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MEDICINAL CHEMISTRY RESEARCH

Novel Pirfenidone Analogs as Antifibrotic Agents: Synthesis and Antifibrotic Evaluation of 2-Pyridones and Fused Pyridones

Magda M. F. Ismail[†] * and Eman Noaman[‡]

[†]Department of Pharmaceutical Chemistry, College of Pharmacy (Girls), Al-Azhar University, Nasr City, Cairo, Egypt.

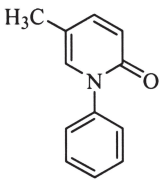
[‡]Department of Radiation Biology, Natural Center for Radiation Research and Technology, Atomic Energy Authority, Cairo, Egypt.

Abstract. A new series of substituted 1-(2-ethylphenyl)-2-oxo-1,2-dihydropyridine-3-carbonitriles have been synthesized. Moreover, substituted bicyclic derivatives *e.g.* thieno[3,4-*c*]pyridone, pyrido[3,4-*c*]pyridone, benzo[*c*]pyridone and tricyclic derivatives, chromeno[3,4-*c*]pyridones have been prepared and evaluated for their antifibrotic activity. Among the tested compounds, compounds **4d** and **5a** exhibited potent antifibrotic activity without harmful side effects on liver and kidney functions. Detailed synthesis, spectroscopic and biological data are reported.

*Corresponding author: email: magda_f_ismail@yahoo.com

Fibrosis is a debilitating condition for which there is currently no effective therapy and for which patient prognosis is poor. Numerous agents cause lung damage that can progress to fibrosis. It is believed that injury to the epithelium and basement membranes is a requisite step in the etiology of pulmonary fibrosis, after which several cell types, including inflammatory and immune cells as well as fibroblasts, migrate to and / or proliferate in areas of injury and release numerous cytokines that lead to further cell recruitment, inflammation and eventual matrix remodeling. This culminates in an overproduction of collagen and other matrix components characteristic of fibrosis. [1] Pirfenidone (PFD), 5-methyl-1-phenyl-2(1*H*) pyridone is an agent with demonstrated antifibrotic activity in several organs in experimental animals, including the lung, kidney and uterus. A phase II clinical study showed PFD to be a promising agent for treatment of idiopathic pulmonary fibrosis, initiated in mice treated with cyclophosphamide, [2] amiodarone [3] or bleomycin. [4-9] PFD may prove beneficial for a range of fibrotic conditions through both anti-inflammatory and antifibrotic mechanisms. [1] The reported antifibrotic activity of PFD prompted us to synthesize new series of 2-pyridones and fused pyridones (bi- and tricyclic derivatives) as analogs to PFD.

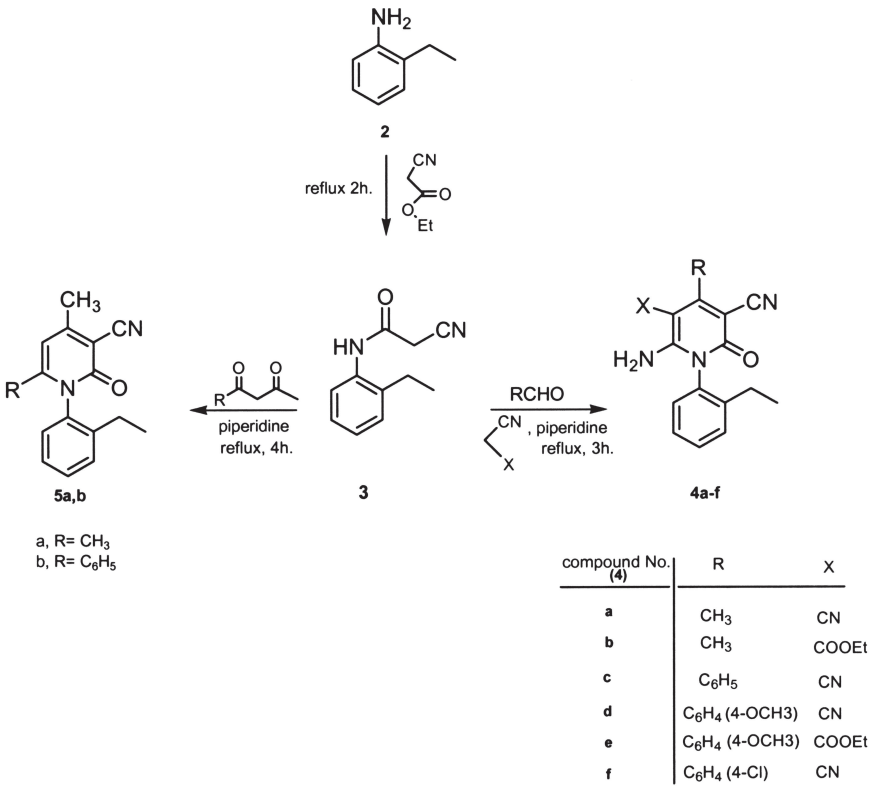
Herein, we report the preparation and pharmacological evaluation of PFD analogs to explore and compare the antifibrotic activity of 2-pyridone and fused pyridone derivatives on lung fibrosis of rats. Another objective was to assess the effect of 4-alkyl/aryl substituent and to determine the electronic effect of 4'-substituent on the 4-phenyl group of pyridone nucleus to assess a correlation with the antifibrotic activity.



Pirfenidone 1

Materials and Methods

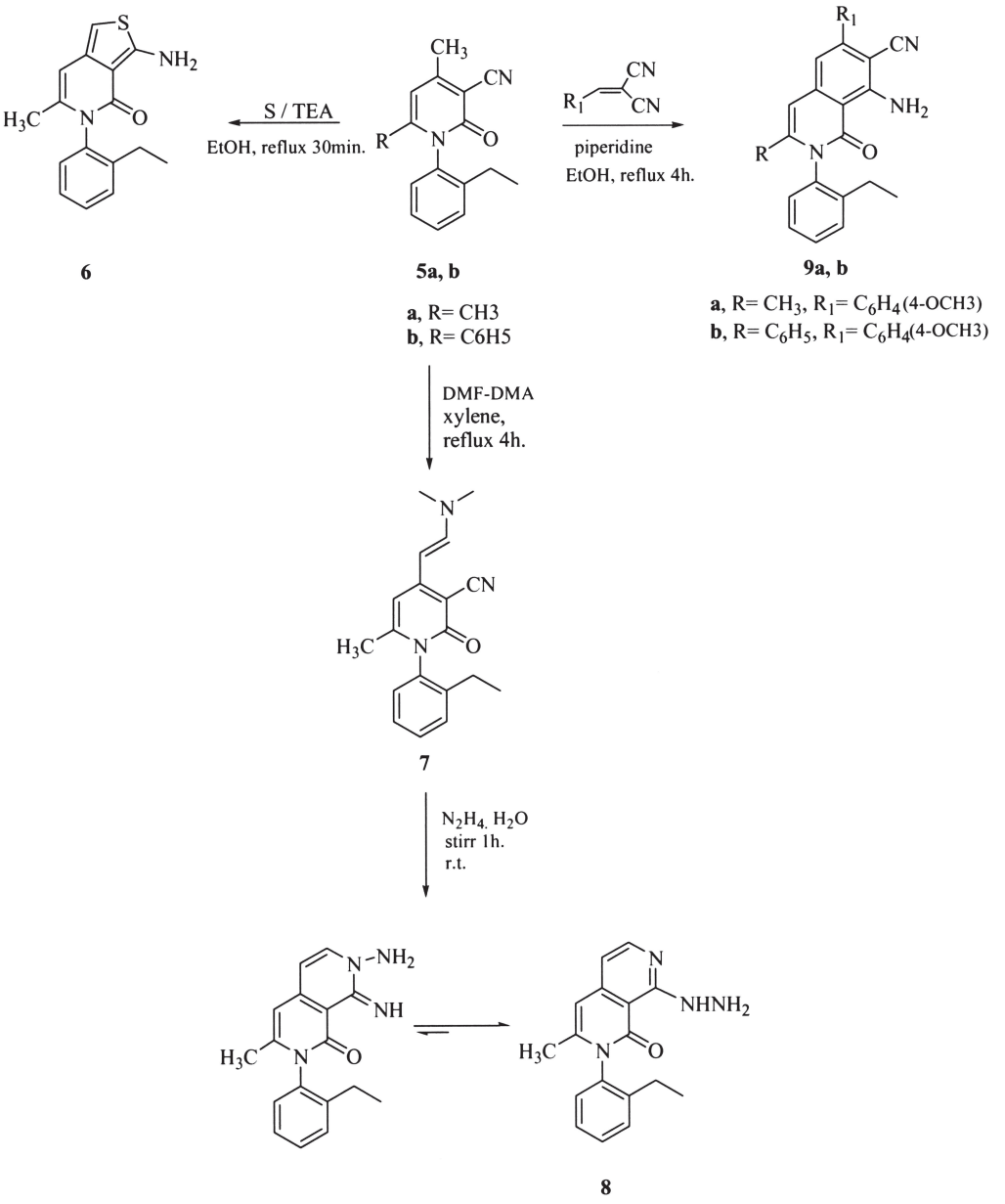
Scheme 1 outlines the synthetic pathway used to obtain compounds **4a-f** and **5a, b.** The starting material, 2-ethyl cyanoacetanilide **3** was prepared via reaction of 2-ethylaniline **2** with ethyl cyanoacetate. Upon treatment of compound **3** with appropriate aldehyde and ethyl cyanoacetate or malononitrile in the presence of catalytic amount of piperidine, afforded 6- amino - 4 - alkyl / aryl - 5 - substituted - 2- pyridone - 3 – carbonitriles **4a-f**. Cyclocondensation of compound **3** with acetylacetone or benzoylacetone in the presence of catalytic amount of piperidine yielded 4, 6-disubstituted -2-pyridone-3-carbonitriles **5a, b** in good yields (Scheme 1, Table 1**).** Reaction of **5a** with elemental sulfur in ethanolic triethylamine solution furnished thieno[3,4-*c*]pyridone **6**. Treatment of **5a** with dimethylformamide- dimethylacetal (DMF-DMA) in xylene produced enamine derivative **7** which underwent hydrazinolysis followed by cycloaddition reaction to yield pyrido[3,4-*c*]pyridone **8**. Application of Michael addition of **5a,b** to 4-methoxy-benzylidenemalononitrile in the presence of piperidine afforded benzo[*c*]pyridone **9a,b** via loss of HCN molecule [10] (Scheme 2, Table 1). Furthermore, Perkin reaction was carried out by reacting **3** with salicylaldehyde in Ac_2O/NaOAc to give the corresponding chromen-2-one **10**, while reaction of **3** with salicylaldehyde in NH_4OAc yielded 2-iminochromene **11**. The chromene derivatives, **10** and **11** were further reacted with malononitrile in NH_4OAc to afford the corresponding chromeno[3,4-*c*]pyridones **12a,b** (Scheme 3, Table1**).**



Scheme 1. Synthesis of cyano derivatives.

Antifibrotic Evaluation

Finding an optimum substitution on pyridin-2-one nucleus of the active lead compound (PFD) has much value in drug design for maximization of drug potency and reduction of side effects. Lung content of hydroxy proline (HP) was determined as a biochemical index of fibrosis (Figure 1). Results are expressed as umol of HP / lung of pulmonary fibrotic models pre- and post-treated with our synthesized PFD analogs: **4c, 4d, 4f, 5a, 6** and **12a.**



6 **5a, b** **9a, b**

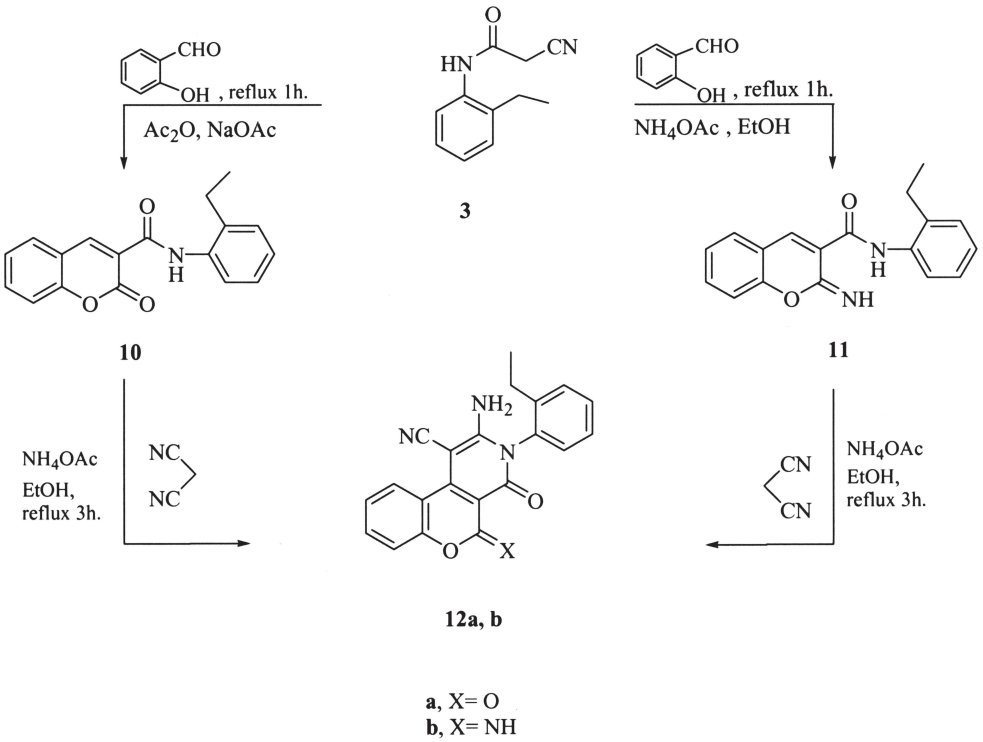
a, R= CH3
b, R= C6H5

a, R= CH_3, R_1= C_6H_4(4-OCH3)
b, R= C_6H_5, R_1= C_6H_4(4-OCH3)

7

8

Scheme 2. Synthesis of benzo[*c*]pyridone



Scheme 3. Synthesis of chromeno[3,4-*c*]pyridones

Table 1. Physical properties and molecular formulae of the synthesized compounds.

Comp.	X	R	Solvent	M.p.(°C)	Yield %	Formulae*
3	-	-	Benzene-Pet ether	130-133	80	$C_{11}H_{12}N_2O$
4a	CN	CH_3	Ethanol	213-215	45	$C_{16}H_{14}N_4O$
4b	$COOC_2H_5$	CH_3	n.Hexane	50-53	85	$C_{18}H_{19}N_3O_3$
4c	CN	C_6H_5	Ethanol	240-243	70	$C_{21}H_{16}N_4O$
4d	CN	C_6H_4-4-OCH_3	Ethanol	233-235	52	$C_{22}H_{18}N_4O_2$
4e	$COOC_2H_5$	C_6H_4-4-OCH_3	Benzene-Pet. ether	110-112	55	$C_{24}H_{23}N_3O_4$
4f	CN	C_6H_4-4-Cl	Ethanol	290-293	64	$C_{21}H_{15}ClN_4O$
5a	-	CH_3	Methanol	178-180	80	$C_{16}H_{16}N_2O$
5b	-	C_6H_5	Methanol	155-157	50	$C_{21}H_{18}N_2O$
6	-	-	$CHCl_3$-Pet.ether	115-117	55	$C_{16}H_{16}N_2OS$
7	-	-	Methanol	140-143	66	$C_{19}H_{21}N_3O$
8	-	-	Ethanol	188-190	40	$C_{17}H_{18}N_4O$

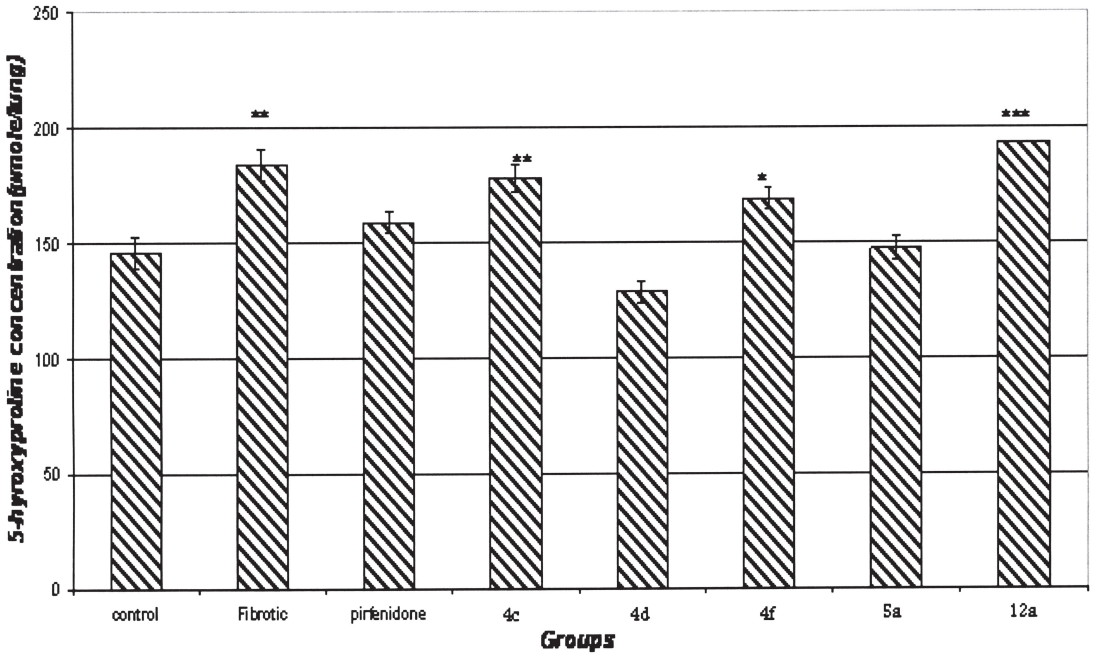
Continues.

Table 1. Continued.

9a	-	CH_3	Methanol	153-155	73	$C_{26}H_{23}N_3O_2$
9b	-	C_6H_5	Benzene-Pet. ether	120-122	54	$C_{31}H_{25}N_3O_2$
10	-	-	Methanol	140-142	64	$C_{18}H_{15}NO_3$
11	-	-	Methanol	120-122	69	$C_{18}H_{16}N_2O_2$
12a	O	-	Dioxane	>300	70	$C_{21}H_{15}N_3O_3$
12b	NH	-	Dioxane	>300	79	$C_{21}H_{16}N_4O_2$

*Analyzed for C, H, N; results were within $\pm$ 0.4% of the theoretical values for formulae given

Figure (1): represented 5-hyroxyproline (µmole/Lung) in different groups of animals



*Significant difference from control group at, $p < 0.05$.

**Significant difference from control group at, $p < 0.01$.

***Significant difference from control group at, $p < 0.001$.

With regard to the 4 substituent, it was found that compound **4d** possessed 4-methoxyphenyl group displayed the most potent antifibrotic activity compared to PFD and the negative control group which is proved by the histopathological study (figure 2). Their counterparts **4c** and **4f** carrying 4-phenyl and 4-chlorophenyl group respectively demonstrated nonsignificant antifibrotic activity (HP=168.8-178.03umol/lung). In case of 4-phenyl-pyridin-2-one **4c**, it is not only lacked the antifibroic activity but also elicited undesirable side effects as shown from liver and kidney functions (Table 2).

Table 2: Liver and kidney function parameters in rats under different treatment conditions:

Group	AST (ug/l)	ALT (ug/l)	Alk.phosphatase (ug/l)	Creatinine (mg/dL)	Urea (mg/dL)	Lactate dehydrogenase (LDH) (ug/l)
Control	182.40 ±14.54 100%	30.00±3.51 100%	1060.75 ± 107.41 100%	0.58±0.03 100%	40.75±1.89 100%	929.60±67.63 100%
Fibrotic	128.67± 19.37 70.54%	21.00±3.06 70%	971.00±98.88 91.54%	** 0.43± 0.03 74.14%	47.67±6.74 116.98%	802.33± 228.05 86.31%
4c	*** 557.00±32.00 305.37%	*** 75.50±9.50 251.67%	871.33±27.28 82.14%	0.70±0.15 120.69%	43.00±13.65 105.52%	811.00± 81.00 87.24%
4d	** 91.33±8.76 50.07%	** 9.00±2.08 30.00%	* 661.00±54.00 62.31%	0.63±0.09 108.62%	*** 15.00±2.89 36.81%	850.00±15.00 91.44%
4f	138.33±6.67 75.84%	21.67±1.67 72.23%	1192.25±127.96 112.39%	0.75±0.06 129.31%	39.00±6.92 95.71%	1080.00±104.76 116.18%
5a	144.00±16.26 78.95%	29.00±4.58 96.67%	1222.00±222.00 115.28%	0.60±0.00 103.45%	*** 24.33±2.33 59.71%	861.00±72.02 92.62%
12a	*** 139.00±0.00 76.21%	*** 26.00±0.00 88.67%	*** 1065.00±0.00 100.40%	0.40±0.00 68.97%	26.00±0.00 63.80%	*** 1300.00±0.00 139.85%

Data is representing 6 animals/ group (mean ± S.E.). *Significant difference from control group at, $p < 0.05$.

**Significant difference from control group at, $p < 0.01$. ***Significant difference from control group at, $p < 0.001$.

In our study and with these results we suggest that 4-methoxyphenyl group, i.e. electron-releasing group played a significant role in the antifibrotic activity of **4d** (HP=128.3umol / lung) whereas in compound **4f** having 4-chlorophenyl moiety, the electron-withdrawing property of Cl group may increase the hydroxy proline level (HP=168.8umol / lung). Other derivative which possessed an impressive antifibrotic activity (HP=147.1umol / lung) was the N-aryl-4, 6-dimethyl-2-oxo-pyridine -3-carbonitrile **5a**. Once again the electron-releasing effect of 4- methyl group confers enhanced antifibrotic activity comparable to that of PFD group (HP=158.39 umol / lung).

Molecular modifications of pyridine-2-one nucleus led to bicyclic and tricyclic PFD analogs by ring condensation at 3 and 4 positions e.g. thieno[3,4-*c*]pyridone **6** which demonstrated high rate of mortality and was excluded from the biochemical evaluation, tricyclic PFD analog, chromeno[3,4-*c*]pyridine **12a** unfortunately showed high fibrotic potential where HP=193.07 umol/lung. With the exception of compound **12a**, all the tested compounds revealed non toxic results on liver and kidney functions. Analysis of BALF 4 days after bleomycin administration revealed severe inflammatory response characterized by an increase in LDH activity and protein content along with slight increase in macrophages, neutrophils and eosinophils (measured as total cell count) that are indicative of alveolar damage. With the exception of protein content, PFD showed mild improvement in the other parameters. All the tested compounds displayed reduction in LDH activity, total cell count and protein concentration more than the reference PFD (Table 3).

Experimental

Synthesis

Elemental analyses (C, H, N) were performed on Perkin-Elmer 2400 analyzer (Perkin-Elmer, Norwalk, CT, USA) at the Microanalytical Unit of Cairo University. All compounds were within $\pm$ 0.4% of the theoretical values. Melting points were determined in open capillaries on an electrothermal LA 9000 Series (Electrothermal Engineering Ltd., Essex, UK) and are uncorrected. TLC chromatography was performed on precoated silica gel 60F 254 plates (Merck CO., Sofia, Bulgaria). Infrared spectra were recorded on Pye

Table 3: BALF measurements following intratracheal administration of water or bleomycin to rats under different conditions

Groups	LDH activity Levels (U/ L)	Total cells count (log_{10}) # (Macrophages+Neutrophils+Eosinophils)	Protein concentration (µg/ml)
Control	25.00 ± 3.52	5.6 ± 0.34	87± 5.3
Fibrotic	141.67± 21.1***	6.24 ± 0.25	233± 6.2***
Pirfenidone	132.52± 22.2***	6.05 ± 0.11	159± 5.5***
4c	33.67 ±0.84*	5.84 ± 0.14	74± 4.4
4d	36.00 ± 2.22**	5.82 ± 0.16	83± 8.4***
4f	26.00 ± 1.44	5.78 ± 0.09	80± 5.3***
5a	67.3 ± 8.90*	5.76 ± 0.08	130± 6.1***
12a	88.25 ± 1.12***	6.08 ± 0.02	147± 7.1***

Data under log_{10}

*Significant difference from control group at, $p < 0.05$.

**Significant difference from control group at, $p < 0.01$.

***Significant difference from control group at, $p < 0.001$

Unicam SP 1000 IR spectrophotometer (Thermoelectron CO., Egelsbach, Germany). ^{1}HNMR spectra were recorded on Varian Gemini EM-300 MHz NMR spectrophotometer (Varian CO., Fort Collins, USA). DMSO-d_6 was used as solvent, TMS was used as internal standard and chemical shifts were measured in δ ppm. Mass spectra were recorded on Varian MAT 311-A 70 *e.v.* (Varian CO., Fort Collins, USA).

PFD was obtained from Marnac, Inc. (Dallas, TX); trans-4-hydroxy-*L*-proline (Sigma, Germany) and bleomycin sulphate (Nippon kayoku, Tokyo, Japan). Unless otherwise stated, all other chemicals and reagents were of analytical grade and were obtained from Sigma Chemical Co., Germany.

2-Cyano-N-(2-ethylphenyl) acetamide (3)

A mixture of 2-ethyl aniline (**2**) (1.21 g., 0.01mol) and ethyl cyanoacetate (1.13 g, 0.01mol) was refluxed for two h., concentrated and cooled. The obtained solid was filtered and crystallized with benzene and pet. ether 3:2, 50ml) (Table 1). IR (cm^{-1}): 3180 (NH), 2255 (CN), 1703 (CO). ^{1}HNMR (DMSO-d_6, 300 M*Hz*): δ 1.09 (t, 3H, CH_2CH_3), 2.23 (q, 2H, CH_2CH_3), 3.85 (s, 2H, CH_2CN), 7.3-7.5 (m, 4H, aromatic), 9.45 (s, 1H, NH D_2O exchangeable).

6-Amino-5-cyano- or ethoxycarbonyl-1-(2-ethylphenyl)-2-oxo-4-substituted-1,2-dihydropyridine-3-carbonitrile (4a - f)

A mixture of the starting material **3** (1.88 g, 0.01 mol), appropriate aldehyde (0.01 mol) and malononitrile or ethyl cyanoacetate (0.01 mol) in ethanol (30ml) with catalytic amount of piperidine was refluxed for 3 h. The resulting solid was filtered and crystallized with appropriate solvent (Table 1).

4a: IR (cm^{-1})**:** 3324, 3215 (NH_2), 2218 (CN), 1670 (CO); ^{1}HNMR (DMSO-d_6, 300 M*Hz*): δ 1.07 (t, 3H, CH_2CH_3), 2.24 (q, 2H, CH_2CH_3), 2.43 (s, 3H, CH_3), 7.20-7.49 (m, 4H, Ar-H), 7.73 (s, 2H, NH_2).

4b: IR (cm^{-1})**:** 3356, 3200 (NH_2), 2211 (CN), 1742 (COO), 1635 (CON); ^{1}HNMR (DMSO-d_6, 300 M*Hz*): δ 0.99 (t, 3H, CH_3-ester, *J*= 6.9*Hz*), 1.09 (t, 3H, CH_2CH_3), 2.10 (s, 3H,

CH_3), 2.59 (q, 2H, CH_2CH_3), 4.27 (q, 2H, CH_2-ester, *J*= 6.9*Hz*), 7.17-7.52 (m, 4H, Ar-H), 9.69 (s, 2H, NH_2).

4c; IR (cm^{-1})**:** 3423, 3353 (NH_2), 2202 (CN), 1684 (CO); ^{1}HNMR (DMSO-d_6, 300 M*Hz*): δ 1.13 (t, 3H, CH_2CH_3), 2.39 (q, 2H, CH_2CH_3), 7.10-8.20 (m, 9H, Ar-H), 9.78 (s, 2H, NH_2).

4d: IR (cm^{-1})**:** 3423, 3353 (NH_2), 2202 (CN), 1684 (CO); ^{1}HNMR (DMSO-d_6, 300 M*Hz*): δ 1.14 (t, 3H, CH_2CH_3), 2.59 (q, 2H, CH_2CH_3), 3.87 (s, 3H, OCH_3), 7.15 (d, 2H, Ar-H, *J* = 9 *Hz*), 8.01 (d, 2H, Ar-H, *J* = 9 *Hz*), 7.24-8.22 (m, 4H, Ar-H), 9.80 (s, 2H, NH_2).

4e: IR (cm^{-1})**:** 3413, 3282 (NH_2), 2212 (CN), 1717 (COO), 1636 (CON); ^{1}HNMR (DMSO-d_6, 300 M*Hz*)**:** δ 1.12 (t, 3H, CH_2CH_3), 1.17 (t, 3H, CH_3-ester, *J*= 6.9 *Hz*), 2.59 (q, 2H, CH_2CH_3), 3.87 (s, 3H, OCH_3), 4.31 (q, 2H, CH_2-ester, *J*= 6.9*Hz*), 7.12 (d, 2H, Ar-H, *J* = 9*Hz*), 8.05 (d, 2H, Ar-H, *J* = 9*Hz*), 6.98-8.30 (m, 4H, Ar-H), 8.60 (s, 2H, NH_2).

4f: IR (cm^{-1})**:** 3324, 3215 (NH_2), 2205 (CN), 1667 (CO).

1-(2-Ethylphenyl)-4-methyl-2-oxo-6-substituted-1,2-dihydropyridine-3-carbonitriles (5a,b)

Equimolar amounts of compound **3** (1.88 g, 0.01 mol) and acetylacetone (1.00 g, 0.01mol) or benzoylacetone (1.62 g, 0.01mol) were refluxed in ethanol (25ml) in the presence of piperidine (0.5ml) for four h. After trituration with ethanol, the solid was filtered and recrystallized with methanol (Table 1).

5a: IR (cm^{-1})**:** 2220 (CN), 1650 (CO); ^{1}HNMR (DMSO-d_6, 300 M*Hz*)**:** δ 1.07 (t, 3H, CH_2CH_3), 1.91(s, 3H, CH_3), 2.28 (q, 2H, CH_2CH_3), 2.41 (s, 3H, CH_3), 6.49 (s, 1H, pyridone-H), 7.19-7.47 (m, 4H, Ar-H).

5b: IR (cm^{-1})**:** 2214 (CN), 1651 (CO); ^{1}HNMR (DMSO-d_6, 300 M*Hz*)**:** δ 1.12 (t, 3H, CH_2CH_3), 2.01 (s, 3H, CH_3), 2.33 (q, 2H, CH_2CH_3), 6.70 (s, 1H, pyridone-H), 7.20-7.72 (m, 9H, Ar-H).

***3-Amino-5-(2-ethylphenyl)-6-methyl-4-oxo-4,5-dihydro-thieno[3,4-c]pyridine* (6)** [11]

The isolated solid was recrystallized with mixture of chloroform and petroleum ether (4:1, 40 ml).

IR (cm^{-1})**:** 3397, 3232 (NH_2), 1650 (CO); ^{1}HNMR (DMSO-d_6, 300 M*Hz*): δ 1.07 (t, 3H, CH_2CH_3), 2.28 (q, 2H, CH_2CH_3), 2.40 (s, 3H, CH_3), 6.49 (s, 1H, H-7), 7.19-7.47 (m, 7H, 5Ar-H + 2H, NH_2).

***1-(2-Ethylphenyl)-4-(2-diethylaminovinyl)-6-methyl-2-oxo-1,2-dihydropyridine-3-carbonitriles* (7)**

To a solution of compound **5a** (2.52 g, 0.01mol) in xylene (30ml), DMF-DMA (1.19 g, 0.01mol) was added. The mixture was refluxed for 4h, cooled and solid was filtered and crystallized with methanol (Table 1).

IR (cm^{-1})**:** 2193 (CN), 1633 (CO); ^{1}HNMR (DMSO-d_6, 300 M*Hz*): δ 1.05 (t, 3H, CH_2CH_3), 2.25 (q, 2H, CH_2CH_3), 2.49 (s, 3H, CH_3), 2.66 (s, 6H, N $(CH_3)_2$), 3.85 (d, 1H, CH=*CH*-N), 5.06 (d, 1H, *CH*=CH-N), 6.56 (s, 1H, pyridone-H), 6.99-7.76 (m, 4H, Ar-H).

***3-Amino-6-(2-ethylphenyl)-4-imino-7-methyl-5-oxo-5,6-dihydropyrido[3,4-c]pyridine* (8)**

To a solution of compound **7** (3.07 g, 0.01mol) in absolute ethanol (30ml), hydrazine hydrate (2ml, 99%) was added with stirring for 1 h at room temperature, the obtained product was filtered, dried and crystallized with ethanol (Table 1). IR (cm^{-1})**:** 3404, 3295, 3197 (NH_2,NH), 1655 (CO), 1613 (C=N); ^{1}HNMR (DMSO-d_6, 300 M*Hz*): δ 1.18 (t, 3H, CH_2CH_3), 1.69 (bs, 2H, NH_2), 1.87(s, 3H, CH_3), 2.40 (q, 2H, CH_2CH_3), 3.01 (bs, 1H, NH), 5.41 (d, 1H, H-2), 6.15 (s, 1H, H-8), 7.07 (d, 1H, H-1), 7.27-7.45 (m, 4H, Ar-H).

***3-Amino-4-cyano-1-(2-ethylphenyl)-5-(4-methoxyphenyl)-8-substituted-2-oxo-1,2-dihydrobenzo[c]pyridine* (9a,b)**

A mixture of compound **5a** or **5b** (0.01mol), 4-methoxy-benzylidene-malononitrile (1.84g, 0.01mol) and piperidine (0.5ml) in ethanol (30ml) was refluxed for 4h. The reaction mixture was cooled and poured onto crushed ice acidified with HCl. The solid product was filtered off and crystallized with the appropriate solvent (Table 1). **9a**: IR (cm^{-1})**:** 3343,

3213 (NH_2), 2217 (CN), 1652 (CO); ^{1}HNMR (DMSO-d_6, 300 M*Hz*): δ 1.21 (t, 3H, CH_2CH_3), 2.16 (q, 2H, CH_2CH_3), 2.49 (s, 3H, CH_3), 3.79 (s, 3H, OCH_3), 6.21 (s, 1H, H-7), 6.84 (s, 1H, H-6), 7.21-7.67 (m, 8H, Ar-H), 7.92 (s, 2H, NH_2). **9b**: IR (cm^{-1})**:** 3340, 3212 (NH_2), 2215 (CN), 1656 (CO); ^{1}HNMR (DMSO-d_6, 300 M*Hz*) δ 1.12 (t, 3H, CH_2CH_3), 2.33 (q, 2H, CH_2CH_3), 3.86 (s, 3H, OCH_3), 6.10 (s, 1H, H-7), 6.70 (s, 1H, H-6), 6.90-7.71 (m, 13H, Ar-H), 8.00 (s, 2H, NH2); **MS:** m/z 473 (M+2, 1.8%), 432 (43.2%), 415 (100%).

3-[N-(2-Ethylphenyl)-carboxamido] - chromene-2-one **(10)**

To a solution of **3** (1.88 g, 0.01mol) in acetic anhydride (20ml), salicylaldehyde (1.22 g, 0.01mol) and fused sodium acetate (0.8 g, 0.01 mol) were added. The mixture was refluxed for 1h, cooled and the solid was filtered and crystallized with methanol (Table 1). IR (cm^{-1})**:** 3476 (NH), 1751 (COO), 1672 (CON); ^{1}HNMR (DMSO-d_6, 300 M*Hz*): δ 1.05 (t, 3H, CH_2CH_3), 2.35 (q, 2H, CH_2CH_3), 7.20-7.63 (m, 8H, Ar-H), 7.71(s, 1H, H-4), 10.08 (s, 1H, NH).

3-[N-(2-Ethylphenyl)-carboxamido] -2-imino-2H-chromene **(11)**

A mixture of compound **3** (1.88 g, 0.01mol), salicylaldehyde (1.22 g, 0.01mol) and anhydrous ammonium acetate (1.15 g, 0.015mol) was refluxed in ethanol (30ml) for 1h. The solid product was filtered and crystallized with methanol (Table 1). **IR** (KBr, cm^{-1})**:** 3299, 3117 (2 NH), 1677 (CO), 1610 (C=N); **^{1}HNMR** (DMSO-d_6, 300 MHz): δ 1.18 (t, 3H, CH_2CH_3), 2.67 (q, 2H, CH_2CH_3), 7.08-8.24 (m, 9H, 8 Ar-H + NH), 8.59 (s, 1H, H-4), 9.29 (bs, 1H, NH).

2-Amino-3-(2-ethylphenyl)-4,5-dioxo-4,5-dihydro-3H-chromeno[3,4-c]pyridine-1-carbonitrile **(12a) and** ***2-amino-3-(2-ethylphenyl)-5-imino-4-oxo-4,5-dihydro-3H-chromeno[3,4-c]pyridine-1-carbonitrile*** **(12b)**

Equimolar amounts of compounds **10** or **11**, malononitrile (0.66 g, 0.01mol) and anhydrous ammonium acetate (1.15 g, 0.015mol) were refluxed in ethanol (30ml) for 3h. The solid product was separated by filteration and crystallized with dioxane (Table 1). **12a:** IR (cm^{-1}): 3339, 3189 (NH_2), 2208 (CN), 1700(COO), 1647 (CON); ^{1}HNMR (DMSO-d_6, 300

MHz): δ 1.15 (t, 3H, CH_2CH_3), 2.43 (q, 2H, CH_2CH_3), 6.27 -7.75 (m, 8H, Ar-H), 8.97 (s, 2H, NH_2); MS: *m/z* 355(M-2, 0.49%), 300 (1.63%), 198 (100%). **12b:** IR (cm^{-1}): 3386, 3348, 3181 (NH_2, NH), 2200 (CN), 1680 (CO), 1644 (C=N); ^{1}HNMR (DMSO-d_6, 300 MHz): δ 1.10 (t, 3H, CH_2CH_3), 2.42 (q, 2H, CH_2CH_3), 6.27-7.58 (m, 8H, Ar-H), 7.79 (s, 2H, NH_2), 8.96 (s, 1H, NH); MS: *m/z* 355 (M-1, 0.99%), 306 (11.43%), 251 (100%).

Antifibrotic activity

Male Swiss albino rats (100-110 gm) were obtained from the Egyptian Organization for Biological Product and Vaccines at Giza, Egypt and were used throughout the present experiments. Animals were housed in cages under good ventilation and illumination conditions; they had access to unlimited water and standard rodent chow or the same chow containing 0.5% PFD (w/w). Animal maintenance and treatments were conducted in accordance with the National Institute of Health Guide for Animal, as approved by Institutional Animal Care and Use Committee (IACUC). To produce pulmonary fibrosis, animals received endotracheally, by the transoral route, a single sublethal dose of bleomycin dissolved in 0.9% NaCl (0.9 unit per animal). Control animals were subjected to the same protocol but received the same volume of intratracheal saline instead of bleomycin. Tracheal instillation was carried out under light anesthesia. Seven days after endotracheal bleomycin and/or compounds administration, the animals were scarified after light anesthesia with diethyl ether to collect blood by heart puncture. Bronchoalveolar lavage was performed and lungs were weighed and processed separately for biochemical and histological studies as indicated below. The 7 day after bleomycin was selected as the approximate time for maximal rate of collagensynthesis.
The animals were randomly divided into nine groups .Group (1) vehicle; group (2): vehicle + bleomycin; group (3) **PFD** + bleomycin group (4) **4c** + bleomycin group (5): **4d**+bleomycin; group (6) **4f** + bleomycin; group (7) **5a** + bleomycin; group (8) **6** + bleomycin; group (9) **12a** + bleomycin. Treatments (vehicle, PFD or tested compounds) were administered orally by gavage on a daily basis (at 9:00 h) started 3 days prior to intratracheal instillation of bleomycin or saline up to the end of the experiment (7 days

postinstillation). Oral compounds **4c**, **4d**, **4f**, **5a**, **6** and **12a** administered by stomach intubation tube. The oral dose was 0.04g/ day for 7 doses for the tested compounds and 0.5% (w/w) for PFD, given as repeated doses in a final volume of 1 mL of 1% carboxymethyl cellulose suspension as vehicle.

Measurement of the Biochemical Parameters

Animals were fasted overnight prior to collection of each sample. Samples were collected after 7 days from beginning of treatments. The whole blood was collected by heart puncture after light anesthesia using heparinized syringes. The separated plasma from heparinized blood was used for the determination Liver function parameters including, alanine aminotransferase (ALT), [12] aspartate aminotransferase (AST), [13] alkaline phosphatase (ALP) [14] and lactate dehydrogenase (LDH) [15] as well as creatinine and urea concentration were estimated in plasma according to the methods of Lustgasten *et al* [16] and Kaplan *et al* [17] respectively (Table 2).

Preparation of lung tissue

For determination of indicators of pulmonary fibrosis, each animal was anesthetized with light anesthesia, chest was opened and blood was collected by heart puncture at 7 days post- tested compounds treatment, a thoracotomy was performed, and the trachea was exposed and cannulated. The right bronchus was ligated, and the right lung was removed, weighed, frozen in liquid nitrogen, and stored at -80°C until determination of hydroxyproline content. The left lung was inflated with 10% neutral-buffered formalin to a pressure of 20-cm H_2O for 1 h. The trachea was then ligated, and the lung was removed and placed in formalin. Sections from the upper, middle, and lower portions of the lung were dehydrated and embedded in paraffin, and 5-µm sections were cut and stained with hematoxylin and eosin for histological evaluation.

Histopathology

To evaluate morphological damage, a disease index was computed for each animal as described in Card *et al* [18] with the evaluator unaware of the animal treatments. The disease

index, which quantified thickening and cellular infiltration, was calculated as the mean of the values for equal numbers of sections taken from the upper, middle, and lower lung (Figure 2).

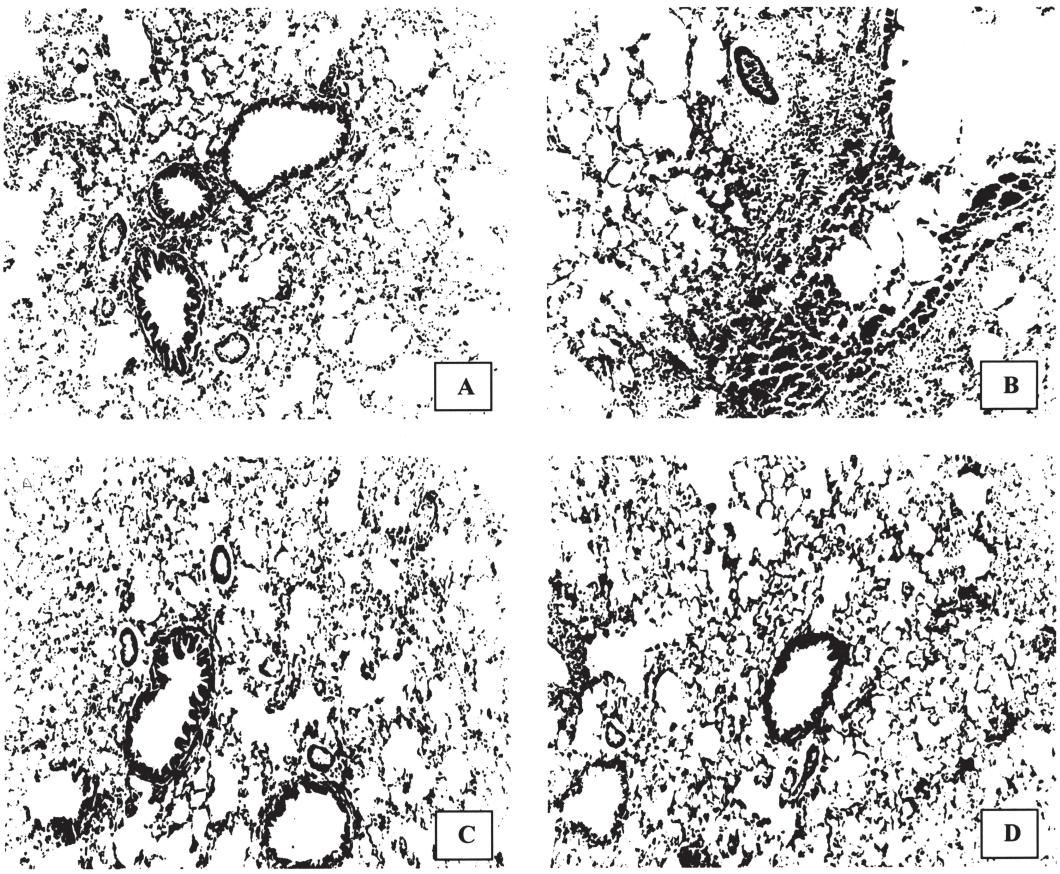


Figure 2. Hematoxylin-and-eosin-stained rat lung sections.
(**A**) Dietary controlled rats.
(**B**) Rats treated with Bleomycin.
(**C**) Rats treated with Bleomycin and drug **IIc.**
(**D**) Rats treated with Bleomycin and drug **IVb**.
Scale bars= 100 µm.

Hydroxyproline measurement.

The lung content of hydroxyproline was determined as a biochemical index of fibrosis (figure 1). Lung hydroxyproline content was measured as follows: Samples were homogenized and then hydrolyzed in 5 ml of 6 N HCl for 72 h at 110°C. The hydrolysate was then neutralized with 2.75ml of 10 M NaOH. Aliquots (0.2 mL) were analysed for hydroxyproline content after the addition of 0.6 mL of chloramineT, 0.6 ml of Ehlrich's reagent . Samples were read for absorbance at 550 nm in a spectrophotometer. Results are expressed as μg of hydroxyproline per lung according to method of Lindenschmidt and Witschi. [19]

Bronchoalveolar lavage.

For determination of inflammation following bleomycin administration, each animal was killed by injection of sodium pentobarbital (300 mg/kg i.p.), the lungs and trachea were removed en bloc, and two successive 5.0-ml aliquots of warm (37°C) phosphate-buffered saline (PBS, pH 7.4) were infused and slowly withdrawn from the lungs through a cannula inserted into the trachea. Recovered bronchoalveolar lavage fluid (BALF) volumes routinely measured between 8.0- and 9.0-ml. BALF were centrifuged at 1000 x g for 10 min at 4°C, after which the supernatants were removed and saved for determination of lactate dehydrogenase (LDH) activity and protein content. The cell pellets were resuspended in PBS, and total cell counts were determined on a haemocytometer. Concurrently, alveolar macrophage viabilities were assessed by trypan blue exclusion. In addition, BALF cell samples were centrifuged onto microscope slides using a Shandon Cytospin 2 centrifuge (Shandon Southern Products Limited, Runcorn, Cheshire, England), stained with Wright's stain, and differential cell counts determined under a light microscope by counting 300 to 400 cells per animal. Absolute cell numbers were $\log_{10}$ transformed for data analysis and presentation The LDH activity of the BALF supernatants was determined using a commercially available kit (Sigma LDH procedure No. DG1340-UV), and the BALF protein content was determined by the method of Lowry *et al* [20] using bovine serum albumin (BSA) as the standard.

Statistical Analysis Student's *t* test was used for analysis of the biochemical parameters. The data were expressed as mean ± standard error. Statistical analysis was done according to Snedecor and Cochron. [21]

In conclusion, among the tested compounds, compounds **3d** and **4a** exhibit more potent antifibrotic activity than the reference pirfenidone in the rat model of bleomycin-induced pulmonary fibrosis which is substantiated by the histopathological study. Rather they-induced decrease in LDH activity, total cell count (macrophages, neutrophils and eosinophils) and protein concentration. Their antifibrotic activity is supported by their acceptable results of liver and kidney functions.

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