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Synthesis and antioxidant activity evaluation of new 7-aryl or 7-heteroarylamino-2,3-dimethylbenzo[*b*]thiophenes obtained by Buchwald–Hartwig C–N cross-coupling

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Abstract—New 7-aryl or 7-heteroarylamino-2,3-dimethylbenzo[*b*]thiophenes were prepared by palladium-catalyzed Buchwald–Hartwig cross-coupling of 7-bromo or 7-amino-2,3-dimethylbenzo[*b*]thiophenes, previously prepared by us, with substituted (4-methoxy or 3,4-dimethoxy) anilines and 3-aminopyridine or with substituted (3-methoxy or 4-cyano) bromobenzenes and 2-bromopyridine, respectively, using $Pd(OAc)_2$, *rac*-BINAP or Xantphos as ligands, and Cs_2CO_3 as base. Their antioxidant properties were evaluated by several methods: reducing power, scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, inhibition of erythrocyte hemolysis and inhibition of lipid peroxidation using the β-carotene linoleate system. EC_{50} values for all the methods were determined and it was possible to establish some structure–activity relationships (SARs) based on the presence and position of different substituents on the phenyl ring (1 or 2 OMe and C≡N), on the presence of a pyridine ring and on the position of its nitrogen atom relative to the N–H bond.

1. Introduction

Free radical formation is associated with the normal natural metabolism of aerobic cells. The oxygen consumption inherent in cell growth leads to the generation of a series of oxygen free radicals. The interaction of these species with lipidic molecules produces new radicals: hydroperoxides and different peroxides.[1,2] This group of radicals (superoxide, hydroxyl, and lipoid peroxides) may interact with biological systems in a cytotoxic manner. Free radicals and their uncontrolled production, in fact, are responsible for several pathological processes, such as certain tumours (prostate and colon cancers) and coronary heart disease.[3]

The reducing properties of diarylamines make them very important as antioxidants, especially as radical scavengers.[4] In fact most representative examples of antioxidants are hindered phenols and diphenylamine derivatives.[5] The reaction of $RO_2^{\cdot}$ radicals with secondary amines seems to proceed according to the mechanism proposed by Thomas,[6] the H-transfer reaction from the N–H bond to peroxyls occurring in a first step leads to aminyl radicals ($RR'N^{\cdot}$) which react again with $RO_2^{\cdot}$ giving nitroxide radicals ($RR'NO^{\cdot}$) in a second step. The latter were detected by ESR (electron spin resonance) in the inhibiting action of diphenylamine in the oxidation of cumene.[7]

Recently we have reported the antioxidant properties of some diarylamines in the benzo[*b*]thiophene series, by evaluation of their free radical scavenging activity and reducing power, and it was possible to establish some structure–activity relationships based on the position of arylamination (either on the benzene or on the thiophene ring) and on the presence of different substituents on both rings.[8]

Herein, we report the synthesis of new 7-aryl or 7-heteroarylamino-2,3-dimethylbenzo[*b*]thiophenes by C–N

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palladium-catalyzed cross-coupling[9] of 7-bromo or 7-amino-2,3-dimethylbenzo[*b*]thiophenes with methoxylated anilines and 3-aminopyridine or substituted (3-methoxy or 4-cyano) bromobenzenes and 2-bromopyridine, respectively. The antioxidant properties of the di(hetero)arylamines obtained were evaluated by their reducing power, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging activity, inhibition of erythrocyte hemolysis and inhibition of lipid peroxidation using the β-carotene linoleate system.

2. Results and discussion

2.1. Synthesis

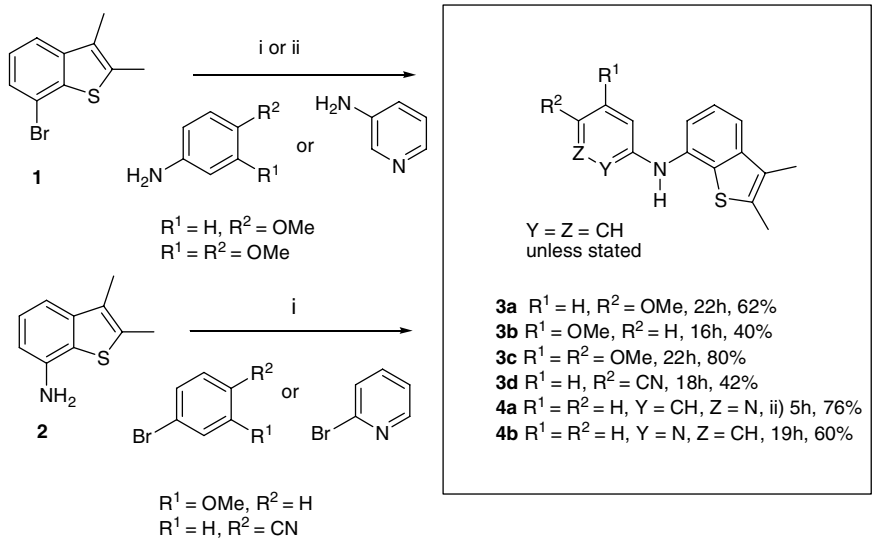
Differently substituted 7-di(hetero)arylamine derivatives of 2,3-dimethylbenzo[*b*]thiophene were obtained by C–N palladium-catalyzed cross-couplings in moderate to high yields (40–80%) using either 7-bromobenzo[*b*]thiophene **1**[10] or 7-aminobenzo[*b*]thiophene **2**,[11] previously prepared by us, as coupling components (Scheme 1). The coupling conditions were the same (i conditions) except for the coupling of 3-aminopyridine where Xantphos was used as ligand instead of BINAP, and dioxane as solvent instead of toluene (ii conditions). These coupling conditions are required for the C–N coupling of heteroaromatic amines as already reported by others[12] and verified by us in an earlier work.[13]

The methoxylated coupling products **3a** and **3c** were obtained in good to high yields from bromo compound **1** and methoxylated anilines but when 3-methoxybromobenzene was coupled with amine **2** the yield for **3b** was only 40%. Almost the same yield (42%) was observed in the synthesis of **3d** from amine **2** and 4-bromobenzonitrile. In the latter cases the starting materials were recovered. These results show that the aminated component needs to bear electron-donating groups to give the products in good yields and that amine **2** is not very activated because even when reacting with a bromo component bearing an electron withdrawing group (C≡N) the yield was only moderate. In our first C–N couplings in the benzo[*b*]thiophene series, methyl groups were also present in the benzene ring of the system and they increased the reactivity of the 6-aminobenzo[*b*]thiophenes used and decreased that of the corresponding 6-bromo compounds.[14] The pyridine derivatives **4a** and **4b** were prepared using compound **1** and 3-aminopyridine or compound **2** and 2-bromopyridine, respectively, in good to high yields (Scheme 1). In the latter case it was possible to use the same conditions as those for bromobenzenes to obtain the product in good yield (60%) due to the high reactivity of 2-bromopyridine.

The antioxidant activity of the di(hetero)arylamines bearing electron-donating methoxy substituents in the *para* (**3a**), *meta* (**3b**) or in both positions (**3c**), an electron-withdrawing cyano group in the *para* position relative to the N–H bond (**3d**) and, of the pyridine derivatives **4a** and **4b** was evaluated by several methods in order to compare the results and to establish some structure–antioxidant-activity relationships for each method.

2.2. Antioxidant activity studies

2.2.1. Reducing power assay. Table 1 shows the reducing power of methanolic solutions of diarylamines **3a–d**, **4a**, and **4b** examined as a function of their concentration. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending upon the reducing power of each compound. The presence of reducers (i.e., antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form giving,



i) $Pd(OAc)_2$ (3-5 mol%), *rac* BINAP (4-7 mol%) Cs_2CO_3 (1.4-1.8 equiv.), toluene, 100 °C, Ar
ii) $Pd(OAc)_2$ (6 mol%), Xantphos (8mol%) Cs_2CO_3 (1.8 equiv.), dioxane, 110 °C, Ar

Scheme 1. Synthesis of 7-aryl or 7-heteroarylamino-2,3-dimethylbenzo[*b*]thiophenes **3a–d**, **4a**, and **4b**.

Table 1. Reducing power (Abs at 700 nm) of diarylamines **3a–d**, **4a**, and **4b**

Compound	Concentration (g/L)				
	0.0156	0.0312	0.0625	0.125	0.250
3a	0.4556	0.5707	0.6350	0.7839	1.0928
3b	0.2532	0.3019	0.4296	0.5513	0.7204
3c	0.4410	0.5789	0.6143	0.6803	0.9757
3d	0.1743	0.1856	0.1907	0.2132	0.2225
4a	0.1677	0.1811	0.2036	0.3078	0.3610
4b	0.1308	0.1560	0.1630	0.1982	0.2330

BHA 0.25 g/L, 1.80; BHT 0.25 g/L, 1.33; ascorbic acid 0.25 g/L, 1.72.

after the addition of trichloroacetic acid and ferric chloride, the Perl's Prussian blue that can be monitored at 700 nm.

The reducing power of the standards 2-*tert*-butyl-4-methoxyphenol (butylated hydroxyanisole, BHA), 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene, BHT) and ascorbic acid at 0.25 g/L was determined, presenting 1.80, 1.33, and 1.72 as absorbance values, respectively.

The reducing power of diarylamine solutions in methanol increases with the increase of the concentration (Table 1). For compounds **3d**, **4a**, and **4b** the absorbance values are very low while for compounds **3a–c** are higher than 0.7 at 0.25 g/L, but lower than the standards. The presence of a methoxy group (electron-donating group) in the *para* position (**3a**) was much better than a cyano group (electron-withdrawing group) in the same position (**3d**), giving **3a** higher reducing power values. The introduction of an additional methoxy group in the *meta* position (**3c**) resulted in similar values of reducing power comparing with **3a**, which is in agreement with our earlier results.[8] The presence of only a methoxy group in the *meta* position gave lower reducing values for compound **3b** than those obtained for **3a** and **3c**. Thus, the methoxy group in the *para* position to the N–H is the most important feature for the good results of reducing power of these compounds.

2.2.2. Radical scavenging activity (RSA) assay. Free radical scavenging is one of the best known mechanisms by which antioxidants inhibit lipid oxidation. DPPH radical scavenging activity evaluation is a standard assay in antioxidant activity studies and offers a rapid technique for screening the radical scavenging activity (RSA) of specific compounds or extracts.[15] The RSA of diarylamines **3a–d**, **4a**, and **4b** was tested using a methanolic solution of the 'stable' free radical, DPPH. A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally fades/disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a colourless/bleached product (i.e., 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm. Hence, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the compound.

The RSA values of methanolic solutions of diarylamines **3** and **4** were examined and compared (Table 2); results are expressed as a percentage of the ratio of the decrease in absorbance at 517 nm to the absorbance of DPPH solution in the absence of diarylamines at 517 nm. From analysis of Table 2 we can conclude that the diarylamines' scavenging effects on DPPH radicals increase with the concentration and were excellent for compounds with one or two OMe, **3a** and **3c** (93.3% and 94.7% at 0.25 g/L, respectively), even higher than RSA values for the standards BHA (84.0%) and BHT (83.0%) at the same concentration. The RSA drastically decreased to 28.4% at 0.25 g/L for compound **3b** bearing a OMe group in the *meta* position. The presence of a cyano group (**3d**) instead of a methoxy in the *para* position (**3a**) was inadequate for this activity. Compounds with a pyridine ring **4a** and **4b** showed very low RSA values.

2.2.3. Assay for erythrocyte hemolysis. The oxidative hemolysis in erythrocytes induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) has been extensively studied as model for the peroxidative damage in biomembranes.[16] AAPH generates peroxyl radicals by thermal decomposition which attack the erythrocytes causing the chain oxidation of lipid and protein, disturbing the membrane organization and eventually leading to hemolysis.[17] In this study, the diarylamines' protective effect on hemolysis mediated by peroxyl radical was investigated. Table 3 shows inhibition percentage of hemolysis, as a result of protection against the oxidative damage of cell membranes of erythrocytes from ram, induced by AAPH.

Table 2. Scavenging activity (%) on DPPH radicals of diarylamines **3a–d**, **4a**, and **4b**

Compound	Concentration (g/L)				
	0.0156	0.0312	0.0625	0.125	0.250
3a	55.8	85.8	88.4	91.1	93.3
3b	7.9	13.4	13.5	22.2	28.4
3c	47.3	72.4	86.9	92.1	94.7
3d	0	0	0	0.5	3.1
4a	2.5	2.8	5.5	9.0	15.5
4b	0.4	2.6	4.9	8.8	9.0

BHA 0.25 g/L, 84.0%; BHT 0.25 g/L, 83.0%.

Table 3. Hemolysis inhibition (%) of diarylamines **3a–d**, **4a**, and **4b**

Compound	Concentration (g/L)				
	0.0156	0.0312	0.0625	0.125	0.250
3a	43.4	61.2	65.1	65.9	66.7
3b	40.3	44.3	58.0	63.1	66.7
3c	47.8	58.3	61.2	62.1	62.9
3d	48.3	59.4	62.1	62.6	66.4
4a	41.8	56.8	66.9	69.3	74.1
4b	38.4	44.5	53.0	64.6	69.5

Ascorbic acid 1 g/L, 94.6%.

The compounds inhibit hemolysis of erythrocytes in a concentration-dependent manner (Table 3). The pyridine derivatives **4a** and **4b** show slightly higher inhibition percentage of hemolysis (74.1% for **4a** and 69.5% for **4b** at 0.25 g/L) than the other diarylamines. The introduction of different substituents in the benzene ring had no significant influence in the good results obtained.

2.2.4. Lipid peroxidation inhibition using the β-carotene linoleate system. Table 4 shows the antioxidant activity of diarylamines measured by the bleaching of β-carotene. The antioxidant activity of carotenoid is based on its radical adducts with free radicals from linoleic acid. The linoleic acid free-radical attacks the highly unsaturated β-carotene models. The presence of antioxidants can decrease the extent of β-carotene bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system.[18] Accordingly, the absorbance decreases rapidly in samples without antioxidant, whereas in the presence of an antioxidant, they retain the colour for a longer time. The antioxidant activity of the di(hetero)arylamines prepared increases with the concentration and is excellent for compounds with one or two methoxy groups **3a** and **3c** (100% at 0.0625 g/L), presenting higher values than *tert*-butylhydroquinone (TBHQ) standard (82.2% at 2 g/L). For the other compounds the lipid peroxidation inhibition is also very good except for the pyridine derivative **4b** which presents only moderate values. The position of the nitrogen atom in the pyridine derivatives **4a** and **4b** relative to the N–H bond is very important, resulting in different results for lipid peroxidation inhibition.

2.2.5. EC_{50} values for the different antioxidant activity assays. For an overview of the results, Table 5 presents the EC_{50} values for the antioxidant activity assays performed on the synthesized di(hetero)arylamines and some structure–antioxidant-activity relationships were established. The presence of a methoxy group (**3a**) instead of a cyano group (**3d**) in the *para* position increases the reducing power, the radical scavenging capacity and the lipid peroxidation inhibition (lower EC_{50} values). Nevertheless, compound **3d** gives good results for erythrocyte hemolysis inhibition. The introduction of an additional methoxy group in the *meta* position (**3c**) increases the RSA values and the lipid peroxidation inhibition, despite the slightly decrease in the reducing power. The presence of only one methoxy group in the *meta* position (**3b**) lowers significantly the reducing power, the RSA capacity and the lipid peroxidation inhibition (much higher EC_{50} values). Although the presence of a pyridine ring is not favourable for the reducing power and RSA properties, the pyridine derivatives **4a** and **4b** show a good protective effect against hemolysis of erythrocytes and in the case of **4a** also good values for the lipid peroxidation inhibition. Thus, the position of the nitrogen atom in the pyridine ring relative to the N–H affects the latter activity.

Table 4. Lipid peroxidation inhibition (%) of diarylamines **3a–d**, **4a**, and **4b** by β-carotene bleaching method

Compound	Concentration (g/L)				
	0.0156	0.0312	0.0625	0.125	0.250
3a	92.3	96.8	99.7	100	100
3b	56.8	62.9	68.9	82.6	84.8
3c	89.3	91.4	100	100	100
3d	49.6	67.3	69.0	71.0	79.6
4a	48.1	65.4	70.9	76.1	86.2
4b	40.7	42.9	45.8	45.9	47.2

TBHQ 2 g/L, 82.2%.

3. Conclusions

Several new 7-aryl and 7-heteroarylamino-2,3-dimethylbenzo[*b*]thiophenes were prepared by palladium-catalyzed Buchwald–Hartwig cross-coupling (C–N) of 7-bromo or 7-amino-2,3-dimethylbenzo[*b*]thiophenes with 4-methoxy or 3,4-dimethoxyanilines and 3-aminopyridine or 3-methoxy or 4-cyanobromobenzenes and 2-bromopyridine, respectively, in moderate to high yields.

The antioxidant properties of the di(hetero)arylamines obtained were evaluated by several methods. The methoxylated compounds **3a** and **3c** revealed high RSA and lipid peroxidation inhibition values, even higher than the standards. The presence of a methoxy group (electron-donating group) in the *para* position relative to the NH bond is the most important feature for the antioxidant activity of the diarylamines studied. Nevertheless, compound **3d** bearing an electron-withdrawing group (cyano group) in the *para* position relative to the NH or the pyridine derivatives give also good results for the erythrocyte hemolysis inhibition.

Table 5. EC_{50} values (g/L) of diarylamines **3a–d**, **4a**, and **4b** in the antioxidant activity evaluation assays

Compound	Reducing power (EC_{50})[a]	DPPH (EC_{50})[b]	Hemolysis inhibition (EC_{50})[c]	β-Carotene bleaching (EC_{50})[d]
3a	0.0216	0.0140	0.0214	0.00845
3b	0.0987	>0.250	0.0442	0.0137
3c	0.0227	0.0173	0.0189	0.00873
3d	>0.250	>0.250	0.0180	0.0160
4a	>0.250	>0.250	0.0241	0.0173
4b	>0.250	>0.250	0.0514	>0.250

[a] EC_{50} (g/L): effective concentration at which the absorbance is 0.5.
[b] EC_{50} (g/L): effective concentration at which 50% of DPPH radicals are scavenged.
[c] EC_{50} (g/L): effective concentration at which 50% of the erythrocyte hemolysis is inhibited.
[d] EC_{50} (g/L): effective concentration at which the lipid peroxidation inhibition is 50%.

The pyridine compound **4a** gives much better results than **4b** for the lipid peroxidation inhibition, which is due to the fact that its nitrogen atom is in the 3-position relative to the NH bond.

4. Experimental

4.1. Synthesis

Melting points were determined on a Stuart SMP3 apparatus and are uncorrected. The ^{1}H NMR spectra were measured on a Varian Unity Plus at 300 MHz. Spin–spin decoupling was used to assign the signals. The ^{13}C NMR spectra were measured in the same instrument at 75.4 MHz (using DEPT θ 45°). IR spectrum of **3d** was recorded as Nujol mull on a Perkin-Elmer 1600-FTIR spectrophotometer.

Elemental analyses were performed on a LECO CHNS 932 elemental analyser. Mass spectra (EI) and HRMS were made by the mass spectrometry service of University of Vigo-Spain.

Column chromatography was performed on Macherey–Nagel silica gel 230–400 mesh. Petroleum ether refers to the boiling range 40–60 °C. Ether refers to diethyl ether. When solvent gradient was used the increase of polarity was done gradually from neat petroleum ether to mixtures of ether/petroleum ether increasing 5% or 10% of ether until the isolation of the product.

The ligands *rac*. BINAP [2,2′-bis(diphenylphosphane)-1,1′-binaphtyl, 97%] and Xantphos [4,5-bis(diphenylphosphane)-9,9-dimethylxanthene, 97%] and the base Cs_2CO_3 were purchase from Aldrich.

4.1.1. General procedure for the synthesis of di(hetero)arylamines. A dry Shlenk tube was charged, under Ar, with dry toluene or dry dioxane (3–5 mL), the arylbromide or bromo compound **1**, $Pd(OAc)_2$, *rac*. BINAP, Cs_2CO_3 or $Pd(OAc)_2$, Xantphos, Cs_2CO_3 (conditions (a) (b) or (c)), the aniline, amine **2** or the aminopyridine, and the mixture was heated at 100 or 110 °C for several hours. The reactions were followed by TLC. After cooling, water and ether were added. The phases were separated, the aqueous phase was extracted with more ether and the organic phase was dried ($MgSO_4$) and filtered. Removal of the solvent gave an oil which was submitted to column chromatography to give products **3a**–**d**, **4a**, and **4b**.

Conditions used in the experiments: (a) $Pd(OAc)_2$ (3 mol%), *rac*. BINAP (4 mol%), Cs_2CO_3 (1.4 equiv) toluene, 100 °C; (b) $Pd(OAc)_2$ (5 mol%), *rac*. BINAP (7 mol%), Cs_2CO_3 (1.8 equiv) toluene, 100 °C; (c) $Pd(OAc)_2$ (6 mol%), Xantphos (8 mol%), Cs_2CO_3 (1.8 equiv), dioxane 110 °C.

4.1.2. 7-(4-Methoxyphenyl)amino-2,3-dimethylbenzo[*b*] thiophene (3a). From bromo compound **1** (150 mg, 0.622 mmol) and 4-methoxyaniline (92.0 mg, 0.747 mmol), using conditions (b), heating for 22 h and using solvent gradient in the column chromatography from neat petroleum ether to 10% ether/petroleum ether, compound **3a** was obtained as a white solid (109 mg, 62%). Crystallization from ether/petroleum ether gave white crystals, mp 103–105 °C. ^{1}H NMR: ($CDCl_3$) 2.32 (3H, s, Me), 2.51 (3H, s, Me), 3.82 (3H, s, OMe), 5.40 (1H, br s, N–H), 6.89 (2H, d, J = 9Hz, Ar-H), 6.94 (1H, dd, J = 7 and 1.2 Hz, 6-H), 7.10 (2H, d, J = 9.0 Hz, Ar-H), 7.18–7.28 (2H, m, ArH) ppm. ^{13}C NMR: ($CDCl_3$) 11.65 (CH_3), 13.84 (CH_3), 55.56 (OCH_3), 109.73 (CH), 113.87 (CH), 114.58 (2× CH), 122.26 (2× CH), 125.04 (CH), 127.13 (C), 128.22 (C), 132.68 (C), 135.60 (C), 139.35 (C), 142.57 (C), 155.33 (C) ppm. Anal. Calcd for $C_{17}H_{17}NOS$: C, 72.05; H, 6.05; N, 4.94; S, 11.31. Found: C, 71.73; H, 6.10; N, 4.96; S, 11.30.

4.1.3. 7-(3-Methoxyphenyl)amino-2,3-dimethylbenzo[*b*]-thiophene (3b). From amine **2** (100 mg, 0.570 mmol) and 3-methoxybromobenzene (106 mg, 0.570 mmol), using conditions (a) and heating for 16 h and using solvent gradient in the column chromatography from neat petroleum ether to 5% ether/petroleum ether, compound **3b** was obtained as a white solid (65.0 mg, 40%). Crystallization from ether/petroleum ether gave white crystals, mp 102–103 °C. ^{1}H NMR: ($CDCl_3$) 2.32 (3H, s, Me), 2.50 (3H, s, Me), 3.79 (3H, s, OMe), 5.60 (1H, br s, N–H), 6.49–6.54 (1H, m, Ar-H), 6.61–6.67 (2H, m, Ar-H), 7.16–7.34 (4H, m, Ar-H) ppm. ^{13}C NMR: ($CDCl_3$) 11.64 (CH_3), 13.84 (CH_3), 55.20 (OCH_3), 103.43 (CH), 106.22 (CH), 110.28 (CH), 113.39 (CH), 115.61 (CH), 124.95 (CH), 128.14 (C), 129.97 (C), 130.00 (CH), 133.07 (C), 136.88 (C), 142.69 (C), 144.55 (C), 160.62 (C) ppm. Anal. Calcd for $C_{17}H_{17}NOS$: C, 72.05; H, 6.05; N, 4.94; S, 11.31. Found: C, 72.17; H, 6.10; N, 4.96; S, 11.15.

4.1.4. 7-(3,4-Dimethoxyphenyl)amino-2,3-dimethylbenzo-[*b*]thiophene (3c). From bromo compound **1** (150 mg, 0.622 mmol) and 3,4-dimethoxyaniline (105 mg, 0.684 mmol), using conditions (a), heating for 22 h and using solvent gradient in the column chromatography from neat petroleum ether to 25% ether/petroleum ether, compound **3c** was obtained as a white solid (156 mg, 80%). Crystallization from ether/petroleum ether gave white crystals, mp 126–128 °C. ^{1}H NMR: ($CDCl_3$) 2.31 (3H, s, Me), 2.50 (3H, s, Me), 3.85 (3H, s, OMe), 3.89 (3H, s, OMe), 5.42 (1H, br s, N–H), 6.68 (1H, dd, J = 8.4 and 2.1 Hz, 6′-H), 6.75 (1H, d, J = 2.1 Hz, 2′-H), 6.84 (1H, d, J = 8.4 Hz, 5′-H), 6.99 (1H, br d, J = 7.5 Hz, 6-H), 7.18–7.30 (2H, m, 4 and 5-H) ppm. ^{13}C NMR: ($CDCl_3$) 11.63 (CH_3), 13.82 (CH_3), 55.85 (OCH_3), 56.25 (OCH_3), 105.44 (CH), 110.20 (CH), 112.04 (CH), 112.24 (CH), 114.07 (CH), 125.03 (CH), 127.44 (C), 128.19 (C), 132.75 (C), 136.11 (C), 139.04 (C), 142.60 (C), 144.72 (C), 149.55 (C) ppm. Anal. Calcd for $C_{18}H_{19}NO_2S$: C, 68.98; H, 6.11; N, 4.47; S, 10.23. Found: C, 68.83; H, 6.16; N, 4.53; S, 10.22.

4.1.5. 7-(4-Cyanophenyl)amino-2,3-dimethylbenzo[*b*]thiophene (3d). From amine **2** (76.0 mg, 0.430 mmol) and 4-bromobenzonitrile (78.0 mg, 0.430 mmol), using conditions (a), heating for 18 h and using solvent gradient

in the column chromatography from neat petroleum ether to 25% ether/petroleum ether, compound **3d** was obtained as a white solid (50.0 mg, 42%). Crystallization from ether/petroleum ether gave white crystals, mp 226–228 °C. IR: $\bar{\nu}$ 3335 (N–H), 2210 (C≡N) cm^{-1}. ^1H NMR ($CDCl_3$): 2.33 (3H, s, 3-Me), 2.49 (3H, s, 2-Me), 6.00 (1H, s, N–H), 6.88 (2H, d, $J = 8.6$ Hz, 2′ and 6′-H), 7.22 (1H, br d, $J = 7.5$ Hz, 6-H), 7.40 (1H, apparent t, $J = 7.5$ Hz, 5-H), 7.44–7.52 (3H, m, 4, 3′ and 5′-H) ppm. ^{13}C NMR ($CDCl_3$): 11.64 (CH_3), 13.84 (CH_3), 101.58 (C), 115.04 (2× CH), 117.68 (CH), 118.38 (CH), 119.89 (C), 125.03 (CH), 128.19 (C), 133.02 (C), 133.66 (2× CH), 133.77 (C), 133.91 (C), 143.04 (C), 148.02 (C) ppm. MS: *m/z* (%) (EI) 280.09 (M^++2, 6), 279.09 (M^++1, 21), 278.09 (M^+, 100). HRMS: M^+ Calcd for $C_{17}H_{14}N_2S$ 278.0878. Found 278.0877.

4.1.6. *N*-(2,3-dimethylbenzo[*b*]thien-7-yl)pyridin-3-amine (4a). From bromo compound **1** (100 mg, 0.420 mmol) and 3-aminopyridine (40.0 mg, 0.420 mmol), using conditions (c), heating for 5 h and using solvent gradient in the column chromatography from neat petroleum ether to 60% ether/petroleum ether, compound **4a** was obtained as a white solid (81 mg, 76%). Crystallization from ether/petroleum ether gave white crystals, mp 161–163 °C. ^1H NMR: ($CDCl_3$) 2.33 (3H, s, Me), 2.50 (3H, s, Me), 5.66 (1H, br s, N–H), 7.13–7.20 (2H, m, Ar-H), 7.28–7.39 (3H, m, Ar-H), 8.19 (1H, dd, $J = 4.7$ and 1.2 Hz, Ar-H), 8.39 (1H, d, $J = 2.4$ Hz Ar-H) ppm. ^{13}C NMR: ($CDCl_3$) 11.65 (CH_3), 13.85 (CH_3), 113.76 (CH), 116.54 (CH), 123.56 (CH), 123.59 (CH), 125.02 (CH), 128.22 (C), 130.47 (C), 133.45 (C), 135.84 (C), 139.80 (C), 139.97 (CH), 141.93 (CH), 142.90 (C) ppm. Anal. Calcd for $C_{15}H_{14}N_2S$: C, 70.83; H, 5.55; N, 11.01; S, 12.61. Found: C, 70.90; H, 5.75; N, 10.74; S, 12.52.

4.1.7. *N*-(2,3-Dimethylbenzo[*b*]thien-7-yl)pyridin-2-amine (4b). From amine **2** (100 mg, 0.570 mmol) and 2-bromopyridine (90.0 mg, 0.570 mmol), using conditions (a), heating for 19 h and using solvent gradient in the column chromatography from neat petroleum ether to 25% ether/petroleum ether, compound **4b** was obtained as a white solid (87.0 mg, 60%). Crystallization from ether/petroleum ether gave white crystals, mp 161–163 °C. ^1H NMR: ($CDCl_3$) 2.33 (3H, s, Me), 2.50 (3H, s, Me), 6.66 (1H, br s, N–H), 6.73–6.81 (2H, m, Ar-H), 7.34–7.52 (4H, m, Ar-H), 8.24 (1H, br d, $J = 4.8$ and 0.9 Hz, Ar-H) ppm. ^{13}C NMR: ($CDCl_3$) 11.63 (CH_3), 13.83 (CH_3), 108.32 (CH), 115.13 (CH), 116.51 (CH), 117.31 (CH), 124.91 (CH), 128.07 (C), 132.19 (C), 133.53 (C), 134.35 (C), 137.64 (CH), 142.71 (C), 148.48 (CH), 156.15 (C) ppm. Anal. Calcd for $C_{15}H_{14}N_2S$: C, 70.83; H, 5.55; N, 11.01; S, 12.61. Found: C, 70.76; H, 5.65; N, 10.74; S, 12.49.

4.2. Antioxidant activity assays

Standards BHA, BHT, TBHQ and L-ascorbic acid were purchased from Sigma (St. Louis, MO, USA). DPPH was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methanol was obtained from Pronalab (Lisbon, Portugal). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

4.2.1. Reducing power assay. The reducing power was determined according to the method of Oyaizu.[19] Various concentrations of methanolic compounds' solutions (2.5 mL) were mixed with 2.5 mL of sodium phosphate buffer (200 mmol/L, pH 6.6, 2.5 mL) and potassium ferricyanide (1%, 2.5 mL). The mixture was incubated at 50 °C for 20 min. After the addition of trichloroacetic acid (10% w/v, 2.5 mL), the mixture was centrifuged at 650 rpm for 10 min. The upper layer (5 mL) was mixed with deionised water (5 mL) and ferric chloride (0.1%, 1 mL), and the absorbance was measured at 700 nm; increase of absorbance of the reaction mixture indicates higher reducing power. Mean values from three independent samples were calculated for each compound and standard deviations were less than 5%. The compound concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against compound concentration. BHA, BHT and ascorbic acid were used as standards.

4.2.2. Radical scavenging activity (RSA) assay. The capacity of compounds to scavenge the 'stable' free radical DPPH was monitored according to the method of Hatano.[20] Various concentrations of methanolic compounds solutions (0.3 mL) were mixed with methanolic solution containing DPPH radicals (6×10^{-5} mol/L, 2.7 mL). The mixture was shaken vigorously and left to stand for 60 min in the dark (until stable absorption values were obtained). The reduction of the DPPH radical was determined by measuring the absorption at 517 nm. The RSA was calculated as a percentage of DPPH discolouration using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the compound has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. Mean values from three independent samples were calculated for each compound and standard deviations were less than 5%. The compound concentration providing 50% inhibition (EC_{50}) was calculated from the graph of RSA percentage against compound concentration. BHA and BHT were used as standards.

4.2.3. Assay for erythrocyte hemolysis mediated by peroxyl free radicals. The antioxidant activity of the methanol solutions from compounds was measured as the inhibition of erythrocyte hemolysis.[21] Blood was obtained from male ram (churra galega transmontana) of body weight ~67 kg. Erythrocytes separated from the plasma and the buffy coat were washed three times with 10 mL of 10 mM phosphate-buffered-saline (PBS; 10 mM, 10 mL) at pH 7.4, prepared by mixing NaH_2PO_4 and Na_2HPO4 (10 mM) and NaCl (125 mM) in 1 L of distilled water, and centrifuged at 1500*g* for 5 min. During the last washing, the erythrocytes were obtained by centrifugation at 1500*g* for 10 min. A suspension of erythrocytes in PBS (20%, 0.1 mL) was added to AAPH solution in PBS (200 mM, 0.2 mL) and

the compound methanolic solution (0.1 mL) at different concentrations. The reaction mixture was shaken gently while being incubated at 37 °C for 3 h. The reaction mixture was diluted with PBS (8 mL) and centrifuged at 1041*g* for 10 min; the absorbance of its supernatant was then read at 540 nm by a spectrophotometer. The percentage hemolysis inhibition was calculated by equation % hemolysis inhibition = $[(A_{AAPH} - A_S)/A_{AAPH}] \times 100$, where A_S is the absorbance of the sample containing the compound solution, and A_{AAPH} is the absorbance of the control sample containing no compound solution. Mean values from three independent samples were calculated for each compound and standard deviations were less than 5%. The compound concentration providing 50% inhibition (EC_{50}) was calculated from the graph of hemolysis inhibition percentage against compound concentration. L-Ascorbic acid was used as standard.

4.2.4. Lipid peroxidation inhibition using the β-carotene linoleate system. Lipid peroxidation inhibition was evaluated by the β-carotene linoleate model system.[17] A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution was pipetted into a 100 mL round-bottomed flask. After the chloroform was removed at 40 °C under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg) and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing 0.2 mL of different concentrations of the compounds. The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. Absorbance readings were then recorded at 20-min intervals until the control sample had changed colour. A blank, devoid of β-carotene, was prepared for background subtraction. Antioxidant activity was calculated using the following equation: antioxidant activity = (β-carotene content after 2 h of assay/initial β-carotene content) × 100. Mean values from three independent samples were calculated for each compound and standard deviations were less than 5%. The compound concentration providing 50% lipid peroxidation inhibition (EC_{50}) was calculated from the graph of antioxidant activity percentage against compound concentration. TBHQ was used as standard.

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