Novel approach to bis(indolyl)methanes: *De novo* synthesis of 1-hydroxyiminomethyl derivatives with anti-cancer properties

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**A R T I C L E  I N F O**

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**A B S T R A C T**

A versatile and broad range approach to previously unknown bis(indolyl)methane oximes based on two consecutive hetero Diels–Alder cycloaddition reactions of electrophilic conjugated nitrosoalkenes with indoles is disclosed. The cytotoxic properties and selectivity of some adducts against several human cancer cell lines pointing to a promising role in the development of anti-tumoural drugs, in particular for leukaemia and lymphoma.

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**1. Introduction**

Bis(indolyl)methanes (BIMs) isolated from marine or terrestrial matrices exhibit a wide range of biological activities including anticancer activity against various types of tumour cells [1] (Scheme 1). Naturally occurring BIMs such as vibrindole (1) are useful in the treatment of fibromyalgia, chronic fatigue, and irritable bowel syndrome [2–4]. Furthermore, various studies point out that 3, 3'-diindolylmethane (DIM) (2) possesses protective cancer activity, especially against mammary and prostate tumour cells [5]. Additionally, DIM inhibits mammary tumour growth in rodents [6] and also exhibits potent antiproliferative and anti-androgenic properties in androgen-dependent human prostate cancer cells [7,8].

Synthetic bis(indolyl)methanes have been used not only in biological applications but also as dyes and colorimetric sensors [19–26]. Consequently, a great deal of interest and effort has been put into the development of efficient synthetic protocols for the preparation of these molecules. The vast majority of synthetic routes involve the reaction of indole with various carbonyl compounds or their synthetic equivalents in the presence of acid, base or metal catalyst (Scheme 2) [27–44].

Although several elegant, efficient and environment friendly methods producing good yields and selectivity have been reported, in most of the above reactions the resulting BIMs generally lacked functionalities, especially at the methylene bridge, which could allow an easy manipulation or conversion into more elaborate compounds, eventually of greater interest and value.

Conjugated nitrosoalkenes, unsubstituted or bearing C- or P-bonded functional groups at the 4-position, have been at the basis of the preparation of an impressive plethora of new heterocyclic systems, used either as electron-deficient heterodienes in cycloaddition or as Michael-type acceptors in conjugate 1,4-addition reactions [45–50].

Although in a much smaller extent, nitrosoalkenes bearing a good leaving group at the 4-position have also been explored. The presence of such as functionality provided an extra feature and was of added value in the subsequent manipulation of adducts and cycloadducts so formed [51–54]. Indeed, we recently described a novel route to 5-substituted dipyrromethanes by bis-hetero-

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Diels–Alder reaction of conjugated azo- and nitrosoalkenes with pyrrole. Base-mediated dehydro-halogenation of α,α′-dihaloaldenones or α,α′-dihaloaldoximes in the presence of pyrrole led to two consecutive Diels–Alder reactions giving the target dipyrromethanes [51]. As part of our continuing interest and investigation on hetero-Diels-Alder reactions of electron-deficient conjugated nitrosoalkenes [55–60], this work describes the possibility of synthesising BIM oximes, a new class of compounds, through a related but versatile and very broad scope approach. The new compounds were assayed for their in vitro cytotoxicity on several human cancer cell lines.

2. Results and discussion

The one-pot synthetic strategy to bis(indolyl)methanes is outlined in Scheme 3. The starting α,α′-dihaloaldenogeno oximes 3 were efficiently prepared from the respective ketones by known procedures [58,61]. These compounds, in the presence of base, were converted, in situ, into the corresponding transient and reactive nitrosoalkenes 4, which were intercepted by a first molecule of the appropriate indole 5 originating the intermediate indole oximes 6. The initially formed tetrahydroxazines undergo ring-opening to the corresponding transient and reactive nitrosoalkenes 7 which reacted with a second molecule of indole, producing the target bis(indolyl)methanes 8.

The results obtained are summarised in Table 1. The reaction yields may be considered generally good, taking into account that the synthetic process involves a sequence of reactions. On the other hand, no other products could be obtained, which indicates that the reactions were regioselective. The results have shown also that both alkyl and aryl oximes can be used in the synthesis of bis(indolyl)methanes. Starting from aryl oximes 9a–f the expected (E)-oximes 9 were obtained as single or major products (Entries 1–11) whereas alkyl oxime 3g reacted with indole to give the (Z)-oxime 10g as the major product (Entries 12–13). The stereochemistry assignment of oximes 9 and 10 was confirmed by analysis of the NOESY spectra of 9d, 9g, 10d and 10g. Connectivity was observed between the hydroxyl proton and the phenyl protons and the methyl protons, respectively, whereas in the case of 9d and 9g no connectivity was observed. Moreover, oximes 9 and 10 are also characterized by 1H NMR spectra with different features. The chemical shift of the methylene proton appears at higher value for (E)-oximes 9 (9b: δ = 6.81 ppm; 9d: δ = 6.82 ppm; 9g: δ = 6.39 ppm) than for the corresponding (Z)-oximes 10 (10b: δ = 5.74 ppm; 10d: δ = 5.77 ppm; 10g: δ = 5.41 ppm).

The synthesis of two isomeric oximes from the reaction of aryl nitrosoethylenes with pyrrole and dipyrromethanes has been previously observed [62]. The process was rationalized considering the conjugate addition of the heterocycle to the nitrosoalkene, at the s-cis or s-trans conformation, followed by rearomatization of the pyrrole unit leading to (E)- and (Z)-oxime, respectively. Thus, the synthesis of the BIM oximes via 1,4-conjugate addition of indole to the nitrosoalkene cannot be ruled out.

The use of water as solvent in Diels–Alder reactions has been shown to be advantageous, not only in environmental terms but also inducing critical improvements in reaction times, yields and selectivity [51,63]. We observed that carrying out the synthesis of bis(indolyl)methanes via 1,4-conjugate addition of indole to the nitrosoalkene cannot be ruled out.

The efficiency of the reaction, using H2O/CH2Cl2 system, amongst the nitrosoalkenes bearing halogenated aryl substituents increases in the order F > Cl > Br > H the order of electron-withdrawing ability and consequently the order of the expected effectiveness for an inverse electron demand Diels–Alder reaction (entries 2, 5, 7 and 9). However, the isolated yields from the reaction carried out in CH2Cl2 do not reflect the expected reactivity, which can be explained considering differences in the efficiency of the purification process.

The cytotoxicity of compounds 9a, 9e and 9d was evaluated in different tumoural cell lines, namely HepG2 (hepatocellular carcinoma), MDA-MB-468 (human breast carcinoma), RAW 264.7 (murine leukaemic monocyte macrophages), THP1 (human acute monocytic leukaemia), U937 (human leukaemic monocytic lymphoma) and EL4 cells (murine T-lymphoma). The compounds’ selectivity towards tumoural cells was assessed determining their cytotoxicity with respect to two non-tumoural derived cell lines S17 (murine bone marrow) and N9 cells (murine microglial).

Results of the half maximal concentrations (IC50) are shown in Table 2 together with the toxicity of etoposide, a known antitumour drug. Compound 9e was considerably less cytotoxic on tumoural cell lines than the other two compounds, with IC50 values ranging from 35.7 (HepG2) to 124 μM (THP1) and was not selective. Compounds 9a and 9d, however, were considerably cytotoxic to all cells tested, with IC50 values ranging from 1.62 (THP1) to 23.9 μM (RAW) and from 10.7 (MDA) to 34.1 μM (U937), respectively. Compound 9a was particularly active against non-adherent cell lines with IC50 values ranging from 1.62 in THP1 to 1.65 μM in EL4.

Some conclusions regarding structure–activity relationships can be redrawn based on the biological evaluation of these bis(indolyl)methanes. There is a dramatic difference in anticancer activity between N-unsubstituted bis(indolyl)methanes 9a and the N-methyl substituted derivative 9e, the latter characterized by high IC50 values. On the other hand, the significantly lower IC50 values observed for 9a for non-adherent cell lines in comparison with the ones obtained for 9d demonstrates that the presence of the bromo substituent leads to higher cytotoxic activity.
The observed high cytotoxicity of compound 9a against THP1, EL4 and U937 cell lines led us to extend the study to BIMs 9c, 9g and 10g (Table 3). Compound 9c, bearing a 4-fluorophenyl substituent, showed moderate anti-cancer activity which reinforces the observation that the 4-bromophenyl group is crucial to ensure low IC50 values. On the other hand, alkyl oximes 9g and 10g were even less cytotoxic against THP1, EL4 and U937 cell lines. None of these compounds were selective towards the tumoural cell lines (selectivity index calculated for non-tumour cell line S17).

In addition to having displayed higher toxicity towards the non-tumoural cell lines than all the studied compounds, compound 9a demonstrated the highest selectivity indexes: 9.86–14.2. Further studies using 9a as scaffold in the development of anti-tumoural drugs for leukaemia and lymphoma is worth pursuing since it presents lower IC50 and higher selectivity than etoposide.

3. Conclusions

The reliable preparation of a variety of unknown BIMs bearing different oxime substituents at the methylene bridge was presented. This strategy, supported on the robust and proved methodology of Diels–Alder cycloaddition reactions of electrophilic nitrosoalkenes with electron rich indoles, may pave the way for the synthesis of a vast library of new compounds. The selection of the

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>X</th>
<th>R1</th>
<th>R2</th>
<th>Solvent</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>p-BrC6H4</td>
<td>Br</td>
<td>H</td>
<td>H</td>
<td>CH2Cl2</td>
<td>9a 34%</td>
</tr>
<tr>
<td>2</td>
<td>p-BrC6H4</td>
<td>Br</td>
<td>H</td>
<td>H</td>
<td>H2O/CH2Cl2</td>
<td>9a 55%</td>
</tr>
<tr>
<td>3</td>
<td>p-ClC6H4</td>
<td>Br</td>
<td>H</td>
<td>H</td>
<td>CH2Cl2</td>
<td>9b 80%</td>
</tr>
<tr>
<td>4</td>
<td>p-ClC6H4</td>
<td>Br</td>
<td>H</td>
<td>H</td>
<td>H2O/CH2Cl2</td>
<td>9b 64%</td>
</tr>
<tr>
<td>5</td>
<td>p-FC6H4</td>
<td>Br</td>
<td>H</td>
<td>H</td>
<td>CH2Cl2</td>
<td>9c 33%</td>
</tr>
<tr>
<td>6</td>
<td>p-FC6H4</td>
<td>Br</td>
<td>H</td>
<td>H</td>
<td>H2O/CH2Cl2</td>
<td>9c 71%</td>
</tr>
<tr>
<td>7</td>
<td>Ph</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>CH2Cl2</td>
<td>9d 44%</td>
</tr>
<tr>
<td>8</td>
<td>Ph</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>H2O/CH2Cl2</td>
<td>9d 42%</td>
</tr>
<tr>
<td>9</td>
<td>p-BrC6H4</td>
<td>Br</td>
<td>Me</td>
<td>H</td>
<td>CH2Cl2</td>
<td>9e 63%</td>
</tr>
<tr>
<td>10</td>
<td>p-BrC6H4</td>
<td>Br</td>
<td>Me</td>
<td>H</td>
<td>H2O/CH2Cl2</td>
<td>9e 70%</td>
</tr>
<tr>
<td>11</td>
<td>p-BrC6H4</td>
<td>Br</td>
<td>H</td>
<td>Br</td>
<td>H2O/CH2Cl2</td>
<td>9f/10f 52%</td>
</tr>
<tr>
<td>12</td>
<td>Me</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>CH2Cl2</td>
<td>9g 9%</td>
</tr>
<tr>
<td>13</td>
<td>Me</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>H2O/CH2Cl2</td>
<td>10g 19%</td>
</tr>
</tbody>
</table>

* Mixture of isomeric oximes.
substitution pattern of the starting oximes and/or indoles allows the easy modulational of the structure.

Furthermore, this class of compounds showed very interesting anti-cancer activity in particular against leukaemia and lymphoma cell lines. Interestingly, the most active bis(indoly)methane studied presents lower IC50 and higher selectivity for these cell lines than etoposide.

The preparation of unsymmetrical BIMs by the reported methodology as well as the tuning of structural modifications, imparting enhanced biological activity, are under investigation.

4. Experimental

4.1. Chemistry

4.1.1. General considerations

4.1.2. General procedure for the synthesis of BIM oximes

4.1.2.1. Method A

4.1.2.2. Method B

4.1.2.4. (E)-1-((4-Chlorophenyl)-1-hydroxyiminomethyl)bis(1H-indol-3-yl)methane (9b).

4.1.2.5. (E)-1-(4-Fluorophenyl)-1-hydroxyiminomethyl)bis(1H-indol-3-yl)methane (9c).

4.2. Spectroscopic data

4.3. Biological activity

4.3.1. Cytotoxicity of compounds 9a, 9e and 9d and etoposide (positive control) against several tumoural and non-tumoural cell lines. Results are expressed as IC50 values (mean ± SEM) and the selectivity index was calculated for two non-tumour cell lines (S17 and N9).

<table>
<thead>
<tr>
<th></th>
<th>Etoside</th>
<th>9a</th>
<th>9e</th>
<th>9d</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (μM)</td>
<td>SI (S17)</td>
<td>SI (N9)</td>
<td>IC50 (μM)</td>
<td>SI (S17)</td>
</tr>
<tr>
<td>HepG2</td>
<td>1.40 ± 0.10</td>
<td>8.97</td>
<td>1.71</td>
<td>35.7 ± 0.40</td>
</tr>
<tr>
<td>MDA</td>
<td>6.69 ± 0.44</td>
<td>3.68</td>
<td>0.88</td>
<td>63.7 ± 0.57</td>
</tr>
<tr>
<td>RAW</td>
<td>0.82 ± 0.03</td>
<td>9.55</td>
<td>1.88</td>
<td>42.2 ± 0.41</td>
</tr>
<tr>
<td>THP1</td>
<td>1.82 ± 0.06</td>
<td>8.55</td>
<td>1.81</td>
<td>124 ± 11.5</td>
</tr>
<tr>
<td>EL4</td>
<td>4.90 ± 0.47</td>
<td>5.47</td>
<td>0.80</td>
<td>106 ± 10.5</td>
</tr>
<tr>
<td>U937</td>
<td>1.10 ± 0.04</td>
<td>9.27</td>
<td>1.85</td>
<td>97.0 ± 4.14</td>
</tr>
<tr>
<td>S17</td>
<td>10.4 ± 0.15</td>
<td>16.3 ± 0.14</td>
<td>51.4 ± 1.07</td>
<td>14.23</td>
</tr>
<tr>
<td>N9</td>
<td>1.95 ± 0.12</td>
<td>23.0 ± 0.34</td>
<td>19.8 ± 0.74</td>
<td>29.2 ± 0.29</td>
</tr>
</tbody>
</table>

4.3.2. Cytotoxicity of compounds 9c, 9g and 10g against two tumoural cell lines (THP1, EL4 and U937). Results are expressed as IC50 values (mean ± SEM) and the selectivity index was calculated for non-tumour cell line S17.

<table>
<thead>
<tr>
<th></th>
<th>9c</th>
<th>9g</th>
<th>10g</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (μM)</td>
<td>SI (S17)</td>
<td>IC50 (μM)</td>
<td>SI (S17)</td>
</tr>
<tr>
<td>THP1</td>
<td>23.0 ± 0.9</td>
<td>0.89</td>
<td>30.7 ± 0.1</td>
</tr>
<tr>
<td>EL4</td>
<td>16.9 ± 1.6</td>
<td>1.47</td>
<td>23.9 ± 0.9</td>
</tr>
<tr>
<td>U937</td>
<td>28.0 ± 1.6</td>
<td>1.08</td>
<td>42.5 ± 0.5</td>
</tr>
<tr>
<td>S17</td>
<td>24.9 ± 0.1</td>
<td>26.1 ± 0.4</td>
<td>35.8 ± 0.4</td>
</tr>
</tbody>
</table>
10.88 (br s, 2H), 11.64 (s, 1H) ppm; \(^{13}C\) NMR (100 MHz, DMSO-d6) \(\delta = 30.3, 111.5, 113.2, 114.3\) (d, \(J = 2.2\) Hz) 118.4, 118.7, 121.1, 123.9, 126.9, 129.7 (d, \(J = 8.1\) Hz), 132.9 (d, \(J = 2.8\) Hz), 136.2, 156.4, 161.8 (d, \(J = 244\) Hz) ppm. MS (ESI): \(m/z (%): 384\) (100) [MH+]. HRMS (ESI): calcld. for \(m/z\) C24H20N3O [MH+] 384.15030, found 384.15067.

4.2.1.6. (E)-1-((1-Oxidooximino-phenyl)methyl)bis(1H-indol-3-yl)methane (9d) and (Z)-1-((1-Oxidooximino-phenyl)methyl)bis(1H-indol-3-yl)methane (10d). Yield: 42% 9d and 12% 10d (Method A), 44% 9d (Method B). IR (KBr) \(v_{\text{max}}\) = 3410, 1705, 1458, 1373, 1249, 1045, 744, 696 cm\(^{-1}\). Data for 9d (isomer E): white solid, m.p. 183.4–184.8 °C (from dichloromethane). \(^1H\) NMR (400 MHz, DMSO-d6) \(\delta = 6.82\) (s, 1H), 6.88 (br s, 2H), 6.94 (t, \(J = 7.2\) Hz, 2H), 7.08 (t, \(J = 7.2\) Hz, 2H), 7.14–7.17 (m, 3H), 7.37 (d, \(J = 8.0\) Hz, 2H), 7.40–7.42 (m, 2H), 7.45 (d, \(J = 8.0\) Hz, 2H), 10.85 (br s, 2H), 11.59 (s, 1H) ppm; \(^{13}C\) NMR (100 MHz, DMSO-d6) \(\delta = 30.3, 111.5, 113.4, 118.3, 118.7, 121.1, 123.9, 126.9, 127.5, 127.6, 127.9, 136.1, 136.6, 157.2 ppm. Data for 10d (isomer Z): white solid, m.p. 202.3–203.4 °C (from dichloromethane). \(^1H\) NMR (400 MHz, DMSO-d6) \(\delta = 5.77\) (s, 1H), 6.96 (d, \(J = 7.2\) Hz, 2H), 7.00 (t, \(J = 7.2\) Hz, 2H), 7.11 (t, \(J = 7.2\) Hz, 7.29–7.32 (m, 4H), 7.39 (d, \(J = 8.0\) Hz, 2H), 7.63 (d, \(J = 8.0\) Hz, 2H), 8.04 (d, \(J = 8.0\) Hz, 2H), 10.44 (s, 1H) ppm; \(^{13}C\) NMR (100 MHz, DMSO-d6) \(\delta = 40.2, 114.2, 114.8, 114.8, 118.2, 120.8, 123.9, 126.9, 127.5, 127.7, 128.1, 135.1, 136.4, 156.6 ppm. MS (ESI): \(m/z\) (%): 366 (100) [MH+]. HRMS (ESI): calcld. for \(m/z\) C24H20N3O [MH+] 366.1601, found 366.1592.

4.2.1.7. (E)-1-((4-Bromophenyl)-1-hydroximino)methyl)bis(1H-indol-3-yl)methane (9e) and (Z)-1-((4-Bromophenyl)-1-hydroximino)methyl)bis(1H-indol-3-yl)methane (10e). The product was obtained as a mixture of (E) and (Z) isomers. Yield: 52% (Method B); light brown solid, m.p. 161.3–162.7 °C (from dichloromethane). IR (KBr) \(v_{\text{max}}\) = 3276, 3224, 1485, 1331, 1011, 939 cm\(^{-1}\). \(^1H\) NMR (400 MHz, DMSO-d6) \(\delta = 3.70\) (s, 3H), 6.78 (s, 1H), 6.96–7.00 (m, 4H), 7.14 (t, \(J = 7.2\) Hz, 2H), 7.34–7.45 (m, 9H), 11.76 (s, 1H) ppm; \(^{13}C\) NMR (100 MHz, DMSO-d6) \(\delta = 30.2, 124.3, 109.7, 112.3, 116.8, 117.0, 121.3, 121.4, 127.2, 128.2, 129.5, 130.5, 135.4, 136.6, 156.1 ppm. MS (ESI): \(m/z\) (%): 471 (100) [MH+]. HRMS (ESI): calcld. for \(m/z\) C26H23BrN3O [MH+] 471.0946, found 471.0953.

4.2.1.8. (E) and (Z)-1-((4-Bromophenyl)-1-hydroximino)methyl)bis(5-bromo-1H-indol-3-yl)methane (9f) and (10f). Where VNT and VT indicate IC50 modulated by the compound on the end of the incubation period, 20 µL of MTT (5 mg/mL in PBS) were added to each well and further incubated at 37 °C in humid atmosphere with 5% CO2.

4.2.2. Cytotoxicity assay

The cytotoxic activity of the extracts was determined by the MTT colorimetric assay [64]. Briefly, exponentially growing cells were plated in 96-well tissue plates at a density of 5 × 10^3 cells/well (HepG2, MDA-MB-468 and S17) or 1 × 10^4 cells/well (THP1, U937, RAW 264.7 and N9). Adherent cell lines were previously incubated for 24 h to ensure adhesion to the wells. Extracts were applied at various concentrations (ranging between 0.078 and 200 µM) and control cells were treated with DMSO at the highest concentration used in test wells (0.5%). Cell viability was determined after 72 h of incubation with the extracts/DMSO. 2 h prior to the end of the incubation period, 20 µL of MTT (5 mg/mL in PBS) were added to each well and further incubated at 37 °C. 150 µL of DMSO were afterwards added to each well in order to dissolve the formazan crystals and absorbance was measured at 590 nm (Biotek Synergy 4). Results were expressed in half maximal inhibitory concentration (IC50, µM). The selectivity of the extracts was estimated using the following equation: Selectivity = IC50 VNT/IC50 VT, where VNT and VT indicate IC50 modulated by the compound on non-tumour and tumour cells, respectively.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.01.050.


