{Ar\text{-}pyrazolinyl}$), 7.35 (d, J = 7.6 Hz, 2H, $H_{Ar\text{-}pyrazolinyl}$), 7.29 (t, J = 7.4 Hz, 1H, $H_{Ar\text{-}pyrazolinyl}$), 5.51 (br s, 1H, $NH_{alanine}$), 3.91 (d, J = 5.2 Hz, 1H, $CH_{alanine}$), 3.07 (s, 3H, N-$Me_{pyrazolinyl}$), 2.18 (s, 3H, C-$Me_{pyrazolinyl}$), 1.49 – 1.38 (overlapped m, 12H, 3xMe_{Boc} & $Me_{alanine}$).^{13}C NMR (101 MHz, $CDCl_3$) δ 169.29 ($C{=}O_{amide}$), 161.92 ($C{=}O_{pyrazolinyl}$), 155.96 ($C{=}O_{Boc}$), 150.30, 134.32, 129.27, 127.19, 124.64, 107.74, 79.69, 43.98 (NH-**C**$H_{alanine}$), 35.76 (N-**C**$H_{3pyrazolinyl}$), 28.35, 12.03, 12.00. IR (KBr, υ, cm^{-1}) 3495, 3310, 2978, 1751, 1606, 1173, 763. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{19}H_{27}N_4O_4$: 375.2032 $[M + H]^+$, found : 375.2019.

*2.2.4 (S)-tert-Butyl (1-oxo-3-phenyl-1-((2,4,6-trimethoxyphenyl)amino)propan-2-yl)carbamate (**3d**)*

White solid; yield: 48%, mp: 151-153 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (s, 1H, NH_{Ar}), 7.29 – 7.21 (m, 5H, $H_{Ar\text{-}phenylalanine}$), 6.64 (s, 2H, H-$3_{Ar}$ & H-5_{Ar}), 5.24 (d, J = 6.4 Hz, 1H, $NH_{phenylalanine}$), 4.56 – 4.47 (m, 1H, $CH_{phenylalanine}$), 3.77 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 3.06 – 3.16 (m, 2H), 1.39 (s, 9H, 3xMe_{Boc}).^{13}C NMR (101 MHz, $CDCl_3$)

δ 169.70 ($C{=}O_{amide}$), 155.89 ($C{=}O_{Boc}$), 153.09 ($C\text{-}1_{Ar}$), 136.56, 134.70, 133.44, 129.21, 128.76, 126.99, 97.62, 80.64, 60.82 (NH-**C**$H_{phenylalanine}$), 56.03 (O**C**H_3), 55.99 (O**C**H_3), 55.92 (O**C**H_3), 38.44 ($CH_{2phenylalanine}$), 28.25 (CH_{3Boc}). IR (KBr, υ, cm^{-1}) 3495, 3333, 2970, 1686, 1528, 1173, 764. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{23}H_{31}N_2O_6$: 431.2182 $[M + H]^+$, found : 431.2166.

*2.2.5 (S)-tert-Butyl (3-methyl-1-oxo-1-(pyridin-4-ylamino)butan-2-yl)carbamate (**3e**)*

White solid; yield: 46%, mp: 142-145 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.24 (s, 1H, NH_{Ar}), 8.37 (d, J = 4.6 Hz, 2H, H-3_{pridyl} & H-5_{pridyl}), 7.38 (d, J = 4.4 Hz, 2H, H-2_{pridyl} & H-6_{pridyl}), 5.35 (d, J = 8.5 Hz, 1H, NH_{valine}), 4.12 – 4.05 (m, 1H, CH_{valine}), 2.19 – 2.10 (m, 1H), 1.43 (s, 9H, 3xMe_{Boc}), 1.00 (d, J = 5.2 Hz, 3H), 0.98 (d, J = 5.2 Hz, 3H).^{13}C NMR (101 MHz, $CDCl_3$) δ 171.49 ($C{=}O_{amide}$), 150.41 ($C{=}O_{Boc}$), 144.82, 113.60, 113.56, 80.68, 61.02 (NH-**C**H_{valine}), 30.50, 28.29, 19.34, 18.12. IR (KBr, υ, cm^{-1}) 3340, 2978, 2932, 1674, 1528, 1165, 818. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{15}H_{24}N_3O_3$: 294.1817 $[M + H]^+$, found : 294.1807.

*2.2.6 tert-Butyl ((2S,3R)-3-methyl-1-oxo-1-(thiazol-2-ylamino)pentan-2-yl)carbamate (**3f**)*

White solid; yield: 69%, mp: 163-165 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.49 (s, 1H, NH_{Ar}), 7.65 (br s, 1H, $H_{thiazolyl}$), 7.01 (d, J = 3.6 Hz, 1H, $H_{thiazolyl}$), 5.47 (d, J = 7.7 Hz, 1H, $NH_{isoleucine}$), 4.45 – 4.36 (m, 1H, $CH_{isoleucine}$), 1.88 – 1.81 (m, 1H), 1.41 (s, 9H, 3xMe_{Boc}), 1.26 – 1.07 (m, 2H), 0.90 (d, J = 6.4 Hz, 3H), 0.83 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.62 ($C{=}O_{amide}$), 159.10 ($C\text{-}2_{Ar}$), 155.67($C{=}O_{Boc}$), 137.07, 113.79, 79.91, 58.98(NH-**C**$H_{isoleucine}$), 38.18, 28.29, 24.53, 15.46, 11.18. IR (KBr, υ, cm^{-1}) 3495, 3340, 2978, 1674, 1520, 1173, 748. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{14}H_{24}N_3O_3S$: 314.1538 $[M + H]^+$, found : 314.1519.

*2.2.7 (S)-tert-Butyl (1-oxo-1-(p-tolylamino)propan-2-yl)carbamate (**3g**)*

White solid; yield: 75%, mp: 143-145 °C, ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.61 (s, 1H, NH_{Ar}), 7.49 (d, J = 8.2 Hz, 2H, H-3_{Ar} & H-5_{Ar}), 7.01 (d, J = 8.1 Hz, 2H, H-2_{Ar} & H-6_{Ar}), 5.51 (br s, 1H, $NH_{alanine}$), 4.06 (d, J = 6.7 Hz, 1H, $CH_{alanine}$), 2.20 (s, 3H, Me_{Ar}), 1.46 –1.43 (overlapped m, 12H, 3xMe_{Boc} & $Me_{alanine}$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.54 ($C{=}O_{amide}$), 156.30 ($C{=}O_{Boc}$), 137.63 ($C\text{-}1_{Ar}$), 133.10 ($C\text{-}4_{Ar}$), 129.97 ($C\text{-}3/5_{Ar}$), 119.95 ($C\text{-}2/C\text{-}6_{Ar}$), 80.16, 49.40 (**C**$H_{alanine}$), 28.30, 20.91 (**C**$H_{3Ar}$), 17.62 (**C**$H_{3alanine}$). IR (KBr, υ, cm^{-1}) 3347,

2966, 2932, 1682, 1528, 1165, 818. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{15}H_{23}N_2O_3$: 279.1708 $[M + H]^+$, found : 279.1688.

2.3 General Synthesis of aminoacyl-aromatic/heteroaromatic amides hydrochloride salts

A solution of dioxane-HCl (5 mL) was added drop-wise to a solution of **3a-g** (1 mmol) dissolved in dioxane at 0 ^oC. The reaction was stirred at room temperature for 2 h and monitored *via* TLC. After the completion, the reaction mixture was concentrated, dried under vacuum, washed with diethyl ether (10 mL x 2) and filtered off to give **4a-g** in pure form.

*2.3.1 (2S,3R)-2-Amino-3-methyl-N-phenylpentanamide hydrochloride (**4a**)*

White solid; yield: 75%, mp: 230-235 °C, ^1H NMR (400 MHz, $CDCl_3$) δ 10.17 (br s, 1H, NH_{Ar}), 8.15 (br s, 3H, NH_3^+), 7.72–7.47 (m, 2H, H-3_{Ar} & H-5_{Ar}), 7.19 – 7.00 (m, 3H, H-2_{Ar} , H-4_{Ar} & H-6_{Ar}), 4.53 – 4.48 (m, 1H, $CH_{isoleucine}$), 2.16 – 1.92 (m, 1H), 1.70 – 1.50 (m, 1H), 1.20 – 1.06 (m, 1H), 1.01 – 0.90 (br s, 3H, $Me_{isoleucine}$), 0.80 – 0.57 (br s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.93 ($C{=}O_{amide}$), 137.21 (C-1_{Ar}), 128.86 (C-$3/5_{Ar}$), 124.82 (C-4_{Ar}), 120.53 (C-$2/6_{Ar}$), 58.58 (NH-**C**$H_{isoleucine}$), 36.98, 24.75, 14.36, 11.36. IR (KBr, υ, cm^{-1}) 3410, 3333, 2970, 1666, 1520, 1173, 1026. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{12}H_{19}N_2O$: 207.1497 $[M - Cl]^+$, found : 207.1484.

*2.3.2 (2S,3R)-2-Amino-N-(benzo[d]thiazol-2-yl)-3-methylpentanamide hydrochloride (**4b**)*

White solid; yield: 88%, mp: 180-183 °C, ^1H NMR (400 MHz, DMSO-d_6) δ 8.55 (br s, 3H, NH_3^+), 7.79 (d, J = 1.4 Hz, 1H, $H_{benzothiazolyl}$), 7.65 (d, J = 3.9 Hz, 1H, $H_{benzothiazolyl}$), 7.38 – 7.27 (m, 1H, $H_{benzothiazolyl}$), 7.25 – 7.14 (m, 1H, $H_{benzothiazolyl}$), 4.06 – 3.93 (m, 1H, $CH_{isoleucine}$), 2.09 – 1.92 (m, 1H,), 1.62– 1.45 (m, 1H), 1.21 – 1.10 (m, 1H), 0.94 – 0.82 (overlapped m, 6H).^{13}C NMR (101 MHz, DMSO-d_6) δ 168.50 ($C{=}O_{amide}$), 157.39 (C-$2_{benzothiazolyl}$), 148.45, 131.80, 126.39, 124.15, 121.71, 121.00, 57.27 (NH-**C**$H_{isoleucine}$), 36.65, 24.51, 14.87, 11.46 . IR (KBr, υ, cm^{-1}) 3340, 3055, 2978, 1674, 1528, 1173. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{13}H_{18}N_3OS$: 264.1170 $[M - Cl]^+$, found : 264.1192.

*2.3.3 (S)-2-Amino-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)propanamide hydrochloride (**4c**)*

Orange solid; yield: 89%, mp: highly hygroscopic,^1H NMR (400 MHz, DMSO-d_6) δ 9.85 (br s, 1H, NH_{Ar}), 8.48 (br s, 3H, NH_3^+), 7.62 – 7.58 (m, 2H, $H_{Ar\text{-}pyrazolinyl}$), 7.46 – 7.41 (m, 3H,

$H_{Ar\text{-}pyrazolinyl}$), 4.15 – 4.07 (m, 1H, $CH_{alanine}$), 3.17 (s, 3H, N-$Me_{pyrazolinyl}$), 2.24 (s, 3H, C-$Me_{pyrazolinyl}$), 1.55 (d, *J* = 7.0 Hz, 3H, $Me_{alanine}$). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.78 ($C{=}O_{amide}$), 160.65 ($C{=}O_{pyrazolinyl}$), 151.76, 134.65, 129.67, 127.50, 124.77, 105.96, 48.69 (NH-**C**$H_{alanine}$), 36.03 (N-**C**$H_{3pyrazolinyl}$), 17.78 (C-**C**$H_{3pyrazolinyl}$), 11.36. IR (KBr, υ, cm^{-1}) 3433, 3325, 2986, 1690, 1597, 1165, 756. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{14}H_{19}N_4O_2$: 275.1508 $[M - Cl]^+$, found : 275.1496.

*2.3.4 (S)-2-Amino-3-phenyl-N-(2,4,6-trimethoxyphenyl)propanamide hydrochloride (**4d**)*

Off-White solid; yield: 85%, hygroscopic, ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.54 (br s, 1H, NH_{Ar}), 8.38 (br s, 3H, NH_3^+), 7.46 – 7.38 (m, 5H, $H_{Ar\text{-}phenylalanine}$), 6.99 (s, 2H, H-$3_{Ar}$ & H-3_{Ar}), 4.31– 4.22 (m, 1H, $CH_{phenylalanine}$), 3.82 (s, 6H, 2xOCH_3), 3.71 (s, 3H, OCH_3), 3.21 – 3.11 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.84 ($C{=}O_{amide}$), 153.14 (C-1_{Ar}), 148.14, 135.18, 134.41, 130.00, 128.96, 127.64, 97.93, 63.19 (NH-**C**$H_{phenylalanine}$), 60.52 (O**C**H_3), 56.24 (O**C**H_3), 54.58 (O**C**H_3), 37.33. IR (KBr, υ, cm^{-1}) 3340, 2978, 2932, 1674, 1528, 1173, 818. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{18}H_{23}N_2O_4$: 331.1657 $[M - Cl]^+$, found : 331.1668.

*2.3.5 (S)-2-Amino-3-methyl-N-(pyridin-4-yl)butanamide hydrochloride (**4e**)*

White solid; yield: 96%, mp: 240-242 °C, ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.91 (s, 1H, NH_{Ar}), 8.68 (d, *J* = 3.5 Hz, 2H, H-3_{pridyl} & H-5_{pridyl}), 8.51 (br s, 3H, NH_3^+), 8.28 (d, *J* = 4.0 Hz, 2H, H-2_{pridyl} & H-6_{pridyl}), 4.21 – 4.14 (m, 1H, CH_{valine}), 1.19 – 1.08 (m, 1H), 1.01 (d, *J* = 6.7 Hz, 3H), 0.96 (d, *J* = 6.7 Hz, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 170.17 ($C{=}O_{amide}$), 152.77, 142.48, 115.35, 58.83 (NH-**C**H_{valine}), 30.26, 18.87, 17.80. IR (KBr, υ, cm^{-1}) 3433, 3310, 3078, 2978, 1651, 1597, 1173, 987, 818. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{10}H_{16}N_3O$: 194.1293 $[M - Cl]^+$, found : 194.1312.

*2.3.6 (2S,3R)-2-Amino-3-methyl-N-(thiazol-2-yl)pentanamide hydrochloride (**4f**)*

White solid; yield: 89%, mp: 238-240 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.14 (br s, 1H, NH_{Ar}), 8.17 (br s, 3H, NH_3^+), 7.70 – 7.58 (m, 1H, $H_{thiazolyl}$), , 7.22 – 7.15 (m, 1H, $H_{thiazolyl}$), 4.55 – 4.48 (m, 1H, $CH_{isoleucine}$), 2.06 – 1.97 (m, 1H), 1.64 – 1.47 (m, 1H), 1.12 (m, 1H), 0.91 (brs, 3H), 0.69 (brs, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.62 ($C{=}O_{amide}$), 155.67 (C-$2_{thiazolyl}$), 137.10, 113.79, 58.98 (NH-**C**$H_{isoleucine}$), 38.18, 24.53, 15.46, 11.18. IR (KBr, υ, cm^{-}

^{1}) 3310, 3086, 2978, 1678, 1620, 1520, 1196, 694. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_9H_{16}N_3OS$: 214.1041 $[M - Cl]^+$, found : 214.1063.

*2.3.7 (S)-2-Amino-N-(p-tolyl)propanamide hydrochloride (**4g**)*

White solid; yield: 79%, mp: 108-110°C, ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6) δ 10.61 (s, 1H, NH_{Ar}), 8.31 (br s, 3H, NH_3^+), 7.49 (d, J = 8.2 Hz, 2H, H-3$_{Ar}$ & H-5$_{Ar}$), 7.01 (d, J = 8.1 Hz, 2H, H-2$_{Ar}$ & H-6$_{Ar}$), 4.10 – 4.02 (m, 1H, $CH_{alanine}$), 2.20 (s, 3H, Me_{Ar}), 1.45 (d, J = 6.8 Hz, 3H, $Me_{alanine}$). ^{13}C NMR (101 MHz, $CDCl_3$ + DMSO-d_6) δ 168.18 ($C{=}O_{amide}$), 136.12 (C-1$_{Ar}$), 133.18 (C-4$_{Ar}$), 129.33 (C-3/5$_{Ar}$), 119.75 (C-2/6$_{Ar}$), 49.42 (**C**H$_{alanine}$), 20.90 (**C**H$_{3Ar}$), 17.66 (**C**H$_{3alanine}$). IR (KBr, υ, cm^{-1}) 3402, 3302, 2978, 2901, 1675, 1628, 1528, 1196, 1026, 694. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{10}H_{15}N_2O$: 179.1184 [M - Cl]+, found : 179.1176.

2.4 General Synthesis of bile acid-aminoacyl aromatic/heteroaromatic amides

To a stirred solution of **5a-b** (0.5 mmol) in DMF, triethyl amine (1.25 mmol) was added at 0 ^{o}C and subsequently EDC.HCl (0.75 mmol) and HOBt (0.5 mmol) was added and the reaction mixture was stirred for 15 min. at 0 ^{o}C. Later the aminoacyl aromatic/heteroaromatic amides hydrochloride salts (**4a-g,** 0.6 mmol) was added and the reaction was stirred at room temperature for 6-8 h. The completion of the reaction was monitored by TLC. After the completion of the reaction, crushed-ice was added and the resulted precipitate was filtered, washed with cold water and recrystallized from ethanol to yield pure **6a-m**.

*2.4.1 (2S,3R)-3-Methyl-N-phenyl-2-((R)-4-((3R,5S,7R,8R,9S,10S,12S,13R,14S,17R)-3,7,12-trihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamido)pentanamide (**6a**)*

White solid; yield: 85%; mp: 133-135 °C, ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.12 (s, 1H, **NH**$_{Ar}$), 8.09 (d, J = 8.4 Hz, 1H, **NH**$_{isoleucine}$), 7.62 (d, J = 7.9 Hz, 2H, H-3$_{Ar}$ & H-5$_{Ar}$), 7.29 (t, J = 7.8 Hz, 2H, H-2$_{Ar}$ & H-6$_{Ar}$), 7.04 (t, J = 7.3 Hz, 1H, H-4$_{Ar}$), 4.36 – 4.26 (m, 2H, $CH_{isoleucine}$ & OH_{CA}), 4.06 – 4.00 (m, 1H, H-12$_{CA}$), 4.03 – 4.15 (m, 1H, H-7$_{CA}$), 3.78 (br s, 1H, OH_{CA}), 3.60 (br s, 1H, OH_{CA}), 3.21 – 3.15 (m, 1H, H-3$_{CA}$), 2.23 – 1.92 (m, 6H), 1.82–1.72 (m, 4H), 1.69 – 1.58 (m, 4H), 1.43 – 1.14 (m, 13H), 0.93 (d, J = 6.2 Hz, 3H, Me-21$_{CA}$), 0.88 – 0.82 (m, 6H, 2x$Me_{isoleucine}$), 0.80 (s, 3H, Me-19$_{CA}$), 0.55 (s, 3H, Me-18$_{CA}$). ^{13}C NMR (101 MHz, DMSO) δ 173.35 (C-24$_{CA}$), 171.16 ($C{=}O_{isoleucine}$), 139.36 (C-1$_{Ar}$), 129.12 (C-3/5$_{Ar}$), 123.70 (C-4$_{Ar}$), 119.71 (C-2/6$_{Ar}$), 71.48 (C-12$_{CA}$), 70.90 (C-3$_{CA}$), 66.70 (C-7$_{CA}$), 58.09

(NH-**C**H$_{isoleucine}$), 46.68, 46.19, 41.98, 41.80, 36.88, 35.77, 35.66, 35.55, 34.85, 32.84, 32.36, 30.85, 28.99, 27.78, 26.66, 25.04, 23.28, 23.08 (C-19$_{CA}$), 17.63 (C-21$_{CA}$), 15.80 & 12.77 (2xMe$_{isoleucine}$), 11.21 (C-18$_{CA}$). IR (KBr, υ, cm^{-1}) 3394, 3310, 3094, 2932, 2870, 1651, 1551. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{36}H_{57}N_2O_5$ [M + H]$^+$: 597.4267, found : 597.4288. $[\alpha]^{20}_D$ = +15.0 (*c* 1.0, MeOH)

*2.4.2 (2S,3R)-2-((R)-4-((3R,5R,8R,9S,10S,12S,13R,14S,17R)-3,12-Dihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamido)-3-methyl-N-phenylpentanamide (**6b**)*

White solid; yield: 82%; mp : 130-131 °C, ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.09 (s, 1H, **NH**$_{Ar}$), 8.04 (d, *J* = 8.5 Hz, 1H, **NH**$_{isoleucine}$), 7.61 (d, *J* = 7.8 Hz, 2H, H-3$_{Ar}$ & H-5$_{Ar}$), 7.30 (t, *J* = 7.8 Hz, 2H, H-2$_{Ar}$ & H-6$_{Ar}$), 7.04 (t, *J* = 7.3 Hz, 1H, H-4$_{Ar}$), 4.49 (d, *J* = 3.7 Hz, 1H, OH$_{DCA}$), 4.29 (t, *J* = 8.2 Hz, 1H, CH$_{isoleucine}$), 4.20 (br s, 1H, H-12$_{DCA}$), 3.79 (br s, 1H, OH$_{DCA}$), 3.29 – 3.22 (m, 1H, H-3$_{DCA}$), 2.26 – 2.00 (m, 4H), 1.81 – 1.74 (m, 5H), 1.66 – 1.58 (m, 4H), 1.53 – 1.44 (m, 4H), 1.37–1.29 (m, 8H), 1.22 – 1.10 (m, 4H), 0.93 (d, *J* = 5.8 Hz, 3H, Me-21$_{DCA}$), 0.86 – 0.84 (m, 6H, 2xMe$_{isoleucine}$), 0.81 (d, *J* = 7.6 Hz 3H, Me-19$_{DCA}$), 0.56 (s, 3H, Me-18$_{DCA}$).^{13}C NMR (101 MHz, DMSO-d_6) δ 173.31 (C-24$_{DCA}$), 171.10 (C=O$_{isoleucine}$), 139.33 (C-1$_{Ar}$), 129.10 (C-3/5$_{Ar}$), 123.69 (C-4$_{Ar}$), 119.68 (C-2/6$_{Ar}$), 71.48 (C-12$_{DCA}$), 70.42 (C-3$_{DCA}$), 58.01 (NH-**C**H$_{isoleucine}$), 47.89, 46.67, 46.43, 46.13, 42.07, 36.93, 36.74, 36.11, 35.57, 34.26, 33.36, 32.76, 32.30, 30.68, 29.74, 29.04, 27.67, 27.45, 26.57, 25.02, 23.98, 23.53 (C-19$_{DCA}$), 17.59 (C-21$_{DCA}$), 15.78 & 12.84 (Me$_{isoleucine}$), 11.21(C-18$_{DCA}$). IR (KBr, υ, cm^{-1}) 3394, 3302, 3140, 3086, 2932, 2870, 1652, 1551. HRMS (ESI): *m/z* calcd for Chemical Formula: $C_{36}H_{57}N_2O_4$: 581.4318 [M + H]$^+$, found : 581.4306. $[\alpha]^{20}_D$ = +25.0 (*c* 1.0, MeOH)

*2.4.3 (2S,3R)-N-(Benzo[d]thiazol-2-yl)-3-methyl-2-((R)-4-((3R,5S,7R,8R,9S,10S,12S,13R,14S,17R)-3,7,12-trihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamido)pentanamide (**6c**)*

White solid; yield: 92%; mp: 139-141 ^{o}C, ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.29 (br s, 1H, **NH**$_{benzothiazolyl}$), 8.09 (d, *J* = 7.9 Hz, 1H, **NH**$_{isoleucine}$), 7.93 (d, *J* = 7.5 Hz, 1H, H$_{benzothiazolyl}$), 7.71 (d, *J* = 8.0 Hz, 1H, H$_{benzothiazolyl}$), 7.40 (t, *J* = 7.4 Hz, 1H, H$_{benzothiazolyl}$), 7.27 (t, *J* = 7.6 Hz, 1H, H$_{benzothiazolyl}$), 4.43 (t, *J* = 8.0 Hz, 1H, CH$_{isoleucine}$), 4.30 – 4.23 (m, 1H, OH$_{CA}$), 4.11 – 4.00 (m, 1H, H-12$_{CA}$), 3.98– 3.90 (m, 1H, H-7$_{CA}$), 3.74 (br s, 1H, OH$_{CA}$), 3.55 (m, 1H,

OH_{CA}), 3.21 – 3.08 (m, 1H, H-3_{CA}), 2.21 – 1.94 (m, 5H), 1.86 – 1.57 (m, 7H), 1.49 – 1.35 (m, 5H), 1.31 – 1.08 (m, 10H), 0.91 (d, J = 6.4 Hz, 3H, Me-21_{CA}), 0.84 – 0.78 (m, 6H, $Me_{isoleucine}$), 0.75 (s, 3H, Me-19_{CA}), 0.49 (s, 3H, Me-18_{CA}).^{13}C NMR (101 MHz, DMSO-d_6) δ 173.58 (C-24_{CA}), 172.42 (C=$O_{isoleucine}$), 149.01 (C-$1_{benzothiazolyl}$), 131.94, 126.47, 123.89, 122.04, 120.90, 71.44 (C-12_{CA}), 70.87 (C-3_{CA}), 66.67 (C-7_{CA}), 57.53 (NH-**C**$H_{isoleucine}$), 46.63, 46.16, 41.76, 41.97, 36.60, 35.75, 35.33, 35.51, 34.82, 32.67, 32.22, 31.38, 30.84, 28.96, 27.71, 26.63, 24.99, 23.22, 23.03, 22.49 (C-19_{CA}), 17.67 (C-21_{CA}), 15.72 & 12.70 (2x$Me_{isoleucine}$), 11.16 (C-18_{CA}). IR (KBr, υ, cm^{-1}) 3394, 3063, 2932, 2870, 1651, 1551. HRMS (ESI): *m/z* $[M+H]^+$ calcd for Chemical Formula: $C_{37}H_{56}N_3O_5S$: 654.3940, found : 654.3922. $[\alpha]^{20}_D$ = +6.0 (*c* 1.0, MeOH).

*2.4.4 (2S,3R)-N-(Benzo[d]thiazol-2-yl)-2-((R)-4-((3R,5R,8R,9S,10S,12S,13R,14S,17R)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamido)-3-methylpentanamide (**6d**)*

White solid; yield: 91%; mp: 119-121 ^oC, ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.45 (br s, 1H, **NH**$_{benzothiazolyl}$), 8.14 (br s, 1H, **NH**$_{isoleucine}$), 7.97 (d, J = 6.7 Hz, 1H, $H_{benzothiazolyl}$), 7.74 (d, J = 6.9 Hz, 1H, $H_{benzothiazolyl}$), 7.54 – 7.37 (m, 1H, $H_{benzothiazolyl}$), 7.33 – 7.27 (m, 1H, $H_{benzothiazolyl}$), 4.45 – 4.42 (m, 2H, $CH_{isoleucine}$ & H-12_{DCA}), 4.21 (br s, 1H, OH_{DCA}), 3.78 (br s, 1H, OH_{DCA}), 3.29 – 3.21 (m, 1H, H-7_{DCA}), 2.34 – 2.01 (m, 4H), 1.81 – 1.59 (m, 9H), 1.54 – 1.43 (m, 4H), 1.34 – 1.15 (m, 12H), 0.93 (d, J = 4.3 Hz, 3H, Me-21_{DCA}), 0.87 (d, J = 18.6 Hz, 6H, $Me_{isoleucine}$), 0.82 (s, 3H, Me-19_{DCA}), 0.53 (s, 3H, Me-18_{DCA}).^{13}C NMR (101 MHz, DMSO-d_6) δ 173.66 (C-24_{DCA}), 172.45 (C=$O_{isoleucine}$),158.17, 149.03, 131.96, 126.52, 123.95, 122.08, 120.96, 71.47 (C-12_{DCA}), 70.42 (C-3_{DCA}), 57.54, 47.86, 46.66, 46.42, 42.07, 36.73, 36.09, 35.60, 34.25, 33.35, 32.61, 32.18, 30.68, 29.02, 27.67, 27.44, 26.56, 25.01, 23.96, 23.52 (C-19_{DCA}), 17.62 (C-21_{DCA}), 15.72 & 12.80 (2x$Me_{isoleucine}$), 11.19 (C-18_{DCA}). IR (KBr, υ, cm^{-1}) 3394, 3310, 3070, 2932, 2870, 1651, 1551. HRMS (ESI): *m/z* $[M+H]^+$ calcd for Chemical Formula: $C_{37}H_{56}N_3O_4S$: 638.3991, found : 638.3976. $[\alpha]^{20}_D$ = +58.0 (*c* 1.0, MeOH)

*2.4.5 (R)-N-((S)-1-((1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)amino)-1-oxopropan-2-yl)-4-((3R,5S,7R,8R,9S,10S,12S,13R,14S,17R)-3,7,12-trihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamide (**6e**)*

Orange solid; yield: 72 %; mp: 152-154 ^oC.^{1}H NMR (400 MHz, DMSO-d_6) δ 9.13 (s, 1H, **NH**$_{pyrazolinyl}$), 8.10 (d, J = 6.9 Hz, 1H, **NH**$_{alanine}$), 7.59 (t, J = 7.9 Hz, 2H, $H_{Ar\text{-}pyrazolinyl}$), 7.41 (dd, J = 16.6, 8.0 Hz, 3H, $H_{Ar\text{-}pyrazolinyl}$), 4.40 (d, J = 4.2 Hz, 1H, OH_{CA}), 4.23 – 4.17 (m , 2H,

$CH_{alanine}$ & H-12_{CA}), 4.10 (d, *J* = 3.0 Hz, 1H, H-7_{CA}), 3.87 (br s, 1H, OH_{CA}), 3.70 (br s, 1H, OH_{CA}), 3.38 – 3.35 (m, 1H, H-3_{CA}), 3.12 (s, 3H, N-$Me_{pyrazolinyl}$), 2.17 (s, 3H, C-$Me_{pyrazolinyl}$), 2.13 – 2.00 (m, 3H), 1.91– 1.83 (m , 4H), 1.77 – 1.68 (m , 4H), 1.54 – 1.45 (m, 6H), 1.36 – 1.29 (m, 7H), 1.01 (d, *J* = 5.6 Hz, 3H, $Me_{alanine}$), 0.98 – 0.91 (m, 3H, Me-21_{CA}), 0.90 (s, 3H, Me-19_{CA}), 0.67 (s, 3H, Me-18_{CA}). ^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 172.98 (C-24_{CA}), 172.63 ($C{=}O_{alanine}$), 171.87 ($C{=}O_{pyrazolinyl}$), 162.11, 152.72, 135.46, 129.48, 126.58, 123.80, 107.79, 71.42 (C-12_{CA}), 70.86 (C-3_{CA}), 66.64 (C-7_{CA}), 46.58 (NH-**C**$H_{alanine}$), 46.19, 41.80, 36.74, 36.49, 35.74, 35.67, 35.65, 35.48, 35.46, 35.30, 34.81, 32.65, 31.99, 31.83, 31.14, 30.83, 28.96, 27.75, 26.64, 23.24, 23.05(C-19_{CA}), 18.79 & 17.59 (C-$Me_{pyrazolinyl}$ & $Me_{alanine}$), 17.36 (C-21_{CA}), 11.57 (C-18 $_{CA}$). IR (KBr, υ, cm^{-1}) 3394, 3086, 3932, 2870, 1751, 1651, 1520. HRMS (ESI): *m/z* $[M+H]^+$ calcd for Chemical Formula: $C_{38}H_{57}N_4O_6$: 665.4278, found : 665.4242. $[\alpha]^{20}_D$ = +18.0 (*c* 1.0, MeOH)

*2.4.6 (R)-4-((3R,5R,8R,9S,10S,12S,13R,14S,17R)-3,12-Dihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)-N-((S)-1-((1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)amino)-1-oxopropan-2-yl)pentanamide (**6f**)*

Orange solid; yield: 67%; mp: 158-160 °C, ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 9.14 (s, 1H, **NH**$_{pyrazolinyl}$), 8.10 (d, *J* = 7.3 Hz, 1H, **NH**$_{alanine}$), 7.58 (t, *J* = 7.7 Hz, 2H, $H_{Ar\text{-}pyrazolinyl}$), 7.38 – 7.43 (m, *J* = 16.7, 7.5 Hz, 3H, $H_{Ar\text{-}pyrazolinyl}$), 4.47 – 4.55 (m, 2H, $CH_{alanine}$ & H-12_{DCA}), 4.28 (br s, 1H, OH_{DCA}), 3.87 (br s, 1H, OH_{DCA}), 3.39 – 3.33 (m, 1H, H-3_{DCA}), 3.12 (s, 3H, N-$Me_{pyrazolinyl}$), 2.17 (s, 3H, C-$Me_{pyrazolinyl}$), 1.92– 1.83 (m, 5H), 1.75 – 1.66 (m, 4H), 1.61 – 1.50 (m, 4H), 1.48 – 1.30 (m, 8H), 1.27 – 1.10 (m, 5H), 1.01 (d, *J* = 6.4 Hz, 3H, $Me_{alanine}$), 0.94 (d, *J* = 9.7 Hz, 6H, Me-19_{DCA} & Me-21_{DCA}), 0.66 (s, 3H, Me-18_{DCA}).^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 173.54 (C-24_{DCA}), 172.99 ($C{=}O_{alanine}$), 172.62 ($C{=}O_{pyrazolinyl}$), 162.12, 152.68, 135.49, 129.46, 126.56, 123.80, 107.82, 71.44 (C-12_{DCA}), 70.39 (C-3_{DCA}), 48.57 (NH-**C**$H_{alanine}$), 47.89, 46.65, 46.41, 42.06, 36.72, 36.48, 36.10, 35.59, 35.55, 34.24, 33.36, 32.63, 31.95, 31.10, 30.66, 29.03, 27.64, 27.43, 26.55, 23.94, 23.51 (C-19 $_{DCA}$), 18.77 & 17.55 (C-$Me_{pyrazolinyl}$ & $Me_{alanine}$), 17.29 (C-21 $_{DCA}$), 11.54 (C-18 $_{DCA}$). IR (KBr, υ, cm^{-1}) 3387, 3063, 2932, 2870, 1750. 1651, 1551. HRMS (ESI): *m/z* $[M+H]^+$ calcd for Chemical Formula: $C_{38}H_{57}N_4O_5$: 649.4328, found : 649.4364. $[\alpha]^{20}_D$ = +58.0 (*c* 1.0, MeOH)

*2.4.7 (R)-N-((S)-1-Oxo-3-phenyl-1-((2,4,6-trimethoxyphenyl)amino)propan-2-yl)-4-((3R,5S,7R,8R,9S,10S,12S,13R,14S,17R)-3,7,12-trihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamide (**6g**)*

White solid; yield: 68%; mp: 151-153 ^{o}C, ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.09 (d, J = 9.6 Hz, 1H), 8.24 (d, J = 8.3 Hz, 1H), 7.38 – 7.33 (m, 5H, $H_{Ar\text{-}phenylalanine}$), 7.06 (s, 2H, H-$2_{Ar}$ & H-6_{Ar}), 4.74 – 4.68 (m, 1H, $CH_{phenylanine}$), 4.40 (d, J = 4.3 Hz, 2H, OH & H-12_{CA}), 4.17 (d, J = 3.5 Hz, 1H, H-7_{CA}), 4.09 (d, J = 3.3 Hz, 2H, 2xOH_{CA}), 3.82 (s, 6H, 2xOCH_3), 3.70 (s, 3H, OCH_3), 3.42 – 3.36 (m, 1H, H-3_{CA}), 2.20 – 2.00 (m, 4H), 1.78 (d, J = 9.3 Hz, 3H), 1.63 (s, 2H), 1.27 (m, J = 32.3, 24.7, 11.7 Hz, 11H), 0.93 (d, J = 6.2 Hz, 3H), 0.88 – 0.83 (m, 6H), 0.80 (s, 3H, Me-19_{CA}), 0.55 (s, 3H, Me-18_{CA}). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.24 (C-24_{CA}), 170.62 ($C{=}O_{alanine}$), 153.08, 138.26, 135.46, 133.85, 129.57, 128.45, 126.71, 97.43, 71.46 (C-12_{CA}), 70.86 (C-3_{CA}), 66.64 (C-7_{CA}), 60.53 (OCH_3), 56.10 (OCH_3), 55.20 (OCH_3), 46.67 (NH-**C**$H_{phenylalanine}$), 46.58, 46.50, 46.25, 46.18, 46.12, 41.94, 41.77, 38.00, 35.81, 35.73, 35.51, 34.81, 32.69, 32.11, 30.83, 28.96, 27.70, 26.62, 23.22, 23.04 (C-19_{CA}), 17.35 (C-21_{CA}), 12.74 (C-18_{CA}). IR (KBr, υ, cm^{-1}) 3394, 3086, 2932, 2870, 1651, 1605. HRMS (ESI): m/z $[M+H]^+$ calcd for Chemical Formula: $C_{42}H_{61}N_2O_8$: 721.4427, found : 721.4396. $[\alpha]^{20}_D$ = +8.0 (c 1.0, MeOH)

*2.4.8 (R)-4-((3R,5R,8R,9S,10S,12S,13R,14S,17R)-3,12-Dihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)-N-((S)-1-oxo-3-phenyl-1-((2,4,6-trimethoxyphenyl)amino)propan-2-yl)pentanamide (**6h**)*

White solid; yield: 62%; mp: 151-153 ^{o}C, ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.09 (s, 1H, **NH**$_{Ar}$), 8.26 (d, J = 7.8 Hz, 1H, **NH**$_{phenylalnine}$), 7.40 – 7.33 (m, 5H, $H_{Ar\text{-}phenylalanine}$), 7.07 (s, 2H, H-$3_{Ar}$ & H-5_{Ar}), 4.71 – 4.67 (m, 1H, $CH_{phenylalnine}$), 4.55 (d, J = 4.1 Hz, 2H, H-12_{DCA} & OH_{DCA}), 4.29 – 4.21 (m , 1H, OH_{DCA}), 3.82 (s, 6H, 2xOCH_3), 3.70 (s, 3H, OCH_3), 3.39 – 3.34 (m, 1H, H-3_{DCA}), 1.82 – 1.73 (m, 5H), 1.65 – 1.53 (m, 7H), 1.42 – 1.35 (m, 8H), 1.14 – 1.03 (m, 6H), 0.93 (d, J = 4.7 Hz, 3H, Me-21_{DCA}), 0.92 (s, 3H, Me-19_{DCA}), 0.62 (s, 3H, Me-18_{DCA}). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.20 (C-24_{DCA}), 170.62 ($C{=}O_{phenylalanine}$), 153.08, 138.26, 135.47, 133.85, 129.56, 128.45, 126.70, 97.44, 71.41(C-12_{DCA}), 70.37 (C-3_{DCA}), 60.47 (OCH_3), 56.10 (OCH_3), 47.86 (NH-**C**$H_{phenylalanine}$), 46.74, 46.55, 46.49, 46.43, 46.37, 42.03, 36.72, 36.08, 35.57, 35.40, 34.24, 33.33, 32.66, 32.06, 30.66, 29.02, 27.60,27.41 26.53, 23.93, 23.51 (C-19_{DCA}), 17.40 (C-21_{DCA}), 12.81 (C-18_{DCA}). IR (KBr, υ, cm^{-1}) 3394, 3317, 2932, 2862, 1651, 1543. HRMS (ESI): m/z $[M+H]^+$ calcd for Chemical Formula: $C_{42}H_{61}N_2O_7$: 705.4478 found : 705.4448. $[\alpha]^{20}_D$ = +29.0 (c 1.0, MeOH)

*2.4.9 (R)-N-((S)-3-Methyl-1-oxo-1-(pyridin-4-ylamino)butan-2-yl)-4-((3R,5S,7R,8R,9S,10S,12S,13R,14S,17R)-3,7,12-trihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamide (**6i**)*

White solid; yield: 60%; mp: 147-148 ^{o}C, ^{1}H NMR (400 MHz, DMSO-d_6) δ δ 10.50 (s, 1H, **NH**$_{pyridyl}$), 8.42 (d, J = 4.7 Hz, 2H, H-3$_{pridyl}$ & H-5$_{pridyl}$), 8.12 (d, J = 7.6 Hz, 1H, **NH**$_{valine}$), 7.58 (d, J = 5.2 Hz, 2H, H-2$_{pridyl}$ & H-6$_{pridyl}$), 4.35 – 4.33 (m, 1H, CH$_{valine}$), 4.29 – 4.23 (m, 1H, OH$_{CA}$), 4.13 – 4.10 (m, 1H, H-12$_{CA}$), 4.05 – 4.10 (m, 1H, H-7$_{CA}$), 3.78 (br s, 1H, OH$_{CA}$), 3.60 (br s, 1H, OH$_{CA}$), 3.24 – 3.13 (s, 1H, H-3$_{CA}$), 2.24 – 1.99 (m, 7H), 1.80 – 1.62 (m, 7H), 1.40 – 1.21 (m, 11H), 0.94 (d, J = 5.8 Hz, 3H, Me-21$_{CA}$), 0.92 – 0.86 (m, 6H, 2xMe$_{valine}$), 0.80 (s, 3H, Me-19$_{CA}$), 0.55 (s, 3H, Me-18$_{CA}$). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.66 (C-24$_{CA}$), 172.37 (C=O$_{valine}$), 150.83, 145.84, 113.70, 71.48 (C-12$_{CA}$), 70.89 (C-3$_{CA}$), 66.69 (C-7$_{CA}$), 59.40 (NH-**C**H$_{valine}$), 46.61, 46.19, 41.97, 41.83, 36.37, 35.35, 35.64, 35.76, 34.84, 32.32, 32.67, 30.70, 30.86, 28.99, 27.75, 26.66, 23.26, 23.09 (C-19$_{CA}$) 19.62 & 18.94 (2xMe$_{valine}$), 17.66 (C-21$_{CA}$), 12.77 (C-18$_{CA}$). IR (KBr, υ, cm^{-1}) 3387, 3094, 2932, 2870, 1651, 1597. HRMS (ESI): *m/z* [M+H]$^{+}$ calcd for Chemical Formula: $C_{34}H_{54}N_3O_5$: 584.4063 found : 584.4077. $[\alpha]^{20}_{D}$ = +68.0 (*c* 1.0, MeOH)

*2.4.10 (R)-4-((3R,5R,8R,9S,10S,12S,13R,14S,17R)-3,12-Dihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)-N-((S)-3-methyl-1-oxo-1-(pyridin-4-ylamino)butan-2-yl)pentanamide (**6j**)*

White solid; yield: 58%; mp: 143-145 ^{o}C, ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.53 (s, 1H, **NH**$_{pyridyl}$), 8.42 (d, J = 2.9 Hz, 2H, H-3$_{pridyl}$ & H-5$_{pridyl}$), 8.15 (d, J = 6.2 Hz, 1H, **NH**$_{valine}$), 7.58 (d, J = 4.2 Hz, 2H, H-2$_{pridyl}$ & H-6$_{pridyl}$), 4.49 (br s, 1H, CH$_{valine}$), 4.17–4.29 (m, 2H, H-12$_{DCA}$ & OH$_{DCA}$), 3.79 (br s, 1H, OH$_{DCA}$), 3.29–3.15 (m, 1H, H-3$_{DCA}$), 2.27 – 2.15 (m, 2H), 2.09 – 1.99 (m, 2H), 1.83 – 1.69 (m, 4H), 1.69 – 1.56 (m, 3H), 1.53 – 1.45 (m,3H), 1.35 – 1.27 (m, 9H), 1.19 – 1.14 (m, 4H), 0.93 (d, J = 5.3 Hz, 3H, Me-21$_{DCA}$), 0.91 – 0.87 (m, 6H, 2xMe$_{valine}$), 0.84 (s, 3H, Me-19$_{DCA}$), 0.56 (s, 3H, Me-18$_{DCA}$).^{13}C NMR (101 MHz, DMSO-d_6) δ 173.62 (C-24$_{DCA}$), 172.36 (C=O$_{valine}$), 150.80, 145.84, 113.71, 71.47 (C-12$_{DCA}$), 70.41 (C-3$_{DCA}$), 59.44 (NH-**C**H$_{valine}$), 47.89, 46.43, 46.66, 42.06, 36.74, 36.10, 35.55, 35.60, 34.27, 33.36, 32.62, 32.28, 30.69, 29.04, 27.65, 27.44, 26.57, 23.97, 23.57 (C-19$_{DCA}$), 19.63, & 18.95 (2xMe$_{valine}$), 17.61 (C-21$_{DCA}$), 12.86 (C-18$_{DCA}$). IR (KBr, υ, cm^{-1}) 3387, 3325, 3094, 2932, 2870, 1651, 1512. HRMS (ESI): *m/z* [M+H]$^{+}$ calcd for Chemical Formula: $C_{34}H_{54}N_3O_4$: 568.4114 found :568.4112. $[\alpha]^{20}_{D}$ = +98.0 (*c* 1.0, MeOH)

*2.4.11 (2S,3R)-3-Methyl-N-(thiazol-2-yl)-2-((R)-4-((3R,5S,7R,8R,9S,10S,12S,13R,14S,17R)-3,7,12-trihydroxy-10,13,14-trimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamido)pentanamide (**6k**)*

White solid; yield: 93%; mp: 158-160 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 (d, J = 7.1 Hz, 1H, **NH**$_{thiazolyl}$), 7.47 (d, J = 3.4 Hz, 1H, H$_{thiazolyl}$), 6.99 (d, J = 3.5 Hz, 1H, H$_{thiazolyl}$), 4.80 – 4.72 (m, 1H, CH$_{isoleucine}$), 4.42 (br s, 1H, **NH**$_{isoleucine}$), 3.99– 3.92 (m, 1H, H-12$_{CA}$), 3.83–3.78 (m, 1H, H-7$_{CA}$), 3.44– 3.35 (m, 1H, H-3$_{CA}$), 2.28 – 1.95 (m, 5H), 1.92 – 1.69 (m, 5H), 1.65 – 1.51 (m, 6H), 1.47 – 1.33 (m, 6H), 1.30 – 0.99 (m, 5H), 0.97 (s, 3H, Me-21$_{CA}$), 0.89 (d, J = 5.5 Hz, 3H, Me$_{isoleucine}$), 0.86 (d, J = 5.9 Hz, 6H, Me$_{isoleucine}$ & Me-19$_{CA}$), 0.58 (s, 3H, Me-18$_{CA}$). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.30 (C-24$_{CA}$), 171.85 (C=O$_{isoleucine}$), 137.90, 113.14, 71.46 (C-12$_{CA}$), 70.89 (C-3$_{CA}$), 66.69 (C-7$_{CA}$), 57.66 (NH-**C**H$_{isoleucine}$), 46.64, 46.18, 41.97, 41.80, 36.95, 35.76, 35.62, 35.34, 34.84, 32.80, 32.28, 30.85, 29.00, 27.78, 26.65, 25.01, 23.29, 23.09 (C-19$_{CA}$), 17.61 (C-21$_{CA}$), 15.82 & 12.75 (2xMe$_{isoleucine}$), 11.34 (C-18$_{CA}$). IR (KBr, υ, cm^{-1}) 3394, 3078, 2932, 2870, 1651, 1551. HRMS (ESI): *m/z* $[M+H]^+$ calcd for Chemical Formula: $C_{33}H_{54}N_3O_5S$: 604.3784 found: 604.3765. $[\alpha]^{20}_D$ = +18.0 (*c* 1.0, MeOH)

*2.4.12 (2S,3R)-2-((R)-4-((3R,5R,8R,9S,10S,12S,13R,14S,17R)-3,12-Dihydroxy-10,13,14-trimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamido)-3-methyl-N-(thiazol-2-yl)pentanamide (**6l**)*

White solid; yield: 89%; mp: 148-150 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (br s, 1H, **NH**$_{thiazolyl}$), 6.98 (br s, 2H, H$_{thiazolyl}$), 4.82 – 4.69 (m , 1H, CH$_{isoleucine}$), 4.01– 3.90 (m, 1H, **NH**$_{isoleucine}$), 3.65 – 3.54 (m, 1H, H-12$_{DCA}$), 3.41 – 3.23 (m, 1H, H-3$_{DCA}$), 2.33 – 2.13 (m, 3H), 1.99 – 1.64 (m, 10H), 1.56 – 1.34 (m, 11H), 1.28 – 1.03 (m, 7H), 0.91 (overlapped s, 6H, Me$_{isoleucine}$ & Me-21$_{DCA}$), 0.88 (overlapped s, 6H, Me$_{isoleucine}$ & Me-19$_{DCA}$), 0.62 (s, 3H, Me-18$_{DCA}$). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.49 (C-24$_{DCA}$), 171.35 (C=O$_{isoleucine}$), 138.10, 113.84, 71.47 (C-12$_{DCA}$), 70.40 (C-3$_{DCA}$), 57.33(NH-**C**H$_{isoleucine}$), 47.90, 46.67, 46.43, 42.05, 36.74, 36.65, 36.10, 35.60, 35.50, 34.27, 33.36, 32.61, 32.21, 30.68, 29.05, 27.67, 27.44, 26.57, 24.99, 23.98, 23.56 (C-19$_{DCA}$), 17.59 (C-21$_{DCA}$), 15.71 & 12.84 (2xMe$_{isoleucine}$), 11.18 (C-18$_{DCA}$). IR (KBr, υ, cm^{-1}) 3402, 3078, 2932, 2870, 1651, 1551. HRMS (ESI): *m/z* $[M+H]^+$ calcd for Chemical Formula: $C_{33}H_{54}N_3O_4S$: 588.3835 found: 588.3809. $[\alpha]^{20}_D$ = +28.0 (*c* 1.0, MeOH)

*2.4.13 (R)-N-((S)-1-Oxo-1-(p-tolylamino)propan-2-yl)-4-((3R,5S,7R,8R,9S,10S,12S,13R,14S,17R)-3,7,12-trihydroxy-10,13,14-trimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamide (**6m**)*

White solid; yield: 93%; mp: 152-154 °C, ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.89 (s, 1H, **NH$_{Ar}$**), 8.10 (d, J = 7.3 Hz, 1H, **NH**$_{alanine}$), 7.48 (d, J = 8.2 Hz, 2H, H-3$_{Ar}$ & H-5$_{Ar}$), 7.10 (d, J = 8.2 Hz, 2H, H-2$_{Ar}$ & H-6$_{Ar}$), 4.38 – 4.33 (m, 2H, CH$_{alanine}$ & OH$_{CA}$), 4.13 – 4.10 (m, 1H, H-12$_{CA}$), 4.04 – 4.01 (m, 1H, H-7$_{CA}$), 3.78 (br s, 1H, OH$_{CA}$), 3.61 (br s, 1H, OH$_{CA}$), 3.21 – 3.17 (m, 1H, H-3$_{CA}$), 2.25 (s, 3H, Me$_{Ar}$), 2.17 – 2.00 (m, 5H), 1.82 – 1.60 (m, 9H), 1.46 – 1.29 (m, 10H), 1.26 (d, J = 6.9 Hz, 3H, Me$_{alanine}$), 0.93 (d, J = 6.2 Hz, 3H, Me-21$_{CA}$), 0.81 (s, 3H, Me-19$_{CA}$), 0.56 (s, 3H, Me-18$_{CA}$). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.13 (C-24$_{CA}$), 171.70 (C=O$_{alanine}$), 137.00, 132.50, 129.49, 119.59, 71.46 (C-12$_{CA}$), 70.90 (C-3$_{CA}$), 66.70 (C-7$_{CA}$), 49.32 (NH-**C**H$_{alanine}$), 46.59, 46.19, 41.98, 41.80, 35.76, 35.64, 35.35, 34.84, 32.65, 32.07, 30.86, 29.00, 27.78, 26.66, 23.27, 23.07, 20.90 (**C**H$_{3alanine}$), 18.58 (C-19$_{CA}$), 17.64 (C-21$_{CA}$), 12.77 (C-18$_{CA}$). IR (KBr, υ, cm^{-1}) 3394, 3325, 3078, 2932, 2870, 1651, 1520. HRMS (ESI): *m/z* [M+H]$^+$ calcd for Chemical Formula: Chemical Formula: $C_{34}H_{53}N_2O_5$: 569.3954 found : 569.3937. $[\alpha]^{20}_D$ = +28.0 (c 1.0, MeOH)

*2.4.14 (R)-4-((3R,5R,8R,9S,10S,12S,13R,14S,17R)-3,12-Dihydroxy-10,13,14-trimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)-N-((S)-1-oxo-1-(p-tolylamino)propan-2-yl)pentanamide (**6n**)*

White solid; yield: 89%; m.p: 153-155 °C, ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.91 (s, 1H, **NH$_{Ar}$**), 8.13 (d, J = 7.1 Hz, 1H, **NH**$_{alanine}$), 7.48 (d, J = 8.2 Hz, 2H, H-3$_{Ar}$ & H-5$_{Ar}$), 7.10 (d, J = 8.1 Hz, 2H, H-2$_{Ar}$ & H-6$_{Ar}$), 4.52 – 4.45 (m, 1H, CH$_{alanine}$), 4.42 – 4.35 (m, 1H, H-12$_{DCA}$), 4.22 (br s, 1H, OH$_{DCA}$), 3.79 (br s, 1H, OH$_{DCA}$), 3.32 – 3.21 (m, 1H H-3$_{DCA}$), 2.24 (s, 3H, Me$_{Ar}$), 2.18 – 1.96 (m, 3H),1.83 – 1.71 (m, 6H), 1.69 – 1.54 (m, 6H), 1.52 – 1.29 (m, 13H), 1.25 (d, J = 6.9 Hz, 3H, Me$_{alanine}$), 0.93 (d, J = 6.2 Hz, 3H, Me-21$_{DCA}$), 0.84 (s, 3H, Me-19$_{DCA}$), 0.57 (s, 3H, Me-18$_{DCA}$). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.11 (C-24$_{DCA}$), 171.71 (C=O$_{alanine}$), 137.01, 132.48, 129.47, 119.59, 71.47 (C-12$_{DCA}$), 70.42 (C-3$_{DCA}$), 49.35 (NH-**C**H$_{alanine}$), 47.90, 46.65, 46.43, 42.07, 37.21, 36.74, 36.11, 35.55, 35.60, 34.26, 33.37, 32.62, 32.03, 30.68, 29.05, 27.45, 27.67, 26.57, 23.97, 23.53, 20.89 (NH-**C**H$_{alanine}$), 18.57 (C-19$_{CA}$), 17.60 (C-21$_{CA}$), 12.86 (C-18$_{CA}$). IR (KBr, υ, cm^{-1}) 3394, 3310, 3070, 2932, 2862, 1651, 1512. HRMS (ESI): *m/z* [M+H]$^+$ calcd for Chemical Formula: $C_{34}H_{53}N_2O_4$: 553.4003 found : 553.4029. $[\alpha]^{20}_D$ = +48.0 (c 1.0, MeOH)

2.5 Cytotoxicity Studies

2.5.1 Cell Lines and Cell Culture

The cell lines MDA-MB-231 (human breast adenocarcinoma cell line), HT29 (human colorectal adenocarcinoma cell line), U87 (human glioblastoma cell line) and HEK293T (Human normal cell line) were obtained from the National Centre for Cellular Sciences (NCCS), Pune, India. Cells were cultured either in RPMI -1640 (MDA-MB-231,HEK293T), DMEM (HT-29) or MEM (U87) media, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1 mM $NaHCO_3$, 2 mM-glutamine, 100 units/ml Penicillin and 100 µg/mL Streptomycin. All cell lines were maintained in culture at 37 °C in an atmosphere of 5% CO_2.

2.5.2 Test Concentration

Initially, stock solutions of each test substances were prepared in 100% DMSO with a final concentration of 10 mM. Further dilutions were made with culture medium to obtain experimental stock concentration of 100-0.01 µM. Exactly 100 µL of each diluent was added to 100 µL of cell suspension (total assay volume of 200 µL) and incubated for 48 h at 37 °C in 5% CO_2.

2.5.3 Methodology

Cytotoxicity was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, according to the method of Johan van Meerloo [28]. Briefly, the cells (5×10^3) were seeded in each well containing 0.1 ml of medium in 96 well plates. After overnight incubation at 37 °C in 5% CO_2, the cells were treated with 100 µL of different test concentrations of test compounds (0.01 to 100 µM) at identical conditions with three replicates each. The final test concentrations were equivalent to 0.01 µM to 100 µM. The cell viability was assessed after 48 h, by adding 10 µL of MTT (5 mg/mL) per well. The plates were incubated at 37 °C for additional 2-4 h. The medium was discarded and the formazan violet crystals, which were colored for the viable cells, were dissolved with 100 µL of DMSO. The rate of color formation was measured at 570 nm in a spectrophotometer (Spectra MAX Plus; Molecular Devices; supported by SOFTmax PRO-5.4). The percent inhibition of cell viability was determined with reference to the control values (without test compound). The data were normalised with the lowest and highest values and subjected to non-linear regression analysis to obtain log (concentration) Vs normalised expression (variable slope) using graph pad prism 6. The GI_{50} (inhibition of cell growth) concentrations were further obtained using the Graph pad prism 6.

3. Results & Discussion

3.1 Chemistry

Synthesis of hybrid molecules between two bioactive moieties have emerged as a successful approach for the discovery of novel cytotoxic agents with high potency and lower side effects. In view of this and taking a lead from the existing bile acid amino acid conjugates (Figure 1), we planned to synthesize bile acid-aryl/heteroaryl amides linked *via* amino acids. Two strategies were initially planned for the synthesis of targeted molecules (Figure 2). The first strategy involves the synthesis of CA-amino acid conjugates [29], followed by coupling of *C*-terminus of amino acid residue with an aromatic/heteroaromatic amines, while the second strategy employs the synthesis of amino acid-aryl/heteroaryl amides, followed by its coupling with bile acid.

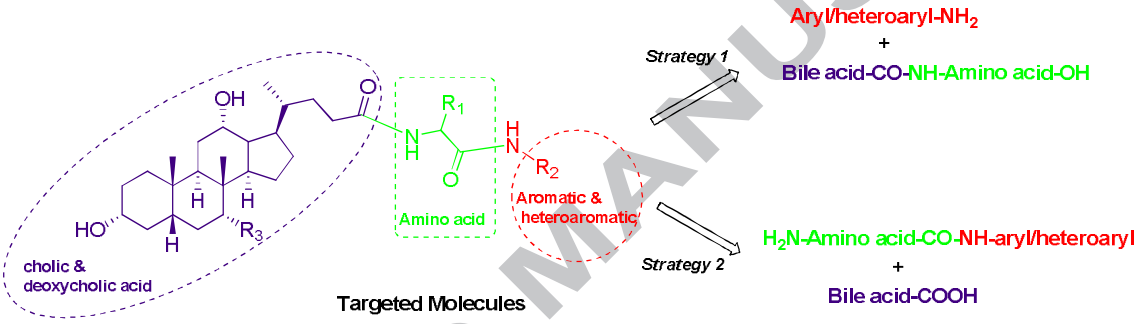
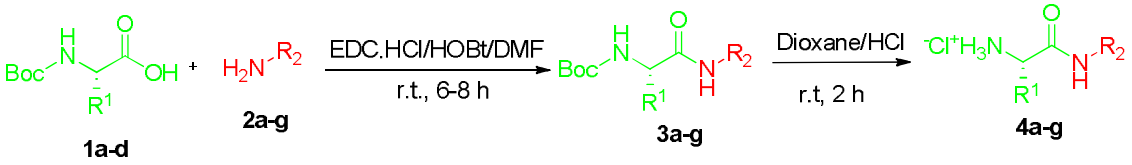


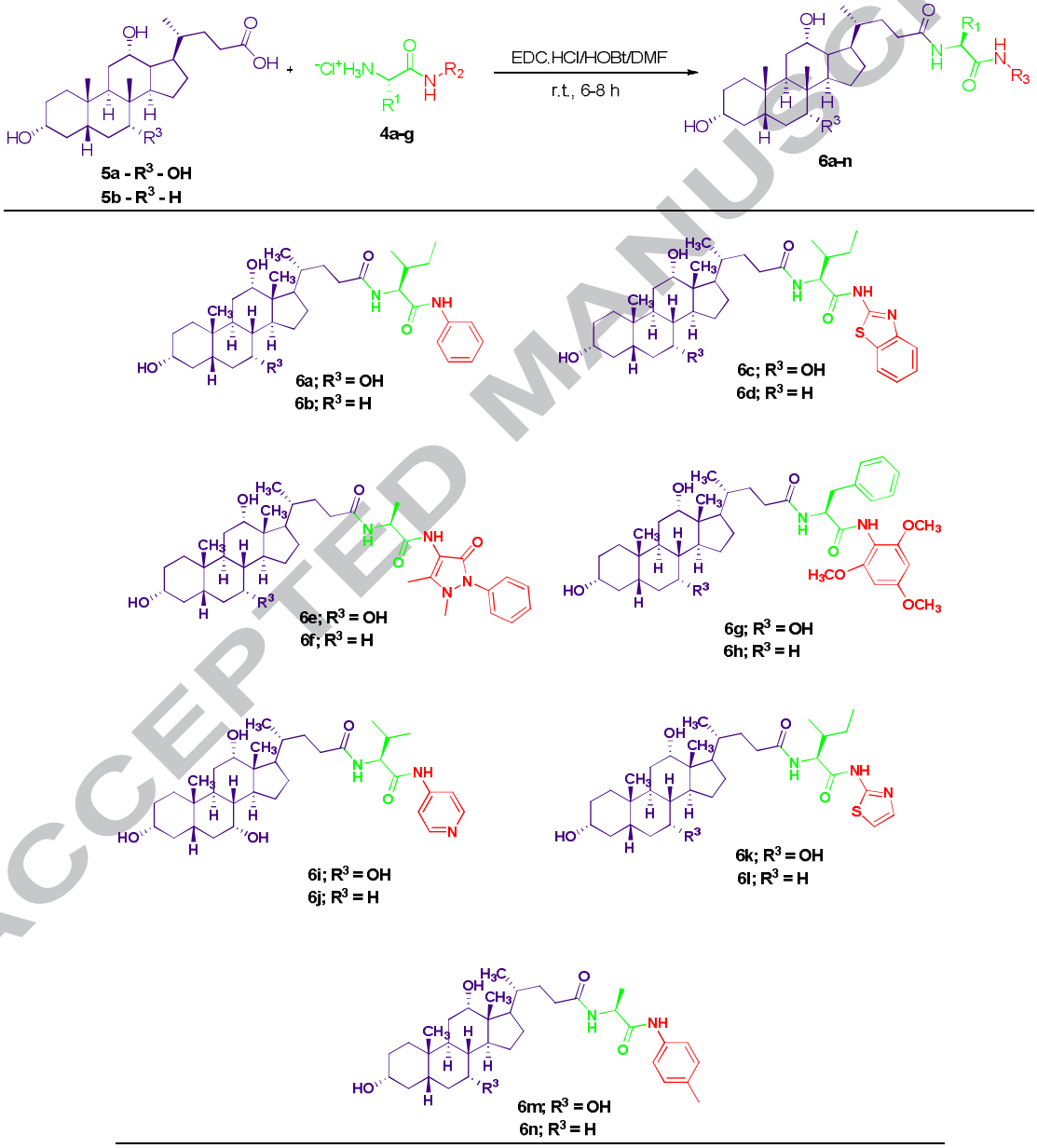
Figure 2. Possible strategies for the synthesis of targeted molecules

We initially attempted the first strategy by synthesizing CA-alanine conjugate in 74% yield by coupling CA and alanine using DCC/HOBt in DMF:H_2O (7:3). However, further coupling of aniline to CA-alanine conjugate proceeded in poor yields using several coupling reagents such as DCC, EDC.HCl, EEDQ. We thus switched towards employing the second strategy. Here, the synthesis commenced with the coupling of protected L-*α*-amino acids (**1a-d**) with different amines (**2a-g**) using EDC.HCl/HOBt in DMF to yield Boc L-aminoacyl aromatic/ heteroaromatic amides (**3a-g**) in 46-81% yield. Deprotection of Boc group in **3a-g** at room temperature yielded unprotected aminoacyl aromatic/herteroaromatic amides (**4a-g**) as hydrochloride salts in quantitative yields (Scheme 1). The structures of the **3** & **4** were characterized on the basis of ^{1}H & ^{13}C NMR and mass spectroscopic studies.



Scheme 1. Synthesis of unprotected L-aminoacyl aromatic & heteroaromatic amides

CA (**5a**) and DCA (**5b**) were reacted with unprotected L-aminoacyl aromatic/heteroaroomatic amides (**4a-g**) using EDC.HCl/HOBt in DMF at room temperature in 6-8 h to yield their corresponding amides (**6a-n**) in excellent yields (Scheme 2). The structures of **6a-n** were characterized on the basis of ^{1}H, ^{13}C NMR, IR and mass spectroscopic studies. In particular, the relative and concordant integrations of aromatic protons of aryl/heteroaryl moiety to that of methine proton on C-3, C-12 (and C-6 in case of) carbons bearing the hydroxyl groups and the three methyl groups of the CA and DCA moiety confirmed the formation of the products.



Scheme 2. Synthesis of CA and DCA aryl/heteroaryl amides

3.2 Cytotoxicity Tests

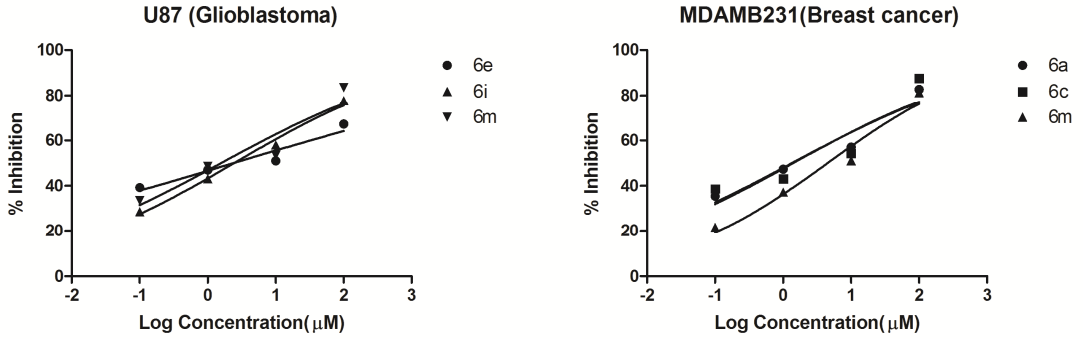
The biological activities of **6a-n** were evaluated to investigate their anti-proliferative/cytotoxic activities in three different types of human cancer cell lines such as colon, breast and glioblastoma cancers (HT-29, MDA-MB-231 & U87) and percentage cytotoxicity on normal cell line (HEK293T) (Table 1). Few compounds showed good activity against breast and glioblastoma cancer cell lines *i.e.,* three (**6a**, **6c**, **6m**) for breast and three (**6e**, **6i**, **6m**) for glioblastoma cell lines, out of total fourteen derivatives tested (Table 1). It is apparent from the results that few derivatives are found to be more potent on breast cancer cell lines than the other two. Even some derivatives were found to be more potent than Doxorubicin and Cisplatin against these three cell lines. For instance, compound **6a** (1.35 µM), **6c** (1.41 µM) and **6m** (4.52 µM) possessing phenyl, benzothiazole and 4-methylphenyl groups showed fairly good activity against the breast cancer cell line with respect to Cisplatin (7.21 µM) and comparable with to Doxorubicin (1 µM). Compound **6e** (2.49 µM), **6i** (2.46 µM) and **6m** (1.62 µM) showed better activity against glioblastoma cancer cell line with respect to both Cisplatin (2.60 µM) and Doxorubicin (3.78 µM) drugs used as standards. Figure 3 display the concentration-responsive curves of the compounds **6e**, **6i**, **6m** on glioblastoma cell lines and **6a**, **6c** and **6m** on breast cancer cell lines. Further testing the compounds on a human normal kidney cell line (HEK293T) indicated that >65% compounds to be safer.

Table -1 *In vitro* anti-cancer activity evaluation[e] of **6a-n** by MTT assay

Compound Code	**GI_{50} [µM]**			**% Cytotoxicity at 25 µM**
	HT-29[a]	MDA-MB-231[b]	U87MG[c]	HEK293T[d]
6a	61.76	**1.35**	66.56	46.54
6b	68.83	17.60	>100	43.27
6c	36.39	**1.41**	>100	62.99
6d	>100	76.29	57.17	34.89
6e	>100	>100	**2.49**	26.21
6f	>100	70	5.50	19.99
6g	47.43	54.95	13.19	13.60
6h	>100	>100	72.29	23.28
6i	>100	75.76	**2.46**	18.76
6j	>100	82.64	23.9	56.40
6k	36.91	36.19	22.15	48.33
6l	44.01	32.64	6.16	53.76
6m	27.86	**4.52**	**1.62**	52.51
6n	>100	45.44	9.65	51.32
Doxorubicin	0.3	1.0	2.60	-
Cisplatin	8.61	7.21	3.78	-

[a] colon cancer, [b] breast cancer, [c] glioblastoma cancer, [d] human normal kidney cell line
[e] All values are with a standard deviation of ±0.55 and were analysed using Graph Pad Prism software.

Figure 3. Log concentration-response curve of the most active compounds on cancer cell lines



3.3 Drug Likliness Properties Evaluation

The synthesized conjugates were evaluated for drug likeliness properties in accordance with Lipinski rule of five and the results are tabulated Table 2.

Table 2. Drug likeliness properties calculation for the Lipinsky rule using molinspiration.

Compound	mi LogP	TPSA	n atoms	MW	n ON	n OHNH	n viol	n rotb	Volume
6a	4.56	118.88	40	554.77	7	5	1	7	546.60
6b	5.47	98.65	39	538.55	6	4	2	7	538.55
6c	6.40	131.77	46	653.93	8	5	2	9	627.33
6d	7.31	111.54	45	637.93	7	4	2	9	619.29
6e	3.94	145.82	48	664.89	10	5	1	8	640.84
6f	4.86	125.59	47	648.89	9	4	1	8	632.80
6g	5.65	146.58	52	720.95	10	5	2	12	694.88
6h	6.56	126.35	51	704.95	9	4	2	12	686.84
6i	4.05	131.77	42	583.81	8	5	1	8	575.83
6j	4.96	111.54	41	567.82	7	4	1	8	567.78
6k	4.89	131.77	42	603.87	8	5	1	9	583.34
6l	5.81	111.54	41	587.87	7	4	2	9	575.30
6m	5.00	118.88	41	568.80	7	5	2	7	563.16
6n	5.92	98.65	40	552.80	6	4	2	7	555.11

The Lipinski rule of five states that a molecule could be a oral drug candidate if it does not violates more than one rule laid down, the rules are a molecule should not have more than five hydrogen bond donor, it should not have more than 10 hydrogen bond acceptors, it should not have molecular weight more than 500 dalton and finally it should not have an octanol-water partition coefficient more than 5. The drug likeliness properties of the synthesized conjugates were evaluated by using mol inspiration [30] and it was found that all the conjugates violated one or more criteria laid down but the compounds which showed fairly good activity with respect to the standard drugs used against one or the other cancer lines used for e.g. compound **6a**, **6e**, **6i** violates only 1 rule and could be considered as ideal candidates for the development of oral drug medications. While compounds **6c** and **6m** showed fairly good activity against the evaluated cancer cell lines but violates 2 rules, thus with further structural modifications and evaluation against other cancer cell lines these compounds could act as potential drug candidates because many natural products for e.g. Vincristine, Vinblastine are there which violates one or more criteria of Lipinski rule but still are potentially active molecules.

3.4 Plausible mode of mechanism

Numerous mechanism have been proposed and proved for the anti-cancer behaviour of bile acid derivatives. The major mechanism for the action of bile acid derivatives is based on apoptosis. For example, DCA is known to induce apoptosis *via* a protein kinase C-dependent signalling pathway, while CA inhibits cell growth [19]. Reports have also indicated the role of bile acids in the removal of damaged cells, maintaining homeostasis and thereby DNA stability and protection against carcinogenesis [5]. Apoptosis has also been induced by bile acid derivatives in human tumour cells *via* caspases, p53 and reactive oxygen species inductions, which thus kills the tumour cells by forming death inducing signalling cascades and by activating caspases 3 and 9 [31, 32].

Based on the literature reports, the following observations could be inferred from our studies:

1. Lower GI_{50} values of CA derivatives (**6a**, **6c**, **6e**, **6i**, **6m**) indicated a crucial role of hydroxyl group at 7^{th} position in the bile acid skeleton in inhibiting the growth of cancer cells [22].

2. CA conjugates possessed lower lipophilicity than their corresponding DCA derivatives suggesting that the importance of lipophilicity of the molecule on activity.

3. Among the CA derivatives, presence of aryl group in the side chain (R^3) yielded comparatively better activity against one or other cell lines than substituted aryl or heteroaryl

group. For instance, **6a** (R^3 = Ph) was found to be most active against breast cancer cell line as compared to **6c** (R^3 = benzothiazolyl)**, 6m** (R^3 = *p*-tolyl). Similarly, **6m** (R^3 = *p*-tolyl) was found to be more active against glioblastoma cancer cell line as compared to **6e** (R^3 = pyrazolinyl), **6i** (R^3 = pyridyl). However, these observations cannot be generalized for all molecules.

3.5 Future Scope of the Present Work

The potent compounds (**6a**, **6c**, **6e**, **6i**, **6m**) will be further studied for their role in growth inhibition of human tumour cells. Importantly, apoptosis-based assays would be performed to know the downstream signalling of the potent compounds. Some of the proceedings in future study include the role of reactive oxygen species, DNA fragmentation and the changes in caspase gene expression.

4. Conclusion

In summary, we have synthesized a series of bile acid-aminoacyl-aryl/heteroaryl amides in excellent yields, the cytotoxicity of these were tested against three cancer cell lines. Many of these derivatives are showing selective activity on one of the cell lines. Though, most of the derivatives are comparatively less potent than the commercially available drug molecule Doxorubicin and Cisplatin; nevertheless slight structural modification of these active derivatives may yield as prospective anticancer drugs. Based on the present results, it is warranted that these derivatives to be further evaluated on other cancer cell lines.

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Appendix A supplementary data

Supplementary data associated with this article can be found, in the online version, at http://

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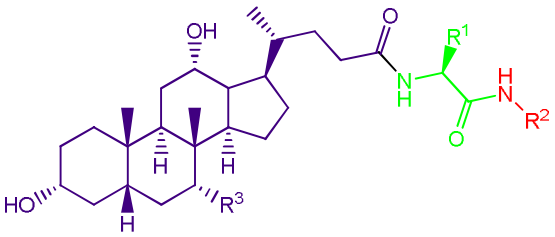

Anti cancer activity: R^3 = OH; R^2 = phenyl, R^1 = $CH(CH_3)CH_2CH_3$; GI_{50} = 1.35 µM
(MDA-MB-231) R^3 = OH; R^2 = 2-benzothiazolyl, R^1 = $CH(CH_3)CH_2CH_3$; GI_{50} = 1.41 µM
R^3 = OH; R^2 = 4-methylphenyl, R^1 = CH_3; GI_{50} = 4.52 µM
Anti cancer activity: R^3 = OH; R^2 = 2,3-dimethyl-1-phenyl-3-pyrazolin-5-onyl, R^1 = CH_3; GI_{50} = 2.49 µM
(U87MG) R^3 = OH; R^2 = 4-pyridyl, R^1 = $CH(CH_3)_2$; GI_{50} = 2.46 µM
R^3 = OH; R^2 = 4-methylphenyl, R^1 = CH_3; GI_{50} = 1.62 µM

- A series of bile acid aryl/heteroaryl amides linked *via* α-amino acid were synthesized in excellent yields.
- Synthesized compounds were tested for their cytotoxic behavior against 3 human cancer cell-lines (HT29, MDAMB231, U87) and 1 human normal cell line (HEK293T).
- Three of the conjugates showed promising results to be new anticancer agents with good *in vitro* activity (GI_{50} = 1.35, 1.41, 4.52 µM) against the breast cancer cell line in comparison to cisplatin (7.21 µM) and doxorubicin (1 µM).
- Another three conjugates showed promising results to be new anticancer agents with good *in vitro* activity (GI_{50} = 2.49, 2.46, 1.62 µM) against the Glioblastoma cancer cell line in comparison to cisplatin (2.60 µM) and doxorubicin (3.78 µM).
- Greater than 65% of the synthesized compounds were found to be safer on human normal cell line.