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Benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-ones electro-deficient heterocyclic compounds with promising anticancer activity

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Abstract: The synthesis and antitumor activity of substituted benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-ones – novel electro-deficient tricyclic compounds – are described. These compounds were prepared by treatment of 3-(2-amino-3-R^2-5-R^3-phenyl)-6-R^1-1,2,4-triazin-5(2*H*)-ones with sodium nitrite in acetic acid. Spectral properties of synthesized compounds were studied and compared with spectral data of known 3-R-8-R^1-10-R^2-2*H*-[1,2,4]triazino[2,3-*c*]quinazolin-2-ones. These compounds are promising antitumor agents. The most active anticancer compound **3d** was studied in dose-depended anticancer activity assay and its selective growth inhibition against breast cancer MDA-MB-468 cell line was established (GI_{50}=0.41 μM).

Keywords: anticancer activity; benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazines; heterocyclic compounds.

Introduction

Derivatives of 1,2,4- and 1,3,5-triazines show anticancer, antibacterial and antifungal activity [1–6]. However, few papers are devoted to the chemistry and evaluation of biological activity of 1,2,3-triazines and their condensed derivatives. Oxidation of *N*-aminopyrazoles is used to synthesize 1,2,3-triazines [7, 8] and treatment of aromatic *o*–aminonitriles and o-aminohydrazides with nitrous acid or butyl nitrite yields condensed derivatives [9–11]. This chemistry was used for the synthesis of novel antifungal agents [12]. Considering our experience in the search for antimicrobial, fungicidal and anticancer agents among [1,2,4]triazino[2,3-*c*]quinazolines [13–16] and the reported efficiency of bioisosteric replacement strategies [17, 18] it was decided to elaborate synthetic protocol for series of 3-R^1-8-R^2-10-R^3-2*H*-benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-ones. These compounds may be regarded as isosteric analogs of previously described heterocyclic compounds with promising antitumor activity [13–16].

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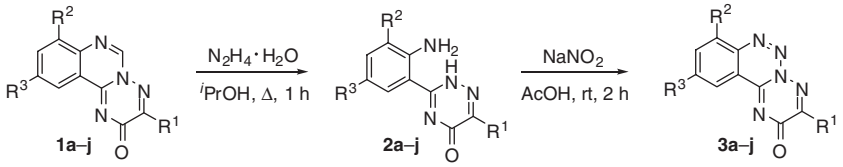
Results and discussion

Chemistry

Substituted 3-(2-amino-3-R^2-5-R^3 phenyl)-6-R1-1,2,4-triazin-5(2*H*)-ones **2a–j** were used as starting materials for synthesis of target 3-R^1-8-R^2-10-R^3-2*H*-benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-ones (**3a–j**, Scheme 1). The starting anilines were obtained according to a known synthetic protocol [19] *via* transformation of 3-R-8-R^1-10-R^2-2*H*-[1,2,4]triazino[2,3-*c*]quinazolin-2-ones **1a–j** [20].

Purity of the synthesized compounds was analyzed by LC-MS (APCI) method and their structure was established by using physicochemical methods including ^{1}H NMR, ^{13}C NMR, IR and mass spectrometry. In particular, the protons in positions 8 and 10 of **3a–j** are significantly deshielded in comparison to the corresponding protons of [1,2,4]triazino[2,3-*c*]quinazoline system [20]. Signals caused by the presence of a substituent in position 3 are also registered in proper fields. The carbons are also more deshielded in comparison to those of previously described [1,2,4]triazino[2,3-*c*]quinazolines [20]. The mass spectrum of compound **3b** is characterized by a molecular ion peak of low intensity (2%). One of the mass fragmentation routes includes elimination of molecular nitrogen (*m/z* 247) from the molecular ion.

The structure of compound **3h** was additionally determined by X-ray diffraction study (Figure 1). The tricyclic fragment is planar within 0.01 Å. The H4…N1 (2.63 Å) and H16c…N2 (2.54 Å) attractive interactions can be seen



a: R^1 = Me; R^2 = R^3 = H
b: R^1 = Ph; R^2 = R^3 = H
c: R^1 = 4-Me-C_6H_4; R^2 = R^3 = H
d: R^1 = 4-^{i}Pr-C_6H_4; R^2 = R^3 = H
e: R^1 = 4-^{t}Bu-C_6H_4; R^2 = R^3 = H
f: R^1 = 4-MeO-C_6H_4; R^2 = R^3 = H
g: R^1 = 4-F-C_6H_4; R^2 = R^3 = H
h: R^1 = Ph; R^2 = Me; R^3 = H
i: R^1 = Ph; R^2 = H; R^3 = Br
j: R^1 = 4-F-C_6H_4; R^2 = H; R^3 = Br

Scheme 1 Synthetic route for 3-R^1-8-R^2-10-R^3-2*H*-benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-ones **3a–j.**

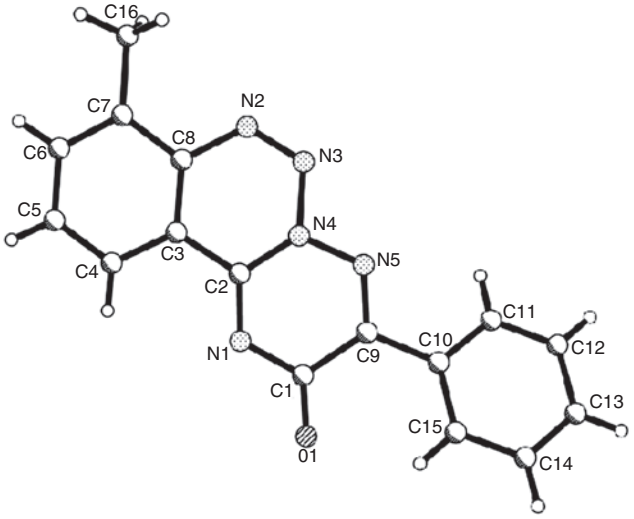


Figure 1 Structure of compound **3h** according to X-ray structural study.

because the van der Waals radii sum is 2.67 Å) [21]. The phenyl substituent is twisted relatively to the triazine ring with the C1-C9-C10-C15 torsion angle of -32.6(2)°. This is probably due by the repulsion between the phenyl group and the adjacent atoms; the shortened intramolecular contacts are 2.49 Å for H11…N5 and 2.87 Å for H15…C1 2.86 Å. It can also be suggested that the repulsion between the phenyl substituent and the triazine moiety promotes the elongation of the C1-C9 bond to 1.497(1) Å as compared with its mean value of 1.46 Å [22].

Anticancer activity

Compounds **3b–d** and **3f–h** were selected by the National Cancer Institute (NCI) Developmental Therapeutic Program (www.dtp.nci.nih.gov) for the *in vitro* cell line screening to investigate their anticancer activity. Anticancer assays were performed according to the US NCI protocol, which was described elsewhere [23–25].

The experimental data show that some of the studied compounds reveal high antitumor activity. Compound **3d** is the most active with a broad cytostatic activity spectrum. The range of cancer cells growth for **3d** is 13–101% with a mean growth of 53.7%. Compounds **3b** (mean growth 92%, range of growth, 56–115%), **3c** (mean growth 91%, range of growth, 29–113%) and **3g** (mean growth 86%, range of growth, 18–108%) show moderate anticancer activity. Compounds **3f** and **3h** do not show significant antitumor activity. It should be noted that antitumor activity of synthesized benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-ones is selective. Breast cancer cell line MDA-MB-468 is the most sensitive. Compounds **3b, 3c** and **3g** inhibit growth of MDA-MB-468 cells line by 43%, 70% and 81%, respectively. The most active compound **3d** shows cytotoxic activity and reduced initial quantity of MDA-MB-468 cells at 13%. Compound **3d** was evaluated for dose-depended antitumor activity according to phase 2 of NCI protocol. Dose-dependency was studied at five concentrations in 10-fold dilution (100–0.01 μM) at 60 lines of nine cancer cell types at 10-fold dilution (100–0.01 μM, Table 1). As can be seen, the most sensitive line is breast cancer line MDA-MB-468. The GI_{50} value for this line is 0.41 μM.

Conclusion

The treatment of 3-(2-amino-4-R^2-phenyl)-6-R^1-1,2,4-triazin-5(2*H*)-ones with sodium nitrite in acetic acid yielded 3-R^1-8-R^2-10-R^3-2*H*-benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-ones derivatives. Some of the synthesized compounds exhibit anticancer activity against human tumor cells, especially breast cancer cell lines. The most active compound **3d** is highly active against breast cancer MDA-MB-468 cell line (GI_{50}=0.41 μM).

Table 1 Anticancer activity of compound **3d** against individual tumor lines (GI_{50}<4.00 μM).

Cell line/cancer type	3d			Erlotinib[b]		
	GI_{50}, μM[a]	TGI, μM[a]	LC_{50}, μM[a]	GI_{50}, μM[a]	TGI, μM[a]	LC_{50}, μM[a]
Leukemia K-562	3.31	> 100	> 100	34.4	> 100	> 100
Leukemia RPMI-8226	1.32	> 100	> 100	26.24	74.64	74.64
Non-small cell lung cancer NCI-H460	3.31	> 100	> 100	5.83	90.36	90.36
Melanoma LOX IMVI	3.89	> 100	> 100	5.83	> 100	> 100
Melanoma UACC-257	3.55	> 100	> 100	97.7	> 100	> 100
Ovarian cancer OVCAR-4	3.55	> 100	> 100	10.00	> 100	> 100
Renal cancer A498	2.95	> 100	> 100	2.27	51.52	51.52
Prostate cancer PC-3	2.51	> 100	> 100	3.64	> 100	> 100
Breast cancer MCF7	2.63	> 100	> 100	100.0	> 100	> 100
Breast cancer T-47D	3.02	> 100	> 100	3.97	> 100	> 100
Breast cancer MDA-MB-468	0.41	> 100	> 100	0.14	3.64	88.71

[a]GI_{50}, Growth inhibition of 50%; TGI, total growth inhibition; LC_{50}, concentration lethal to 50%.
[b]Erlotinib data were obtained from NCI DTP database (https://dtp.cancer.gov).

Experimental

Melting points were determined in open capillary tubes and were uncorrected. The elemental analyses (C, H, N, S) were performed using the ELEMENTAR vario EL Cube analyzer (USA). ^{1}H NMR spectra (400 MHz) and ^{13}C NMR spectra (100 MHz) were recorded on a Varian-Mercury 400 (Varian Inc., Palo Alto, CA, USA) spectrometer with TMS as internal standard in DMSO-d_6–CCl_4 (1:1) mixture. LC-MS were recorded using a chromatography- mass spectrometric system which consisted of high performance liquid chromatograph Agilent 1100 Series (Agilent, Palo Alto, CA, USA) equipped with diode-matrix and mass-selective detector Agilent LC/MSD SL (atmospheric pressure chemical ionization – APCI). Electron impact mass spectra (EI-MS) were recorded on a Varian 1200 L instrument at 70 eV (Varian, USA). Compounds **1a–j** and **2a–j** were obtained according to the described synthetic protocols [19, 20].

General method for the preparation of 3-R^1-8-R^2-10-R^3-2*H*-benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-ones 3a–j

Compound **2a–j** (5 mmol) was suspended in acetic acid (20 mL) and treated with sodium nitrite (0.52 g, 7.5 mmol). The mixture was stirred at room temperature for 2 h, then cooled, and the resultant precipitate was filtered off, washed by water, dried and crystallized from acetic acid.

3-Methyl-2*H*-benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-one (3a) This compound was obtained as white crystalline powder in 38% yield; mp 226–228°C; ^{1}H NMR: δ 8.62 (d, *J* = 7.8 Hz, 1H, H-11), 8.47 (d, *J* = 7.9 Hz, 1H, H-8), 8.26 (t, *J* = 7.5 Hz, 1H, H-9), 8.17 (t, *J* = 7.5 Hz, 1H, H-10), 2.43 (s, 3H, CH_3); ^{13}C NMR: δ 171.9 (C-2), 166.1 (C-11b), 156.0 (C-3), 150.3 (C-7a), 147.3 (C-10), 146.3 (C-9), 140.2 (C-8), 135.3 (C-11), 129.0 (C-11a), 28.9 (CH_3), LC-MS: *m/z* 214. Anal. Calcd for $C_{10}H_7N_5O$: C, 56.34; H, 3.31; N, 32.85. Found: C, 56.38; H, 3.35; N, 32.89.

3-Phenyl-2*H*-benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-one (3b) This compound was obtained as white crystalline powder in 93% yield; mp 230–232°C; IR: 1662, 1605, 1584, 1549, 1529, 1479, 1444, 1359, 1333, 1311, 1281, 1244, 1206, 1185, 1160, 1119, 1069, 1058, 1029, 1001, 945, 931, 896, 840, 814, 773, 752, 687, 677, 642, 616 cm^{-1}; ^{1}H NMR: δ: 8.71 (d, *J* = 7.2 Hz, 1H, H-11), 8.45 (d, *J* = 7.7 Hz, 1H, H-8), 8.38 (d, *J* = 6.5 Hz, 2H, 3-Ph H-2,6), 8.26 (t, *J* = 7.7 Hz 1H, H-9), 8.17 (t, 1H, *J* = 7.2 Hz, H-10), 7.72–7.41 (m, 3H, 3-Ph H-3,4,5); EI-MS: *m/z* 275 (M^+, 2), 247 (6), 219 (10), 191 (14), 190 (39), 144 (10), 119 (10), 117 (7), 116 (53), 107 (7), 103 (30), 102 (27), 90 (11), 89 (100), 88 (50), 77 (18), 76 (78), 75 (28), 74 (9), 64 (9), 63 (49), 62 (18), 52 (8%); LC-MS: *m/z* 276. Anal. Calcd for $C_{15}H_9N_5O$: C, 65.45; H, 3.30; N, 25.44. Found: C, 65.47; H, 3.32; N, 25.45.

3-(*p*-Tolyl)-2*H*-benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-one (3c) This compound was obtained as white crystalline powder in 85% yield; mp 235–237°C; ^{1}H NMR: δ 8.69 (d, *J* = 7.6 Hz, 1H, H-11), 8.44 (d, *J* = 7.7 Hz, 1H, H-8), 8.31 (d, *J* = 7.7 Hz, 2H, 3-Ph H-2,6), 8.28- 8.21 (t, *J* = 7.2 Hz, 1H, H-9), 8.17 (t, *J* = 7.2 Hz, 1H, H-10), 7.36 (d, *J* = 7.6 Hz, 2H, 3 Ph H-3,5), 2.48 (s, 3H, CH_3); LC-MS: *m/z* 290. Anal. Calcd for $C_{16}H_{11}N_5O$: C, 66.47; H, 3.83; N, 24.21. Found: C, 66.50; H, 3.84; N, 24.26.

3-(4-Isopropylphenyl)-2*H*-benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-one (3d) This compound was obtained as white crystalline powder in 93% yield; mp 207–209°C; ^{1}H NMR: δ: 8.70 (d, *J* = 7.2 Hz, 1H, H-11), 8.44 (d, *J* = 7.3 Hz, 1H, H-8), 8.32 (d, *J* = 6.3 Hz, 2H, 3-Ph H-2,6), 8.25 (t, *J* = 6.6 Hz, 1H, H-9), 8.17 (t, *J* = 6.2 Hz, 1H, H-10), 7.40 (d, *J* = 6.3 Hz, 2H, 3-Ph H-3,5), 3.05–2.96 (m, 1H, $\underline{CH}(CH_3)_2$), 1.33 (d, *J* = 4.3 Hz, 6H, $CH(\underline{CH_3})_2$); LC-MS: *m/z* 318. Anal. Calcd for $C_{18}H_{15}N_5O$: C, 68.13; H, 4.76; N, 22.07. Found: C, 68.17; H, 4.79; N, 22.11.

3-(4-(tert-Butyl)phenyl)-2*H*-benzo[*e*][1,2,4]triazino[2,3-c][1,2,3]triazin-2-one (3e) This compound was obtained as white crystalline powder in 83% yield; mp 187–189°C; ^{1}H NMR: δ: 8.68 (d, *J* = 7.7 Hz, 1H, H-11), 8.44 (d, *J* = 7.8 Hz, 1H, H-8), 8.31 (d, *J* = 7.6 Hz, 2H, 3-Ph H-2,6), 8.26 (t, *J* = 7.4 Hz, 1H, H-9), 8.17 (t, *J* = 7.4 Hz, 1H, H-10), 7.56 (d, *J* = 7.7 Hz, 2H, 3-Ph H-3,5), 1.40 (s, 9H, $C(CH_3)_3$); LC-MS: *m/z* 332. Anal. Calcd for $C_{19}H_{17}N_5O$: C, 68.87; H, 5.17; N, 21.13. Found: C, 68.89; H, 5.21; N, 21.15.

3-(4-Methoxyphenyl)-2*H*-benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-one (3f) This compound was obtained as white crystalline powder in 94% yield; mp 246–248°C; ^{1}H NMR: δ: 8.65 (d, *J* = 8.2 Hz, 1H, H-11), 8.50 (d, *J* = 7.8 Hz, 1H, H-8), 8.40 (d, *J* = 8.6 Hz, 2H, 3-Ph H-2,6), 8.27 (t, *J* = 7.5 Hz, 1H, H-9), 8.19 (t, *J* = 7.5 Hz, 1H, H-10), 7.16 (d, *J* = 8.7 Hz, 2H, 3-Ph H-3,5), 3.89 (s, 3H, OCH_3); LC-MS: *m/z* 306. Anal. Calcd for $C_{16}H_{11}N_5O_2$: C, 62.95; H, 3.63; N, 22.94. Found: C, 62.97; H, 3.66; N, 22.95.

3-(4-Fluorophenyl)-2*H*-benzo[*e*][1,2,4]triazino[2,3-c][1,2,3]triazin-2-one (3g) This compound was obtained as white crystalline powder in 88% yield; mp 337–339°C; ^{1}H NMR: δ: 8.70 (d, *J* = 7.8 Hz, 1H, H-11), 8.55–8.37 (m, 3H, H-8, 3-Ph H-2,6), 8.26 (t, *J* = 7.1 Hz, 1H, H-9), 8.18 (t, *J* = 7.1 Hz, 1H, H-10), 7.32 (t, *J* = 8.5 Hz, 2H, 3-Ph H-3,5); LC-MS: *m/z* 294. Anal. Calcd for $C_{15}H_8FN_5O$: C, 61.43; H, 2.75; N, 23.88. Found: C, 61.47; H, 2.76; N, 23.91.

8-Methyl-3-phenyl-2*H*-benzo[*e*][1,2,4]triazino[2,3-c][1,2,3]triazin-2-one (3h) This compound was obtained as white crystalline powder in 54% yield; mp 216–218°C; IR: 1663, 1596, 1541, 1484, 1465, 1444, 1364, 1338, 1316, 1273, 1209, 1162, 1087, 1074, 1038, 1011, 906, 845, 814, 805, 771, 756, 695, 676 cm^{-1}; ^{1}H NMR: δ 8.51 (d, *J* = 6.9 Hz, 1H, H-11), 8.37 (d, *J* = 7.2 Hz, 2H, 3-Ph H-2,6), 8.12 – 7.95 (m, 2H, H-9,10), 7.58 (m, 3H, 3-Ph H-3,4,5), 2.96 (s, 3H, CH_3); ^{13}C NMR: δ 171.1 (C-2), 160.8 (C-11b), 155.4 (C-3), 152.2 (C-7a), 148.2 (C-10), 146.1 (C-9), 142.7 (C-8), 142.7 (3-Ph, C-4), 140.5 (3-Ph, H-3,5), 139.6 (3-Ph, H-2,6), 133.1 (C-11), 128.9 (C-11a), 27.6 (CH_3); LC-MS: m/z 290. Anal. Calcd for $C_{16}H_{11}N_5O$: C, 66.43; H, 3.83; N, 24.21. Found: C, 66.44; H, 3.85; N, 24.22.

10-Bromo-3-phenyl-2*H*-benzo[*e*][1,2,4]triazino[2,3-*c*][1,2,3]triazin-2-one (3i) This compound was obtained as white crystalline powder in 82% yield; mp 248–250°C; ^{1}H NMR: δ: 8.77 (s, 1H, H-11), 8.46–8.24 (m, 1H), 7.66–7.48 (m, 4H, H-8, 9, 3-Ph H-2,6); EI-MS: *m/z* 355 (M^+, 2), 190 (39), 156 (6), 154 (9), 120 (10), 119 (19), 115 (28), 105 (7), 103 (35), 100 (8), 90 (26), 89 (100), 88 (31), 77 (13), 76 (9), 75 (38), 74 (8), 73 (10), 65 (7), 63 (48), 62 (11), 61 (10), 54 (8), 50 (8); LC-MS: *m/z* 355. Anal. Calcd for $C_{15}H_8BrN_5O$: C, 50.87; H, 2.28; N, 19.77. Found: C, 50.89; H, 2.32; N, 19.81.

10-Bromo-3-(4-fluorophenyl)-2*H*-benzo[*e*][1,2,4]triazino[2,3-c][1,2,3]triazin-2-one (3j) This compound was obtained as white crystalline powder in 95% yield; mp 237–239°C; ^{1}H NMR: δ: 8.78 (s, 1H, H-11), 8.55 – 8.46 (m, 2H, 3-Ph H-2,6), 8.44–8.31 (m, 2H, H-8,9), 7.32 (t, *J* = 8.6 Hz, 2H, 3-Ph H-3,5); LC-MS: *m/z* 371. Anal. Calcd for $C_{15}H_7BrFN_5O$: C, 48.41; H, 1.90; N, 18.82. Found: C, 48.44; H, 1.93; N, 18.84.

Cytotoxic activity against malignant human tumor cells

Primary anticancer assay was performed at 60 human tumor cell lines panel derived from nine neoplastic diseases, in accordance with the protocol of the Drug Evaluation Branch, National Cancer Institute, Bethesda [23–25]. Three dose response parameters were calculated for each compound. Growth inhibition of 50% (GI_{50}) – drug concentration resulting in a 50% lower net protein increase in the treated as compared to the net protein increase seen in the control cells, the drug concentration resulting in total growth inhibition (TGI), and LC_{50} concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning. The lowest values were obtained with the most sensitive cell lines.

X-ray crystallographic study

The colorless crystals **3h** ($C_{16}H_{11}N_5O$) are monoclinic. At 193 K, a=7.9168(4), b=11.0274(4), c=15.7059(6) Å, β=102.618(4)°, V=1338.0(1) Å^3, M_r=289.30, Z=4, space group $P2_1/n$, d_{calc}=1.436 g/cm^3, μ(MoK_α)=0.096 mm^{-1}, F(000)=600. Intensities of 12 976 reflections (3889 independent, R_{int}=0.022) were measured on the Xcalibur-3 diffractometer (graphite monochromated MoK_α radiation, CCD detector, ω-scaning, $2\Theta_{max}$=60°).

The structure was solved by direct method using SHELXTL package [26]. Positions of the hydrogen atoms were located from electron density difference maps and refined by a riding model with U_{iso}=nU_{eq} (n=1.5 for methyl group and n=1.2 for other hydrogen atoms) of the carrier atom. Full-matrix least-squares refinement against F^2 in anisotropic approximation for non-hydrogen atoms using 3805 reflections was converged to wR_2=0.125 (R_1=0.043 for 2714 reflections with F>4σ(F), S=1.025). The final atomic coordinates, and crystallographic data for molecule **3h** were deposited to with the Cambridge Crystallographic Data Centre, 12 Union Road, CB2 1EZ, UK (fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk). They are available on request quoting the deposition number CCDC1408960).

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