Synthesis and Biological Activities of C-2, N-9 Substituted 6-Benzylaminopurine Derivatives as Cyclin-Dependent Kinase Inhibitor

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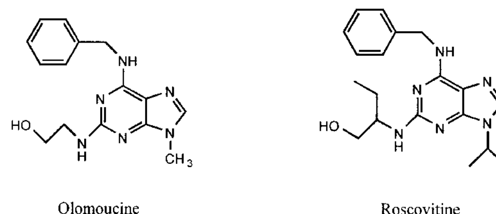
Summary

In this study, C-2, N-9 substituted 6-benzylaminopurine derivatives were synthesized and their inhibitory effects on cyclin-dependent kinase (CDK2) were evaluated. The effect of substituents at the C-2 and N-9 positions of substituted purine was investigated. Among the compounds tested, compound **7b-iii** (6-benzylamino-2-thiomorpholinyl-9-isopropylpurine) was the most active inhibitor (IC_{50} = 0.9 μM). Compound **7b-iii** showed 10-fold higher activity compared to olomoucine and almost the same activity as roscovitine. Results from structure-activity relationship studies should allow the design of more potent and selective CDK inhibitors, which may provide an effective therapy for cancer or other CDK dependent diseases.

Introduction

Most of the extracellular signals are amplified and transduced inside cells by a cascade of various kinases. Protein kinases are involved in essentially all intracellular regulatory pathways. Cyclin-dependent kinase (CDK) is one of the cellular kinases which control the cell division cycle (cdc). Cyclin-dependent kinases constitute a family of highly conserved protein kinases playing essential roles in cell cycle regulation [1,2]. The CDKs are activated through binding to a family of regulatory proteins called cyclins and phosphorylation on a specific threonine residue. The crystal structures of CDK2 [3] and CDK2/cyclinA [4] have recently been determined, allowing a very precise understanding of the mechanisms of enzyme activation and activity. Numerous examples of CDK deregulation in human primary tumors and in tumor cell lines have been described recently [5,6]. It has already been shown in animal models that different inhibitors of CDK show antitumor activity[7]. The search for potent inhibitors of CDKs has led to the discovery of a family of C-2, C-6, N-9-substituted purines with high CDK selectivity. The most studied members of this family, olomoucine [8] and roscovitine [9,10], have been co-crystallised with CDK2 [9,11]. Both molecules interact with CDK2 at the ATP binding site. Analysis of the CDK2/olomoucine and CDK2/roscovitine crystal structures reveals that the C-2 substituents bind to an area of the ATP-binding pocket occupied by the ribose in the CDK2/ATP complex.

Olomoucine, a purine derivative, displays quite a narrow selectivity: among 35 kinases tested, it only inhibits cdc2 (cell division cycle 2, CDK1), CDK2 and CDK5 and ERK-l (endoplasmic reticulum kinase-1) to a lesser extent [8]. The position of olomoucine in the ATP binding pocket of CDK2 has been determined by analysis of olomoucine/CDK2 co-crystal [11]. Recently, olomoucine was found to stimulate massive apoptosis in cells which have been arrested in G2 by the use of DNA-damaging agents[12]. A synthesis of olomoucine, roscovitine, and structurally related compounds as well as their SAR study was recently reported [13]. These encouraging results led us to investigate other C-2, C-6, and N-9-substituted purines as potential CDK inhibitors.



We here report the synthesis of a new potent and selective CDK2 inhibitor, 6-benzylamino-2-thiomorpholinyl-9-isopropylpurine **(7b-iii)**. The structure-activity relationship of the synthesized compounds as well as cytotoxicity using human cell line is reported.

Chemistry

Adenine (**1**) is readily oxidized with hydrogen peroxide in aqueous acetic acid to give adenine 1-*N*-oxide. The deamination of *N*-oxide (**2**) was carried out with sodium nitrite in acetic acid. 2,6-Dichloropurine (**4**) was obtained by treating **3** with phosphoryl chloride in the presence of an organic base. The 2,6-chloropurine derivative was reacted with benzylamine in *n*-butanol at 100 °C to yield 6-benzylaminopurine (**5**). N-9 Alkylation of the purine (**5**) was carried out with alkyl halide in the presence of sodium hydride. The final compound (**7**) was synthesized by the reaction of **6** with cyclic amino

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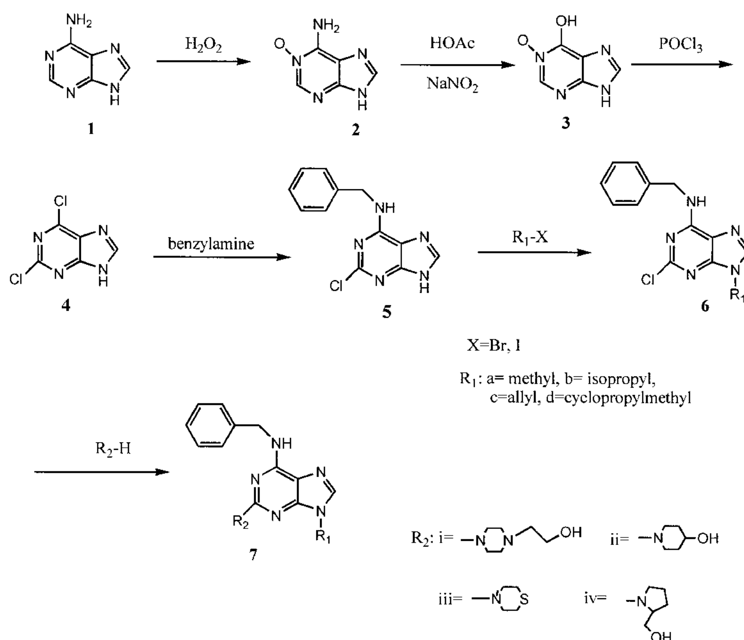


alcohol (4-hydroxypiperidine, 2-hydroxymethylpyrrolidine, etc.) and thiomorpholine in *n*-butanol at 120 °C.

Biological Evaluation

Enzymatic Activity

For CDK2/cyclin A enzyme inhibition studies, CDK2 and cyclin A gene were subcloned into pBacPaK8 vector (Clon Tech, USA) respectively. CDK2 gene was tagged with hexahistidine at the N-terminal for affinity purification of CDK2/cyclin A complex. Active CDK2/cyclin A was purified using His-Resin(Novagen, USA) from sf21 insect cells cotransfected with a 1:1 ratio of CDK2 and cyclin A baculoviral stocks. Enzyme assays were done in 20 µl reaction mixture containing 100 ng of purified CDK2/cyclin A enzyme, 4 µg of histone H1 as substrate, 100 µM ATP, 0.2 µCi γ-P^{32}-ATP, 10 mM $MgCl_2$, 1 mM DPT in 50 mM Tris-HCl (pH 7.5) buffer. The reaction was continued for 10 min at 30 °C in the presence of inhibitor and stopped by adding 80 µl of 12% phosphoric acid. The stopped mixtures were transferred to a 96-well PVDF filter (Millipore, USA) prewetted with 50% EtOH and drained with mild suction. Each well was washed five times with 10 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl to remove free γ-P^{32}-ATP. The filter was briefly dried and exposed to Phosphoimager (Molecular Dynamics, USA) to measure radioactivity.

In this study, C-2, N-9 substituted 6-benzylaminopurine derivatives were synthesized and their structures and enzyme inhibition activities are listed in Table 1.

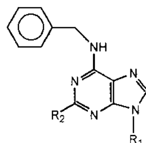
The effect of substituents at the C2 and N9 positions of the purine was investigated. Among the compounds tested, compound **7b-iii** was the most active inhibitor (IC_{50} = 0.9 µM). Compound **7b-iii** showed 10-fold increased activity compared to olomoucine and almost the same activity as roscovitine. With regard to substituents at the N-9 position of purine, it was shown that the isopropyl derivatives are 4–10 times more potent than their corresponding analogues, such as methyl, allyl, and cyclopropylmethyl, respectively. We tried to replace the aliphatic alcoholic group on the C-2 position of purine by cyclic alcohol moieties such as 4-hydroxylpiperidine and 2-(hydroxymethyl)pyrrolidine. It is interesting that replacement of the hydroxy moiety at the C-2 position of the purine by a thiomorpholine led to highly potent inhibition. It may be possible that lone pair electrons on the sulfur atom in the thiomorpholine could be provided for the hydrogen bonding to the residue of CDK2 enzyme.

Cellular Activity

Cytotoxic activity of the anticancer drugs against human cancer cell lines were investigated using the MTT assay. Human lung (A549), human ovarian (SKOV-3), human melanoma (SKMEL-2), human CNS (XF-498), and human colon (HCT15) cancer cell lines were supplied from the College of Medicine, Seoul National University. All cell lines were grown in RPMI 1640 (Gibco BRL) supplemented with 10% (*v/v*) heat inactivated Fetal Bovine Serum (FBS) and maintained at 37 °C in a humidified atmosphere with 5% CO_2. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma. The cells (3–4 × 10^3 cells/well) were seeded into a 96-well plate. Various concentrations of samples were added to each well in duplicate, then incubated at 37 °C with 5% CO_2 for three days such that cells are in the exponential phase of growth at the time of drug addition. Then 50 µl of 0.2% MTT solution was added and incubated in the dark for 4 h at 37 °C. After 4 h incubation, the medium and MTT were removed from the wells, and the water-insoluble MTT-formazan crystals were dissolved in 15 µl DMSO (dimethyl sulfoxide). The plates were then vigorously shaken in order to ensure solubilization of the blue formazan. The optical density was measured using a microplate reader (Vmax, Molecular Devices) with a 540 nm wavelength and the anticancer affective concentration was expressed as an IC_{50} [14–15].

As shown in Table 1, in general, compounds(**7a-iii**, **7a-iv**, **7b-iii**, and **7b-iv**) which were potent inhibitors of CDK, also showed antiproliferative activity, whereas derivatives which

Table 1. Inhibitory activity of 6-benzylamino-purine derivatives against CDK-2 and various human cancer cell lines (IC_{50}, μM).



Compound	R_1	R_2	CDK2 IC_{50} (μM)	Cytotoxicity IC_{50} (μM)				
				A549	SK OV-3	SK MEL-2	XF498	HCT 15
7a-i	methyl	–N N~OH	21	>100	>100	>100	>100	>100
7a-ii	methyl	–N –OH	14	47	71	83	62	59
7a-iii	methyl	–N S	2	29	47	24	29	29
7a-iv	methyl	–N OH	10	15	47	38	38	30
7b-i	isopropyl	–N N~OH	8	13	43	33	30	25
7b-ii	isopropyl	–N –OH	4	25	44	27	25	27
7b-iii	isopropyl	–N S	0.9	11	22	35	27	22
7b-iv	isopropyl	–N OH	1.5	11	27	22	27	22
7c-i	allyl	–N N~OH	31	79	86	99	86	>100
7c-ii	allyl	–N –OH	16	66	71	>100	52	80
7d-i	$–CH_2$–◁	–N N~OH	.>100	66	71	42	83	76
7d-ii	$–CH_2$–◁	–N –OH	79	>100	>100	>100	26	>100
	olomoucine		7	57	>100	87	57	50
	roscovitine		0.5	–	–	–	–	–

were inactive at the enzyme level showed only marginal antiproliferative activity. Compound **7b-iii** was most inhibitory to CDK activity, an effect which could be translated into an antiproliferative effect.

Experimental Part

Melting points (mp) were determined on a Thomas Hoover apparatus and are uncorrected. ^{1}H-NMR spectra: Varian Gemini 300 spectrometer, tetramethylsilane (TMS) as an internal standard. The mass spectrometer was a Hewlett Packard Model HP5989A MS Engine (Palo Alto, CA, USA) spectrometer interfaced with a HP Model 59987A electrospray system.

Adenine 1-N-Oxide (**2**)

Adenine (20.0 g, 0.15 mol) was suspended in 120 ml of acetic acid and was refluxed for 1 h. After the solid was completely dissolved, the mixture was cooled to room temperature. To this solution was added dropwise 74 ml of 30% H_2O_2 and then the solution was allowed to stand for 3 days at room temperature. The precipitate was collected and washed with water to give **2** as a white solid. Yield 15.7 g (70.1%) mp >300 °C (dec.), mp (lit.) 300 °C(dec.)

Hypoxanthine 1-N-Oxide (**3**)

Adenine 1-*N*-oxide (7.19 g, 0.053 mmol) was suspended in a solution containing 33.07 g of $NaNO_2$ (0.48 mol) in 500 ml of water. The mixture was cooled to 10 °C in an ice bath, and 300 ml of 30% aqueous acetic acid was added dropwise with stirring over a period of 30 min. After the addition of acid was complete, the solution was heated at 70–80 °C for 2 h, then cooled to room temperature and allowed to stand for 4 days. The precipitate was collected and washed with water, alcohol, and ether to afford **3** as a yellow solid. Yield 5.3 g (66.2%), mp >300 °C(dec.) mp (lit.) 300 °C (dec.)

2,6-Dichloropurine (**4**)

Hypoxanthine 1-*N*-oxide (2.4 g, 0.016 mol) was suspended in a mixture of 120 ml of phosphoryl chloride and 4 ml of triethylainine and was refluxed for 3 h under N_2. After the mixture was cooled to room temperature, excess phosphoryl chloride was distilled off under reduced pressure. The residue

was dissolved in 100 ml of water, and was extracted with ethyl ether (5 × 100 ml). The organic solvent was evaporated *in vacuo* to give a crude oil, which was chromatographed on silica gel using ethyl acetate/*n*-hexane (1:1) as an eluent to give **4** as a white solid. Yield 1.2 g (39.9%). mp 177–178°C, mp (lit.) 177 °C.

*6-Benzylamino-2-chloropurine (***5***)*

Benzylamine (0.87 ml, 8.0 mmol) and 2,6-dichloropurine (0.5 g, 2.7 mmol) were dissolved in *n*-BuOH (15 ml) and the solution was heated at 100 °C for 5 h. The resulting solution was evaporated and diluted with ethyl acetate. The organic layer was washed with water, 1N HCl, and brine. Evaporation of the solvent *in vacuo* gave a crude solid, which was washed with cold acetone to give **5** as a white solid. Yield 0.56 g (80.5%), mp 205–207 °C. ^{1}H-NMR (DMSO-d$_6$); δ (ppm) = 4.43(d, 2H, *J* = 6.3 Hz, *CH$_2$*Ph), 4.64 (br, 1H, -*NH*CH$_2$-), 6.78 (bs, 1H, N$_9$-H), 7.2-7.35 (m, 5H, phenyl), 7.62 (s, 1H, C$_8$-H).

*6-Benzylamino-2-chloro-9-methylpurine (***6a***)*

To a solution of NaH (0.3 g, 10.5 mmol) in dry DMF (100 ml) at 20 °C was added slowly a solution of **5** (2 g, 8.1 mmol) in DMF under N_2 gas. After 1 h methyl iodide (9.1 g, 32.0 mmol) was added dropwise and the reaction mixture stirred for 24 h. The resulting mixture was diluted with sat. NH_4Cl (30 ml) and ethyl acetate(50 ml). The organic layer was washed with water (3 × 50 ml), brine, and dried over anhydrous Na_2SO_4. Removal of the solvent gave a crude residue, which was chromatographed on silica gel using ethyl acetate as eluent to give **6a** as a pale yellow solid. Yield 1.92 g (90.5%). ^{1}H-NMR (DMSO-d$_6$); δ (ppm) = 3.68 (s, 3H, -NCH$_3$), 4.83 (d, 2H, *J* = 6.33 Hz, *CH$_2$*Ph), 7.24–7.35 (m, 5H, phenyl), 8.13 (s, 1H, C$_8$-H), 8.77 (bs, 1H, -*NH*CH$_2$).

*6-Benzylamino-2-(N-(2-hydroxymethyl)pyrrolidinyl)-9-methylpurine (***7a–iv***)*

To a solution of compound **6a** in *n*-butanol was added 2-(hydroxymethyl)-pyrrolidine and the mixture was heated in an evacuated sealed tube at 155 °C for 3–5 h. After cooling, the solution was diluted with water and ethyl acetate. and the organic layer was washed with water and brine. Evaporation of the solvent *in vacuo* gave a crude solid, which was recrystallized from MeOH/ benzene to give **7–iv** as a white solid, ^{1}H-NMR(CDC13); δ (ppm) =1.65 (m, 1H, pyrrolidine H), 1.89 (m, 2H, pyrrolidine H), 2.14 (m, 1H, pyrrolidine H), 3.58 (s, 3H, -NCH$_3$), 3.60–3.88 (bs, 4H), 4.2l (m, lH, pyrrolidine H), 4.73 (d, 2H, *J* = 6.3 Hz, *CH$_2$*Ph) 5.98 (bs, 1H, -*NH*CH$_2$-) 7.26–7.45 (m, 6H, phenyl, C$_8$-H).– MS: *m/z* 338 (M$^+$ $C_{18}H_{22}N_6O$).

Compounds **7a-i–7d-ii** were prepared by same procedure as described for the preparation of **7a-iv.**

7a-i, mp 165–167 °C.– ^{1}H-NMR(CDCl$_3$); δ (ppm) = 2.58 (m, 6H, *CH$_2$*HN(*CH$_2$*)$_2$-) 3.69 (q, 5H, -N*CH$_3$*, -CH$_2$OH), 3.81 (t, 4H, *J* = 5.0 Hz, -N(CH$_2$)$_2$-), 4.79 (d, 2H, *J* = 5.2 Hz,-NH*CH$_2$*-), 5.87 (bs, 1H, *NH*CH$_2$-) 7.25–7.43 (m, 6H, -CH$_2$Ph , C$_8$-H).– MS *m/z* 367(M$^+$ $C_{19}H_{25}N_7O$).

7a-ii, mp 184–187 °C.– ^{1}H-NMR (CDCl$_3$); δ (ppm) = 1.5l (m, 2H), 1.93(m, 2H), 3.25 (m, 2H, -*CH$_2$*NCH$_2$-), 3.66 (s, 3H, -NCH$_3$), 3.9l (m, 1H, -CHOH), 4.46 (m, 4H, -CH Ph, -CH$_2$N*CH$_2$*-), 4.84 (bs, 1H, -NHCH$_2$-), 7.26–7.4 (m, 5H, phenyl), 7.46 (s, 1H, C$_8$-H).– MS: *m/z* 338 (M$^+$ $C_{18}H_{22}N_6O$).

7a-iii ^{1}H-NMR (CDCl$_3$); δ (ppm) = 2.61 (d, 4H, -S (CH2)2-), 3.66 (s, 3H, -NCH$_3$), 4.13 (q, 4H, -N(CH$_2$)$_2$-), 4.77 (d, 2H, -NHCH$_2$-), 6.39 (bs, 1H, -NHCH$_2$-), 7.22–7.44 (m, 6H, phenyl, C$_8$-H).– MS *m/z* 340(M$^+$ $C_{17}H_{20}N_6S$).

7b-i, mp 104–107 °C.– ^{1}H-NMR (CDCl$_3$) δ (ppm) = 1.54 (d, 6H, *J* = 7.0 Hz, 2CH$_3$) 2.57 (m, 6H, -*CH$_2$*N(*CH$_2$*)$_2$-), 3.66 (t, 2H, *J* = 5.3 Hz, -*CH$_2$*OH), 3.82 (t, 4H, -N(*CH$_2$*)$_2$-), 4.65 (m, 3H, -*CH*(CH$_3$)$_2$, -*CH$_2$*Ph), 7.21–7.36 (m, 5H, phenyl), 8.28 (s, 1H, C$_8$-H).– MS *m/z* 396 (M$^+$ $C_{21}H_{30}N_7O$).

7b-ii, mp 184–188 °C.– ^{1}H-NMR (CDCl$_3$); δ (ppm) =1.5l (m, 8H, -CH$_2$CH*CH$_2$*-, 2CH$_3$), 1.92 (m, 2H, -CH$_2$CH*CH$_2$*-), 3.19 (m, 2H, -*CH$_2$*NCH$_2$), 3.92 (m, 1H, -*CH*OH), 4.48 (m, 2H, -CH$_2$N*CH$_2$*-), 4.68 (m, 1H, -*CH*(CH$_3$)$_2$), 4.78 (d, 2H, -*CH$_2$*Ph), 5.96 (bs, 1H, -NHCH$_2$-), 7.25–7.36 (m, 5H, phenvl) 7.48 (s, 1H. C$_8$-H).– MS *m/z* 367 (M$^+$ $C_{20}H_{27}N_6O$).

7b-iii, mp 137–141 °C.– ^{1}H NMR(CDCl$_3$); δ (ppm) =1.54 (d, 6H, -2CH$_3$), 2.6l (d, 4H, -S(CH$_2$)$_2$-), 4.13 (q,4H, -N(CH$_2$)$_2$-), 4.63 (m, 1H, -C-H(CH$_3$)$_2$) 4.77(d, 2H, -NHCH$_2$-), 6.39(bs, 1H, -NHCH$_2$-), 7.22–7.44 (m, 6H, phenyl, C$_8$-H).– MS *m/z* 369 (M$^+$ $C_{19}H_{25}N_6S$).

7b-iv, mp 58–61 °C.– ^{1}H-NMR(CDCl$_3$); δ (ppm) =1.53 (d, 6H,CH(CH$_3$)$_2$), 1.65 (m, 1H, -NCH*CH$_2$*CH$_2$), 1.89 (m, 2H, -NCHCH$_2$*CH$_2$*-), 2.14 (m, 1H, -NCH*CH$_2$*CH$_2$), 3.57–3.87 (m, 4H, -CH$_2$NCHCH$_2$OH), 4.28 (m, 1H, -CH$_2$OH), 4.59 (m, 1H. CH(CH$_3$)$_2$), 4.79 (d, 2H, -*CH$_2$*Ph), 6.12 (bs, 1H, -*NH*CH$_2$-), 7.26–7.39 (m, 6H, phenyl), 7.47 (s, 1H, C$_8$-H).– MS *m/z* 367 (M$^+$ $C_{20}H_{27}N_6O$).

7c-i, mp 130–133 °C.– ^{1}H-NMR(CDCl$_3$); δ (ppm) = 2.58 (m, 6H, -CH$_2$N(CH$_2$)$_2$-), 3.69 (t, 2H, -CH$_2$OH), 3.85 (m, 4H, -N(*CH$_2$*)$_2$-), 4.66 (d, 2H, *J* = 8.8 Hz, -CHCH$_2$-), 4.79 (d, 2H, *J* = 5.0 Hz, -NH*CH$_2$*-), 5.19–5.29 (dd, 2H, -NCH$_2$-), 5.87–6.08 (m, 2H, *NH*CH$_2$-, -*CH*=CH$_2$), 7.26–7.39 (m, 5H, Phenyl), 7.45 (s, 1H, C$_8$-H).– MS *m/z* 394 (M* $C_{21}H_{28}N_7O$).

7c-ii, mp 184–188 °C.– ^{1}H-NMR (DMSO-d$_6$); δ (ppm) = 1.23 (m, 2H, -*CH$_2$*CHCH$_2$-) 1.69 (m, 2H, -CH$_2$CH*CH$_2$*-), 3.07 (m, 2H, -CHNCH$_2$-), 3.63 (m, 1H, -*CH*OH), 4.26 (m, 2H, 4.61 (d, 4H, -CHCH$_2$, -NH*CH$_2$*-), 5.07–5.25 (dd, 2H, -NCH$_2$-), 6.03(m, 1H, -*CH*CH$_2$), 7.19–7.37 (m, 5H, phenyl), 7.70 (s, 1H, C$_8$-H).– MS *m/z* 365 (M$^+$ $C_{20}H_{25}N_6O$).

7d-i mp 135–138 °C.– ^{1}H-NMR (CDCl$_3$); δ (ppm) = 0.42 (m, 2H, -CH$_2$CH$_2$-), 0.62 (m, 2H, -CH$_2$CH$_2$-), 1.26 (m, 1H, -CH(CH$_2$)$_2$), 2.63 (m, 6H, -*CH$_2$*N(*CH$_2$*)$_2$-), 3.71 (t, 2H, -*CH$_2$*OH), 3.87 (m, 6H, -N(*CH$_2$*)$_2$-, CH(CH$_2$)$_2$), 4.79 (d, 2H, -NH*CH$_2$*-), 6.03 (bs, 1H, *NH*CH$_2$-), 7.25–7.38 (rn. 5H, phenyl), 7.54(s, 1H, C$_8$-H).– MS *m/z* 408 (M$^+$ $C_{22}H_{30}N_7O$).

7d-ii, mp 165–168 °C.– ^{1}H-NMR (CDCl$_3$), δ (ppm) = 0.42 (m, 2H, -CH$_2$CH$_2$-) 0.63 (m, 2H, -CH$_2$CH$_2$-), 1.26 (m, 1H, -CH(CH$_2$)$_2$), 1.59 (m, 2H, -*CH$_2$*CHCH$_2$-) 1.88 (m, 2H, -CH$_2$CH*CH$_2$*-), 3.76 (m, 2H, -CH$_2$N*CH$_2$*-) 3.88 (d, 2H, 1.14 Hz, -CH(CH$_2$)$_2$), 4.65 (d, 2H, -CH$_2$N*CH$_2$*-) 4.79 (d, 2H, -*CH$_2$*Ph), 5.13 (m, 1H, -*CH*OH), 5.83 (br, 1H, -NHCH$_2$-) 7.26–7.36 (m, 5H, phenyl), 7.55 (s, 1H, C$_8$-H).– MS *m/z* 379 (M+ $C_{21}H_{27}N_6O$).

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