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The Synthesis of Novel Methotrexate-like Compounds

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Summary. Methotrexate (*MTX*) is a folate antagonist used in treatment of several chronic inflammatory and neoplastic conditions. In this study, new *MTX*-like compounds that maybe potential anticancer agents were synthesized and their structures were determined by IR, UV, GC-MS, ^{1}H NMR, and ^{13}C NMR spectra. Also, in this study, a series structurally related to *MTX* or folate analogous compounds were evaluated whether they have inhibitory properties on the dihydrofolate reductase activity (DHFR).

Keywords. Methotrexate; *p*-Aminobenzoic acid; *DMAP*; *DCC*; Amino acid esters.

Introduction

Methotrexate (*MTX*; 2,4-diamino-N^{10}-methylpteroylglutamic acid; **I**) is a small molecule inhibitor of the ubiquitous enzyme dihydrofolate reductase (DHFR), which is a requisite component of the biosynthesis pathway for purine and pyrimidine. *MTX* is a classical type of inhibitor of dihydrofolate reductase and is known to be metabolized to polyglutamyl forms after its incorporation into cells [1]. *MTX* is also a potent folic acid antagonist which inhibits the reduction of folic acid to tetrahydrofolic acid, thereby leading to the suppression of the nucleic acid biosynthesis pathway, and cell death. It has been used in the treatment of neoplastic disorders since the late 1940s [2, 3]. As a consequence, *MTX* is used as a drug for the treatment of a wide range of diseases, *e.g.*, for cancer treatment since its development and the identification of its biological activities [4].

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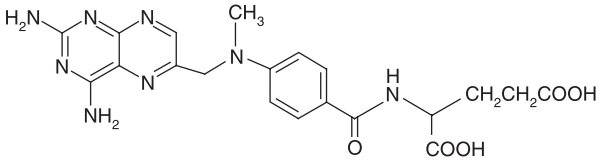
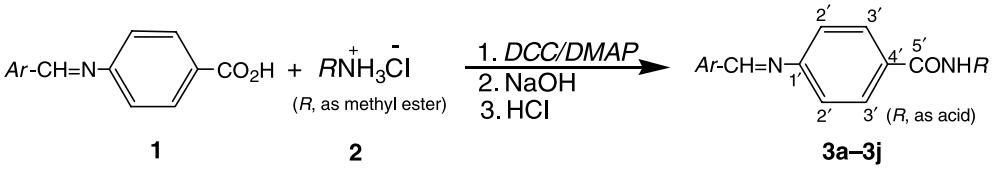
High doses ($>3\ g/m^2$) have been prescribed as a cytostatic agent in oncology since 1953 [5]. Low doses of methotrexate ($5–25\ mg/m^2$ per week) have been used increasingly for the treatment of patients with rheumatoid arthritis (RA) [6, 7], or psoriasis [8–12] and steroid-dependent asthma [13–15]. At the same time *MTX* is widely utilized in the treatment of acute leukemia, carcinoma of the breast, osteosarcoma, ovarian cancer, and other malignant diseases [16].



I

Therapeutic drug monitoring is of importance in order to improve *MTX*–RA therapy because of the severe side-effects of *MTX*, *e.g.*, marrow suppression, gastro-intestinal lesion, renal insufficiency, hepatic failure, hypoalbuminemia, and pancytopenia [17, 18].

MTX is known for its severe serious side effects although it is used in a wide range in medicine. Because of these shortcomings the synthesis of *MTX*-like compounds is pursued. Aim of this work is the synthesis of compounds which can be used instead of *MTX* and having less side effects than *MTX*. Therefore we use some aromatic aldehydes instead of the pterin ring, but did not alter the *PABA* and glutamic acid parts. Then we use β-alanin instead of glutamic acid.



Scheme 1

Results and Discussion

Compounds **3a–3j** were synthesized as depicted in Scheme 1. The −COOH functionalized imines **1** were reacted with amino acids (β-alanine and L-glutamic acid) with *DCC* and *DMAP* as coupling agent.

The IR spectra of these compounds show the C=N bond stretching vibration in the 1625–1635 cm^{-1} and the *Ar*-H bond stretching vibration in the 3101–3037 cm^{-1} region and the strong sharp absorption bands of the amide (NH) appeared at 3307–3435 cm^{-1}. The ^1H NMR spectra of the compounds were recorded in *DMF*-d_7. The ^1H NMR assignments are detailed in the following. The imine protons for compounds **3a–3j** are observed as singlets of 9.08, 9.00, 9.08, 8.60, 9.88, 9.26, 9.00, 9.08, 8.34, and 9.87 ppm.

Also this series of compounds was evaluated whether they have inhibitory properties on the dihydrofolate reductase activity (DHFR).

The result of screening regarding the effect of methotrexate related compounds on DHFR activity is shown in Table 1, listing the compounds in their rank order of potency as inhibitors of DHFR activity. As shown in Table 1, compounds **3g**, **3e**, **3j**, and **3d**, with inhibitions of 84, 83, and 82% (mean), were the most potent inhibitors among the compounds.

Table 1. Maximal inhibition of DHFR by selected compounds **3a–3j** related to methotrexate

Test substance	Maximal inhibition in % (mean)
Methotrexate	84
3a	64
3b	72
3c	78
3d	82
3e	83
3f	70
3g	84
3h	74
3i	78
3j	82

Experimental

Melting points were measured on a Gallenkamp apparatus using a capillary tube. Infrared absorption spectra were recorded from a Mattson 1000-FTIR spectrometer, using KBr pellets. ^1H NMR and ^{13}C NMR spectra were obtained with a Bruker DPX FT-NMR (300 MHz) spectrometer. The chemical schifts are reported in δ/ppm with external *TMS*. Mass spectra were determined on a Thermo Finnigan Trace DSQ. The elemental analyses were performed on a LECO CHNS-932 C, H, N analyzer. All were found to agree favourably with the calculated values. All reagents were of analytical grade. Salicylaldehyde, 2-hydroxy-4-methylbenzaldehyde, thiophene-2-carbaldehyde, pyrrole-2-carbaldehyde, 2-hydroxy-1-naphtaldehyde, 4-aminobenzoic acid, glycine, β-alanine, L-glutamic acid, dichloromethane, thionyl chloride, 4-(dimethylamino)pyridin, and *N,N′*-dicyclohexylcarbodiimid were purchased from Aldrich Chemical Co. Ethanol, methanol, and CH_2Cl_2 were purchased from Merck (Germany). Dichloromethane was distilled from P_2O_5 before use. Amino acid esters were prepared according to published procedures [19, 20]. An improved preparation of these two esters is reported below.

β-Alanine Methyl Ester

Thionyl chloride (16.7 g, 0.14 mol) was added dropwise to a suspension of 8.91 g β-alanine (0.10 mol) in 75 cm^3 methanol at 0°C. The mixture was stirred at room temperature for 18 h and then concentrated using a rotary evaporator. The residual oil occurred was triturated with cold ether and β-alanine methyl ester precipitated as a white powder. The powder was air-dried 12.38 g (89%), mp 103–104°C (Ref. [21] 103–104°C).

Dimethyl L-Glutamate Hydrochloride

Freshly distilled 6.5 cm^3 thionyl chloride (75 mmol) were added dropwise with stirring to cold (at 0°C) anhydrous 20 cm^3 methanol. Dry L-glutamic acid (2.4 g, 14 mmol) was added in portions to this mixture and stirred for 18 h at ambient temperature. On completion of the reaction confirmed by TLC analysis (*n*-butanol:acetic acid:H_2O = 6:2:2), the reaction mixture was concentrated on a rotary evaporator to give a heavy syrup. The syrup was crystallized by the addition of dry diethyl ether to give a fine white powder. Recrystallization from acetone-diethyl ether gave 2.55 g (87%) dimethyl L-glutamate hydrochloride as a colorless solid, mp 77–79°C (Ref. [22] 78–80°C).

*Synthesis of 4-[(2-Hydroxy-4-methoxybenzylidene)amino]-benzoic acid] (**3b**). Typical Procedure*

A mixture of 1.52 g 2-hydroxy-4-methoxybenzaldehyde (10 mmol) in 20 cm^3 *Et*OH and 1.37 g 4-aminobenzoic acid (10 mmol) in 20 cm^3 *Et*OH was refluxed for 2 h and then cooled to room temperature. The solid which precipitated was collected, washed with cold ethanol, and recrystallized from ethanol to give 2.00 g (74%) of a yellow solid, mp 289–290°C.

All imine compounds were prepared according to this procedure.

Coupling of Amino Acids to Imines. General Procedure

A mixture of 4-[(2-hydroxybenzylidene)amino]benzoic acid, dicyclohexylcarbodiimide (*DCC*) and 4-(dimethylamino)pyridine in dichloromethane was stirred at 0°C for 3 h. Then β-alanine methyl ester hydrochloride was added to the mixture. After 2 h, temperature of the solution was allowed to rise to room temperature and the solution was stirred at this temperature for 48 h. The precipitated *N,N′*-dicyclohexylurea was removed by filtration and the filtrate was extracted with water, then cold dilute HCl solution, and then 10% $NaHCO_3$ solution. Evaporation gave a residue which was dissolved in 2 cm^3 ethanol and then hydrolyzed at room temperature with 12 cm^3 cold aqueous alcoholic sodium hydroxide (0.10 *M*) to afford the required acid as a sodium salt. The solution of acid salt was acidified with a dilute hydrochloric acid solution (0.10 *M*) to obtain the product as acid. The precipitate occurred was filtrated off and crystallized from *DMF*-water.

*The Investigation of Inhibitory Properties of **3a–3j** on the Dihydrofolate Reductase (DHFR) Activity*

DHFR and L-7,8-dihydrofolic acid were purchased from Fluka (from bovin liver, 9 U/cm^3) and Sigma, respectively. Methotrexate and related compounds were dissolved in *DMSO* making a stock solution, which was diluted approx. 100 fold in the assay. At this concentration *DMSO* had no effect on DHFR activity as tested in control experiments. All experiments were done in duplicate with 2 parallel samples. Activity and inhibition values were estimated by the linear regression analyses Determination of DHFR activity and the inhibitor studies were carried out as described by *Pastore et al*. [23], and DHFR activities were expressed in IU/cm^3. The experimental results are given below.

N-[4-[[(2-Hydroxyphenyl)methylene]amino]benzoyl]-β-alanin (**3a**)

Yield 36%, mp 158–160°C; MS (EI): $m/z = 313.27$ (molecular weight 312.33); ^{1}H NMR (300 MHz, *DMF*-d_7): $\delta = 2.71$ (t, 2H, H-7′), 3.65 (q, 2H, H-6′), 7.11 (dd, 2H, H-4, H-5), 7.49 (t, 1H, H-6), 7.57 (d, 2H, H-2′, $J = 8.51$ Hz), 7.74 (dd, 1H, H-3), 8.09 (d, 2H, H-3′, $J = 8.51$ Hz), 8.65 (t, 1H, N*H*), 9.08 (s, 1H, C*H*=N), 12.62 (b, 1H, Ar–O*H*), 13.01 (s, 1H, COO*H*) ppm; ^{13}C NMR (75 MHz, *DMF*-d_7): $\delta = 33.87$ (C-7′), 36.08 (C-6′), 112.80 (C-5), 116.83 (C-4), 117.27 (C-1), 121.41 (C-2′), 128.78 (C-3′), 133.11 (C-6), 133.75 (C-3), 150.94 (C-4′), 152.19 (C-1′), 161.11 (C-2), 165.12 (*C*H=N), 166.71 (C-5′), 173.20 (C-8′) ppm; IR (KBr): $\bar{\nu} = 3352$s (CON*H*), 3056s (Ar–H), 2929w (C–H), 1740s (*CO*N), 1625s (C=N) cm^{-1}.

N-[4-[[(2-Hydroxy-4-methoxyphenyl)methylene]amino]-benzoyl]-β-alanin (**3b**)

Yield 32%, mp 212–215°C; MS (EI): $m/z = 343.06$ (molecular weight 342.35); ^{1}H NMR (300 MHz, *DMF*-d_7): $\delta = 2.67$ (t, 2H, H-7′), 3.68 (q, 2H, H-6′), 3.91 (s, 3H, OCH_3), 6.56 (d, 1H, H-3), 6.63 (d, 1H, H-6), 7.53 (d, 2H, H-2′, $J = 8.60$ Hz), 7.64 (d, 1H, H-5), 8.05 (d, 2H, H-3′, $J = 8.60$ Hz), 8.63 (t, 1H, N*H*), 9.00 (s, 1H, C*H*=N), 12.62 (b, 1H, Ar–O*H*), 13.60 (s, 1H, COO*H*) ppm; ^{13}C NMR (75 MHz, *DMF*-d_7): $\delta = 35.78$ (C-7′), 36.05 (C-6′), 55.65 (OCH_3), 107.80 (C-6), 112.80 (C-5), 113.36 (C-1), 121.16 (C-2′), 128.77 (C-3′), 132.60 (C-4′), 134.63 (C-3), 150.91 (C-1′), 152.17 (C-4), 163.83 (C-2), 163.93 (*C*H=N), 166.01 (C-5′), 173.18 (C-8′) ppm; IR (KBr): $\bar{\nu} = 3333$s (CON*H*), 3056s (Ar–H), 2921w (C–H), 1734s (*CO*N), 1635s (C=N) cm^{-1}.

N-[4-(2-Thiophenealdimino)benzoyl]-β-alanin (**3c**)

Yield 33%, mp 168–170°C; MS (EI): $m/z = 302.38$ (molecular weight 302.35); ^{1}H NMR (300 MHz, *DMF*-d_7): $\delta = 2.86$ (t, 2H, H-7′), 3.85 (q, 2H, H-6′), 7.43 (dd, 1H, H-3), 7.54 (d, 1H, H-2′), 7.96 (d, 1H, H-2), 8.09 (d, 1H, H-4), 8.23 (d, 2H, H-3′), 8.79 (t, 1H, N*H*), 9.08 (s, 1H, C*H*=N), 12.73 (b, 1H, COO*H*) ppm; ^{13}C NMR (75 MHz, *DMF*-d_7): $\delta = 34.08$ (C-7′), 36.24 (C-6′), 113.02 (C-3), 121.15 (C-2′), 128.73 (C-3′), 131.78 (C-4′), 132.34 (C-2), 134.37 (C-4), 143.03 (C-1′), 154.01 (C-1), 155.19 (*C*H=N), 166.38 (C-5′), 173.28 (C-8′) ppm; IR (KBr): $\bar{\nu} = 3435$s (CON*H*), 3101s (Ar–H), 2935w (C–H), 1721s (*CO*N), 1633s (C=N) cm^{-1}.

N-[4-(2-Pyrrolaldimino)benzoyl]-β-alanin (**3d**)

Yield 34%, mp 173°C (decomposed); MS (EI): $m/z = 284.80$ (molecular weight 285.31); ^{1}H NMR (300 MHz, *DMF*-d_7): $\delta = 2.86$ (t, 2H, H-7′), 3.83 (q, 2H, H-6′), 6.48 (dd, 1H, H-3), 6.99 (d, 1H, H-2), 7.37 (d, 1H, H-4), 7.43 (d, 2H, H-2′, $J = 8.45$ Hz), 8.17 (d, 2H, H-3′, $J = 8.45$ Hz), 8.60 (s, 1H, C*H*=N), 8.73 (t, 3H, CON*H*CH_2), 12.02 (b, 1H, N*H*), 12.73 (b, 1H, COO*H*) ppm; ^{13}C NMR (75 MHz, *DMF*-d_7): $\delta = 35.82$ (C-7′), 36.04 (C-6′), 112.87 (C-3), 121.85 (C-2′), 128.84 (C-3′), 132.19 (C-4′), 134.21 (C-4), 137.85 (C-2), 144.19 (C-1′), 153.85 (C-1), 155.03 (*C*H=N), 166.23 (C-5′), 173.25 (C-8′) ppm; IR (KBr): $\bar{\nu} = 3371$s (CON*H*), 3070s (Ar–H), 2940w (C–H), 1676s (*CO*N), 1628s (C=N) cm^{-1}.

N-[4-[[(2-Hydroxy-1-naphtyl)methylene]amino]benzoyl]-β-alanin (**3e**)

Yield 41%, mp 235–237°C; MS (EI): $m/z = 363.53$ (molecular weight 362.39); ^{1}H NMR (300 MHz, *DMF*-d_7): $\delta =$ 2.66 (t, 2H, H-7′), 3.63 (q, 2H, H-6′), 7.08 (d, 1H, H-5), 7.40 (t, 1H, H-6), 7.58 (t, 1H, H-7), 7.78 (d, 2H, H-2′, $J = 7.50$ Hz), 7.88 (d, 1H, H-8), 8.02 (d, 1H, H-4), 8.11 (d, 2H, H-3′, $J = 7.50$ Hz), 8.59 (d, 1H, H-3), 8.68 (t, 1H, CON*H*CH_2), 9.88 (s, 1H, C*H*=N), 15.72 (b, 1H, Ar–O*H*) ppm; ^{13}C NMR (75 MHz, *DMF*-d_7): $\delta = 34.04$ (C-7′), 36.24 (C-6′), 109.39 (C-10), 120.59 (C-6), 120.65 (C-2′), 122.16 (C-7), 123.96 (C-5), 127.57 (C-9), 128.50 (C-8), 128.99 (C-3′), 129.40 (C-4), 137.47 (C-3), 157.02 (*C*H=N), 132.68 (C-1), 133.76 (C-4′), 147.50 (C-1′), 166.09 (C-2), 170.73 (C-5′), 173.23 (C-8′) ppm; IR (KBr): $\bar{\nu} = 3352$s

(CONH), 3050s (Ar–H), 2935w (C–H), 1740s (CON), 1631s (C=N) cm^{-1}.

N-[4-[[(2-Hydroxyphenyl)methylene]amino]benzoyl]-L-glutamic acid (**3f**)
Yield 40%, mp 165–167°C; MS (EI): $m/z = 371.53$ (molecular weight 370.37); ^{1}H NMR (300 MHz, *DMF*-d_7): $\delta = 2.41$ (dm, 2H, H-7′), 2.74 (t, 2H, H-8′), 4.87 (m, 1H, H-6′), 7.21 (dd, 2H, H-4, H-5), 7.67 (dd, 1H, H-6), 7.76 (d, 2H, H-2′, $J = 8.46$ Hz), 7.91 (dd, 1H, H-3), 8.31 (d, 2H, H-3′, $J = 8.46$ Hz), 8.90 (d, 1H, CONH), 9.26 (s, 1H, CH=N), 12.88 (b, 1H, Ar–OH), 13.26 (b, 2H, COOH) ppm; ^{13}C NMR (75 MHz, *DMF*-d_7): $\delta = 26.90$ (C-7′), 30.84 (C-8′), 52.88 (C-6′), 112.93 (C-4), 117.01 (C-5), 119.51 (C-6), 119.73 (C-1), 121.59 (C-2′), 129.46 (C-3′), 133.95 (C-3), 133.29 (C-4′), 151.34 (C-1′), 159.66 (C-2), 165.38 (CH=N), 173.78 (C-5′), 174.33 (C-9′), 174.41 (C-10′) ppm; IR (KBr): $\bar{\nu} = 3384$s (CONH), 3080s (Ar–H), 2933w (C–H), 1734s (CON), 1715s (COO) cm^{-1}.

N-[4-[[(2-Hydroxy-4-methoxyphenyl)methylene]amino]-benzoyl]-L-glutamic acid (**3g**)
Yield 36%, mp 222–223°C; MS (EI): $m/z = 401.26$ (molecular weight 400.39); ^{1}H NMR (300 MHz, *DMF*-d_7): $\delta = 2.22$ (dm, 2H, H-7′), 2.54 (t, 2H, H-8′), 3.19 (s, 3H, OCH_3), 4.69 (m, 1H, H-6′), 6.56 (d, 1H, H-3), 6.63 (dd, 1H, H-5), 7.53 (d, 2H, H-2′, $J = 8.53$ Hz), 7.64 (d, 1H, H-6), 8.14 (d, 2H, H-3′, $J = 8.53$ Hz), 8.72 (d, 1H, CONH), 9.00 (s,1H, CH=N), 12.84 (b, 2H, COOH), 13.58 (b, 1H, Ar–OH) ppm; ^{13}C NMR (75 MHz, *DMF*-d_7): $\delta = 26.76$ (C-7′), 35.66 (C-8′), 52.70 (C-6′), 55.60 (OCH_3), 107.30 (C-5), 100.90 (C-6), 121.34 (C-2′), 129.14 (C-3′), 134.82 (C-3), 113.52 (C-4′), 132.32 (C-1′), 151.30 (C-1), 152.39 (C-4), 164.02 (C-2), 164.30 (CH=N), 164.75 (C-5′), 166.92 (C-9′), 174.24 (C-10′) ppm; IR (KBr): $\bar{\nu} = 3371$s (CONH), 3044s (Ar–H), 2947w (C–H), 1708s (CON), 1700s (COO), 1625s (C=N) cm^{-1}.

N-[4-(2-Thiophenealdimino)benzoyl]-L-glutamic acid (**3h**)
Yield 33%, mp 190–193°C; MS (EI): $m/z = 360.74$ (molecular weight 360.39); ^{1}H NMR (300 MHz, *DMF*-d_7): $\delta = 2.34$ (dm, 2H, H-7′), 2.74 (t, 2H, H-8′), 4.83 (m, 1H, H-6′), 7.43 (t, 1H, H-3), 7.54 (d, 2H, H-2′, $J = 8.34$ Hz), 7.96 (d, 1H, H-2), 8.10 (d, 1H, H-4), 8.37 (d, 1H, H-3′, $J = 8.34$ Hz), 8.85 (d, 1H, CONH), 9.08 (s, 1H, CH=N), 12.56 (b, 2H, COOH) ppm; ^{13}C NMR (75 MHz, *DMF*-d_7): $\delta = 26.61$ (C-7′), 30.94 (C-8′), 52.56 (C-6′), 112.90 (C-3), 120.58 (C-2), 121.00 (C-2′), 129.16 (C-3′), 133.30 (C-4′), 134.26 (C-4), 144.19 (C-1′), 154.08 (C-1), 155.12 (CH=N), 166.59 (C-5′), 174.03 (C-9′), 174.22 (C-10′) ppm; IR (KBr): $\bar{\nu} = 3307$s (CONH), 3037s (Ar–H), 2935w (C–H), 1718w (CON) cm^{-1}.

N-[4-(2-Pyrrolaldimino)benzoyl]-L-glutamic acid (**3i**)
Yield 35%, mp 179°C (decomposed); MS (EI): $m/z = 343.47$ (molecular weight 343.34); ^{1}H NMR (300 MHz, *DMF*-d_7): $\delta = 2.03$ (dm, 2H, H-7′), 2.36 (t, 2H, H-8′), 4.40 (m, 1H, H-6′), 6.22 (dd, 1H, H-3), 6.76 (d, 1H, H-2), 7.06 (d, 1H, H-4), 7.25 (d, 2H, H-2′, $J = 8.39$ Hz), 7.90 (d, 2H, H-3′, $J = 8.39$ Hz), 8.34 (s, 1H, CH=N), 8.58 (d, 1H, CONH), 11.85 (b, 1H, NH), 12.42 (b, 2H, COOH) ppm; ^{13}C NMR (75 MHz, *DMF*-d_7): $\delta = 26.44$ (C-7′), 30.88 (C-8′), 52.15 (C-6′), 110.99 (C-3), 112.88 (C-2), 120.98 (C-2′), 129.19 (C-3′), 120.91 (C-4′), 129.48 (C-4), 130.95 (C-1′), 152.29 (C-1), 151.86 (CH=N), 166.96 (C-5′), 174.01 (C-9′), 174.43 (C-10′) ppm; IR (KBr): $\bar{\nu} = 3377$s (CONH) , 3048s (Ar–H), 2947w (C–H), 1727s (CON), 1689s (COO), 1628s (C=N) cm^{-1}.

N-[4-[[(2-Hydroxy-1-naphtyl)methylene]amino]benzoyl]-L-glutamic acid (**3j**)
Yield 40%, mp 181–183°C; MS (EI): $m/z = 420.93$ (molecular weight 420.43); ^{1}H NMR (300 MHz, *DMF*-d_7): $\delta = 2.23$ (dm, 2H, H-7′), 2.57 (t, 2H, H-8′), 4.70 (m, 1H, H-6′), 7.11 (d, 1H, H-4), 7.08 (d, 1H, H-5), 7.44 (t, 1H, H-6), 7.61 (t, 1H, H-7), 7.80 (d, 2H, H-2′, $J = 8.60$ Hz), 7.88 (d, 1H, H-8), 8.19 (d, 2H, H-3′, $J = 8.60$ Hz), 8.61 (d, 1H, H-3), 8.77 (d, 1H, NH), 9.87 (s, 1H, CH=N), 12.80 (b, 2H, COOH), 15.69 (b, 1H, Ar–OH) ppm; ^{13}C NMR (75 MHz, *DMF*-d_7): $\delta = 26.75$ (C-7′), 30.68 (C-8′), 52.71 (C-6′), 109.41 (C-10), 112.92 (C-6), 121.49 (C-2′), 122.16 (C-7), 123.97 (C-8), 124.63 (C-5), 128.50 (C-4), 129.41 (C-3′), 127.58 (C-9), 132.23 (C-4′), 133.77 (C-1), 137.51 (C-3), 147.74 (C-1′), 157.10 (CH=N), 166.46 (C-2), 170.75 (C-5′), 173.78 (C-9′), 174.22 (C-10′) ppm; IR (KBr): $\bar{\nu} = 3423$s (CONH), 3063s (Ar–H), 2947w (C–H), 1721s (CON), 1625s (C=N) cm^{-1}.

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