Turkish Journal of Chemistry

http://journals.tubitak.gov.tr/chem/

Research Article

Turk J Chem
(2019) 43: 963 – 971

doi:10.3906/kim-1812-54

Bisbenzoxazole derivatives had an antiinflammatory effect on in vitro stimulated macrophages

Furkan AYAZ[1,*], **Rusmeenee KHEEREE**[1], **Qadar Ahmed ISSE**[1], **Ronak Haj ERSAN**[2], **Öztekin ALGÜL**[2]

[1]Department of Biotechnology, Faculty of Arts and Science, Mersin University, Mersin, Turkey
[2]Faculty of Pharmacy, Mersin University, Mersin, Turkey

Received: 24.12.2018 • **Accepted/Published Online:** 08.04.2019 • **Final Version:** 11.06.2019

Abstract: Benzoxazoles are DNA base bioisosteres and studies suggest that their derivatives have antiproliferative activities. Based on their antiproliferative activities they have been mostly studied as new generation anticancer drugs. In our study we exploited their antiproliferative effect, aiming to delineate bisbenzoxazole derivatives' (RHE 231 and RHE 238) potential antiinflammatory effect on mouse macrophages that are activated in vitro through danger signal LPS stimulation. RAW 267.4 mammalian macrophages were activated in the presence of our derivatives with or without danger mimic *E. coli* derived LPS. We present data that support the strong antiinflammatory activity of the bisbenzoxazole derivatives RHE 231 and RHE 238 on stimulated mammalian macrophages. There was a significant and substantial decrease in the production levels of TNF-α, IL-1β, and IL-6 proinflammatory cytokines in the presence of RHE 231 and RHE 238. These molecules had an antiproliferative effect on the macrophages and, probably, this was their mechanism of action on the cells to alter their inflammatory functions. Our results show that bisbenzoxazole structures RHE 231 and RHE 238 have potential to be used as antiinflammatory drug agents.

Key words: Bisbenzoxazole, inflammation, macrophage, antiinflammatory, medicinal chemistry, antiproliferative agents

1. Introduction

One of the essential types of cells in the human immune system is macrophages. They play a significant role in the development of a proper immune response. Other than their direct effect via cytokine production and phagocytosis they also activate other immune cells by antigen presentation [1–7].

Macrophages have an active role as the first line of defense against infections in living organisms; these functions include pathogen recognition and enhancement of bactericidal activities [1–7]. Stimulated or activated macrophages produce proinflammatory cytokines in response to danger signals and these signals lead to inflammation in the tissue [1–14]. Inflammation aims to encircle the pathogen or danger molecule in the affected tissue and later eradicate it [1–14]. After elimination of the danger, antiinflammatory cytokines are produced and macrophages produce some of these to clear the tissue and start the wound healing process [1–14].

The aim of inflammatory reactions is to protect endangered tissue. Associated tissue injury is a result of an excess and persistent inflammatory reaction, and this situation can eventually become life-threatening, such as anaphylaxis and septic shock. In addition, patients with chronic inflammation also suffer from certain associated diseases such as obesity, cardiovascular disorders, and cancer [15–29].

*Correspondence: furkanayaz@mersin.edu.tr

It has been shown by other studies that pathogen-associated molecular patterns act as danger signals and can activate the immune cells [1–7]. Lipopolysaccharides (LPSs) from the cell wall of gram-negative bacteria have been a good tool in in vitro studies in order to mimic the danger and activate macrophages to stimulate the production of proinflammatory cytokines [7–14]. These proinflammatory cytokines include tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), granulocyte macrophage stimulating factor (GMCSF), interleukin 12p40 (IL12p40), and interleukin 6 (IL-6) [7–14].

TNF-α was initially described for its ability to induce necrosis and stimulate the acute phase reactions of the immune system; it was known to be the first cytokine type that is released in response to an inflammatory stimulus [7–14]. However, excess amounts of TNF-α can be harmful, since it can endanger the organism with its role in the induction of necrosis, fever, vasodilation, and synthesis of acute phase proteins like C reactive protein (CRP) [7–14]. Similar to TNF-α, IL-1β is also produced and released in the early stages of the immune response. IL-1β leads to secretion of prostaglandins and production of CRP from the liver [7–14]. Moreover, IL-1β can induce the release of histamines from the mast cells, which causes further problems in patients with chronic inflammatory systemic diseases [7–14].

IL-6 differs from TNF-α and IL-1β in terms of its functions [7–14]. It can act in both proinflammatory and antiinflammatory ways during the tissue repair stage at the end of inflammation [7–14]. Similar to TNF-α and IL-1β, IL-6 promotes fever and the synthesis of CRP [7–14]. IL-6 is a soluble cytokine that plays an important role in the migration of monocytes into the inflammatory site and their maturation during this process, and is also associated with T-cell functions, such as maintenance of Th17 cells, inhibition of T-cell apoptosis, and development of Tregs [7–14].

Although inflammatory responses are the first line of defense against infections, excessive and chronic cases turn out to be harmful [15–29]. These inflammatory disorders include common chronic inflammatory systemic diseases (CIDs) and autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis [15–29]. Molecules that can modulate the immune system cells create new alternatives as therapeutics to regulate the immune system cells according to our need in certain disease conditions [15–30].

Previous studies reported antiproliferative, anticancer, and antiinflammatory activities for similar molecules [30–36]. Benzoxazole derivatives interfere with DNA synthesis due to their structural similarities to the nucleotides of DNA [30–36]. RHE 231 and RHE 238 are bisbenzoxazole derivatives that were designed to suppress antiinflammatory activities. In the present study we aimed to decipher the immunomodulatory roles of these benzoxazole derivatives.

2. Results and discussion

2.1. RHE 231 and RHE 238 did not change the live cell percentages in macrophage samples after 24 h of incubation

RHE 231 and RHE 238 were synthesized as specified in the Experimental section and Supplementary Information. Their characterization studies were conducted by taking IR and NMR spectra (S1–S6). In order to assess cell viability in the presence of our chemicals, cell viability measurements were conducted. Under all conditions, including the negative control, which was treated with dimethyl sulfoxide (DMSO), the positive control, which was treated with combination of LPS and DMSO, and 50 μg/mL, 75 μg/mL, and 100 μg/mL of RHE 231 or RHE 238 with or without LPS, macrophages had 100% cell viability (Figure 1). These results support the biocompatibility of our reagents on macrophage cells in terms of cell viability.

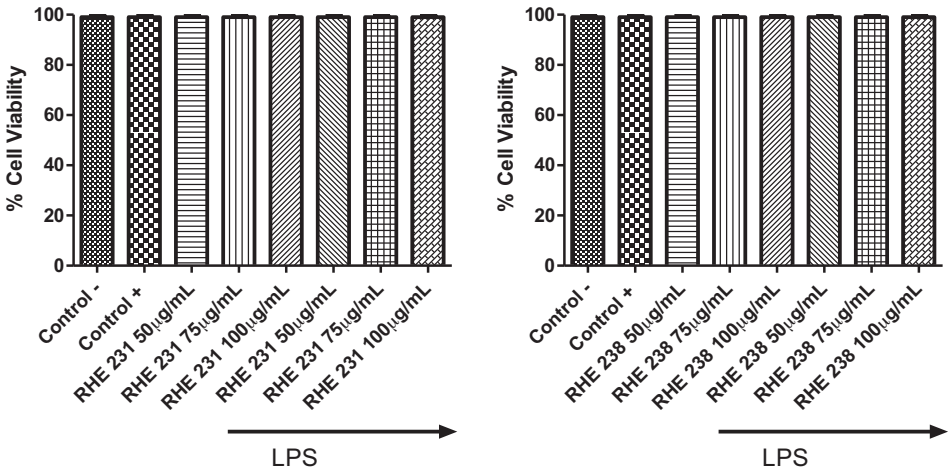


Figure 1. Percentage of RAW 264.7 cell viability in the presence of bisbenzoxazole derivatives. RAW macrophages were stimulated with RHE 231 and RHE 238 for 24 h in different concentrations, including 50, 75, and 100 μg/mL, with or without LPS. DMSO was used for negative control wells, 1 μg/mL LPS and DMSO were used for positive control groups. After 24 h percentage of viable cells was counted with Trypan blue staining.

2.2. RHE 231 and RHE 238 led to decreased cell proliferation in macrophages after 24, 48, and 72 h of incubation

RHE 231 and RHE 238 had a strong antiproliferative effect on mouse macrophages at all concentrations. Even at the earliest time point and the lowest concentrations used of both chemicals, there was a substantial and significant decrease in the cell proliferation of these cells (Figure 2). This implies that our reagents were exerting their antiinflammatory roles possibly by decreasing the cell proliferation and metabolism in the macrophages. Since by Trypan blue staining we did not observe any change in the cell viability percentages, RHE 231 and RHE 238 did not have cytotoxic effect on macrophages but rather they had an antiproliferative effect.

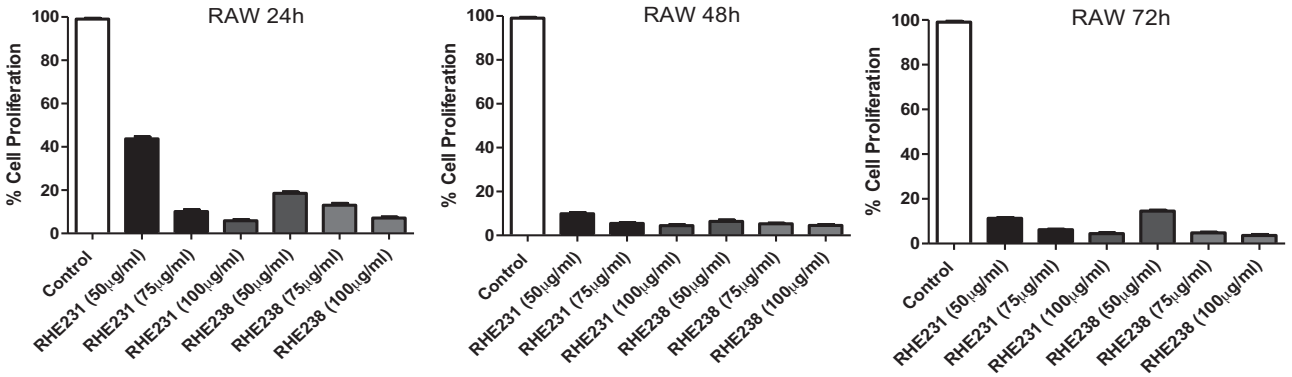


Figure 2. Dose-dependent effect of the compounds RHE 231 and RHE 238 on the proliferation rate of RAW 264.7 macrophage cells after 24 h, 48 h, and 72 h of incubation. Macrophages were treated with RHE 231 and RHE 238 in three different concentrations: 50 μg/mL, 75 μg/mL, and 100 μg/mL for 24 h, 48 h, and 72 h. MTT assay was used to assess the rate of cell proliferation. Two-tailed t-test was applied for the statistical analysis, $P < 0.0001$, $N = 9$.

2.3. RHE 231 and RHE 238 treatment led to complete knockout of TNF-α production

In order to measure TNF-α secretion, macrophages were treated with 50 μg/mL, 75 μg/mL, and 100 μg/mL RHE 231 or RHE 238 with or without LPS and then supernatants were collected for ELISA. There was a significant decrease in TNF-α secretion by the LPS stimulated macrophages in the presence of our chemicals compared to only LPS treated positive control macrophages. In addition, our chemicals did not stimulate macrophages to secrete TNF-α by themselves (Figure 3). Therefore, our reagents had an immunomodulatory role rather than exerting immunostimulatory effects.

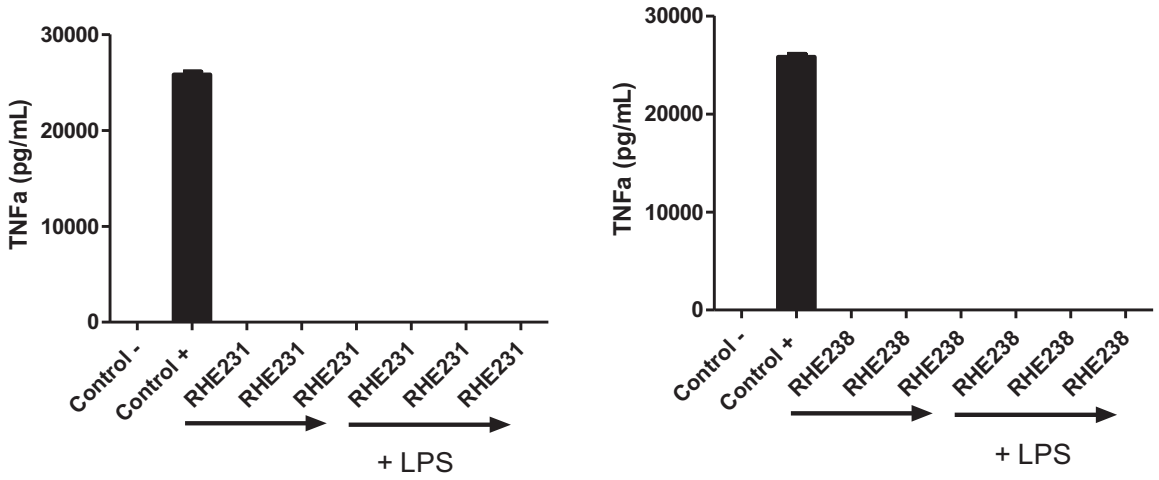


Figure 3. Production of TNF-α. Macrophages were treated with 50, 75, and 100 μg/mL RHE 231 and RHE 238 with or without LPS. DMSO was used in negative control wells, 1 μg/mL LPS and DMSO were used in the positive control groups. TNF-α ELISA was applied to the supernatants to measure the TNF-α production after 24 h of incubation. Two-tailed t-test was applied for the statistical analysis, $P < 0.0001$, $N = 9$.

2.4. Interleukin-1β production was significantly and completely abrogated by RHE 231 and RHE 238 treatment

Similar to changes in the TNF-α production patterns of LPS stimulated macrophages, in the presence of RHE 231 and RHE 238 at all concentrations used there was a significant decrease in IL-1β production compared to positive control wells after 24 h (Figure 4).

2.5. Interleukin-6 production was significantly reduced in the presence of RHE 231 and RHE 238 in LPS activated macrophages but these reagents were able to stimulate IL-6 production by themselves even in the absence of a danger signal

IL-6 cytokine production was stimulated by the presence of our chemicals RHE 231 and RHE 238. These reagents stimulated the macrophages by themselves to produce IL-6. In the presence of an LPS signal our reagents caused a significant drop in the production of IL-6 compared to only LPS treated positive control macrophages (Figure 5). At higher doses of RHE 231 and RHE 238 this immunostimulatory role was suppressed compared to at lower concentrations of the chemicals (Figure 5). Therefore, at higher concentrations our reagents were suppressing their own immunostimulatory functions on macrophages.

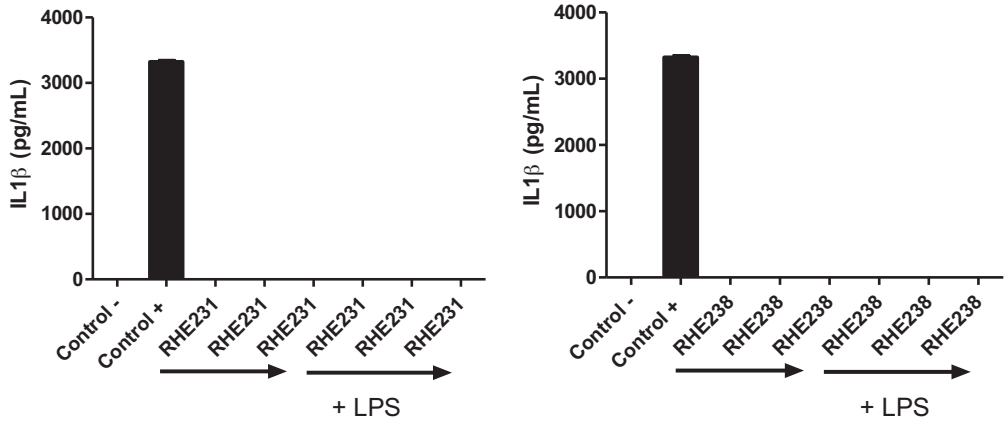


Figure 4. Production of IL-1β. Macrophages were treated with 50, 75, and 100 μg/mL RHE 231 and RHE 238 with or without LPS. DMSO was used in negative control wells, 1 μg/mL LPS and DMSO was used in the positive control groups. IL-1β ELISA was applied to the supernatants to measure the IL-1β production after 24 h of incubation. In order to stimulate the secretion of IL-1β, the cells were treated with 2 mM fresh ATP 2 h before the harvest. Two-tailed t-test was applied for the statistical analysis, $P < 0.0001$, $N = 9$.

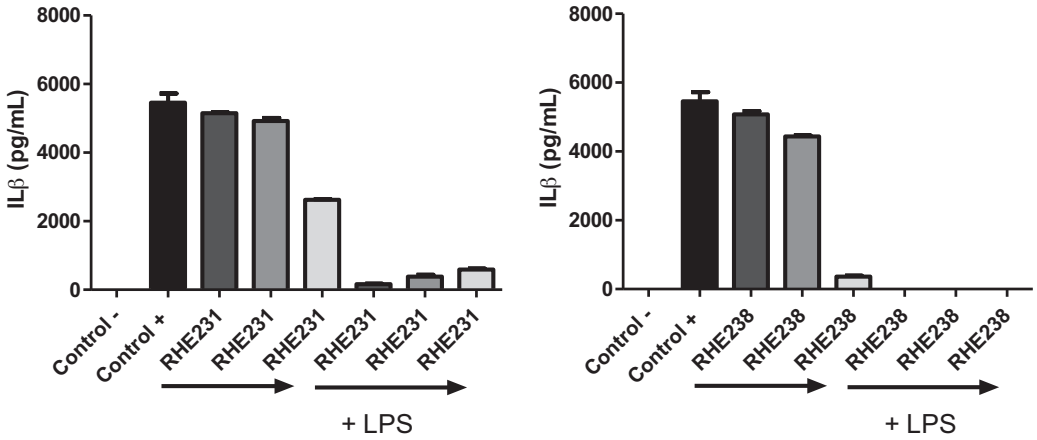


Figure 5. Production of IL-6. Macrophages were treated with 50, 75, and 100 μg/mL RHE 231 and RHE 238 with or without LPS. DMSO was used in negative control wells, 1 μg/mL LPS and DMSO were used in the positive control groups. IL-6 ELISA was applied to the supernatants to measure the IL-6 production after 24 h of incubation. Two-tailed t-test was applied for the statistical analysis, $P < 0.0001$, $N = 9$.

2.6. Discussion

Macrophages are essential due to their efficacy on presenting antigen and producing cytokines [1–14]. Through these functions a proper immune response is developed to fight against a particular danger [1–14]. However, cytokine production becomes a primary concern as in chronic situations stimulated or activated macrophages produce excessive amounts of proinflammatory cytokines and prolonged exposure of the tissue resident cells may eventually lead to tissue injury [15–29]. Undesirable inflammation becomes life-threatening in numerous disease conditions such as CIDs and autoimmune diseases [15–29].

In order to maintain tissue homeostasis and prevent the destructive effects of inflammation, there has been increasing interest in the design and synthesis of new drug candidates that can regulate the activities of immune system cells [15–29]. Previous studies reported an antiproliferative, anticancer, and antiinflammatory role for

bisbenzoxazole-based chemical structures [30–36]. Based on those results, we designed two bisbenzoxazole derivatives and further examined their effect on macrophage activity in the presence of proinflammatory danger signal LPS.

Our study shows that RHE 231 and RHE 238 have an antiinflammatory effect on macrophages, based on TNF-α, IL-6, and IL-1βproinflammatory cytokine production levels. Both reagents led to a significant and substantial decrease in the proinflammatory cytokine secretion levels by LPS stimulated macrophages. There was a significant decrease in TNF-α, IL-1β, and IL-6 production in RHE 231 and RHE 238 treated groups compared to the only LPS treated positive control macrophages. In addition, our chemicals did not stimulate macrophages by themselves for the secretion of TNF-α and IL-1β. Therefore, our reagents had an immunomodulatory role rather than exerting immunostimulatory effects. IL-6 cytokine production was different due to, depending on the case, its ability to act as both a proinflammatory and an antiinflammatory molecule. Our chemicals could stimulate IL-6 production by macrophages even in the absence of the LPS danger signal. However, in the presence of LPS our reagents caused a significant drop in the production of IL-6 in a dose-dependent fashion, compared to LPS only treated positive control macrophages. Although in the absence of a danger molecule our reagents were able to stimulate IL-6 production, which can help with tissue renewal and wound healing, in the presence of a danger signal these same reagents were able to suppress IL-6 production and acted as ultimate antiinflammatory molecules. This property is especially crucial since in the presence of a danger signal IL-6 would act as a proinflammatory molecule. Moreover, our results are in line with the findings of previous studies suggesting that benzoxazole derivatives can antagonize IL-6 production [37].

In order to examine the possible cytotoxicity of our reagents on the immune system cells (macrophages), Trypan blue staining was used. There was no effect on cell viability, whereas based on the MTT assay there was a significant and substantial decrease in cell proliferation in the presence of our reagents. These results suggest antiproliferative roles for the chemicals without causing any cell death on macrophages. RHE 231 and RHE 238 had strong antiproliferative effects on the mouse macrophages in a time- and dose-dependent fashion, but those same macrophages did not cause any decrease in cell viability compared to the control wells.

Previous studies suggest that the benzoxazole structures exert their anticancer and antiinflammatory activities through their antiproliferative activities. Our findings overlap with previous studies. Based on the Trypan blue assay our reagents lacked cytotoxic activities on the macrophages. The MTT assay revealed that the same compounds decreased cell proliferation and this was their mechanism of action to suppress the production of inflammatory cytokines.

Overall, bisbenzoxazole derivatives RHE 231 and RHE 238 have great potential as new drug candidates to modulate immune system cells without causing any cytotoxic effect. Currently, we are focusing on possible signaling pathways that are affected by our reagents.

3. Experimental

3.1. Cell culture

ATCC RAW 264.7 macrophages were cultured in 10% fetal bovine serum and 1% antibiotics (100 μg/mL penicillin and 100 μg/mL streptomycin) containing Roswell Park Memorial Institute medium (RPMI 1640) at 37 °C with 5% CO_2. Then 1 mL/well of cells were seeded in a 24-well plate at a density of 1 $\times$ 10^6 cells/mL.

3.2. Reagents and LPS stimulation

RHE 231 and RHE 238: 2 mg of RHE231 and RHE238 were dissolved in 1000 μL (1 mL) of dimethyl sulfoxide (DMSO). The chemicals were added in different concentrations and conditions after resting the cells overnight

at 37 °C with 5% CO_2 in an incubator: 50 μg/mL RHE 231, 50 μg/mL RHE 231 and LPS treatment, 75 μg/mL RHE 231, 75 μg/mL RHE 231 and LPS treatment, 100 μg/mL RHE 231, 100 μg/mL RHE 231 and LPS treatment. LPS (1 mg/mL, Enzo Life Sciences, *Salmonella minnesota* R595) treatment was given in a final concentration of 1 μg/mL. RHE238 was added in the same order. Moreover, 1 μL of DMSO was added to 1 mL of medium of overnight rested cells. The same volume of only LPS was added to the positive control wells. All treated cells were incubated at 37 °C with 5% CO_2 for 24 h and then the supernatants of each well were collected into Eppendorf tubes and stored at –80 °C. IL-1β secretion was stimulated by adding freshly prepared 5 mM ATP (Fisher Scientific) to RAW 264.7 cell wells 2 h before the harvest.

3.3. Cell viability using Trypan blue staining

After the incubation cells were counted by staining with Trypan blue.

3.4. Cytotoxicity: MTT assay

The cell metabolic activity of cell lines was evaluated using the MTT assay, which is based on the ability of viable cells to metabolize yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenases.

Briefly, cells were seeded at a density of 1×10^5 per well in 96-well plates; subsequently, after 24 h incubation, they were treated with various concentrations (50 μg/mL, 75 μg/mL, and 100 μg/mL) of RHE 231 and RHE 238 for three different durations of incubation at 37 °C with 5% CO_2: 24 h, 48 h, and 72 h. The untreated or DMSO treated well was considered as a negative control, and all were in triplicate.

After 24 h, 48 h, and 72 h of incubation, the instructions for the Roche MTT kit were followed.

3.5. Enzyme-linked immunosorbent assay (ELISA)

Secreted cytokines (TNF-α, IL-1β, and IL-6) were analyzed by ELISA kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

3.6. Statistical analysis

For the statistical analysis of each data set a two-tailed t-test was applied through GraphPad Prism Software version 5.

Acknowledgments

This study was supported by 2017-2-AP-4-2506 and 2018-1-TP3-2911 BAP Projects of Mersin University and the Scientific and Technological Research Council of Turkey (TÜBİTAK, Grant Number: 115S190). Our group greatly appreciates the material support from Prof. Dr. Juan Anguita from CIC bioGUNE.

References

1. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. Frontiers in Immunology 2014; 5 (1): 491.
2. Murray RZ, Stow JL. Cytokine secretion in macrophages: SNAREs, Rabs, and membrane trafficking. Frontiers in Immunology 2014; 5 (1): 538.
3. Kawagishi C, Kurosaka K, Watanabe N, Kobayashi Y. Cytokine production by macrophages in association with phagocytosis of etoposide-treated P388 cells in vitro and in vivo. Molecular Cell Research 2001; 1541 (3): 221-230.

4. Cavaillon JM. Cytokines and macrophages. Biomedicine and Pharmacotherapy 1994; 48 (10): 445-453.
5. Scull CM, Hays WD, Fischer TH. Macrophage proinflammatory cytokine secretion is enhanced following interaction with autologous platelets. Journal of Inflammation 2010; 7 (1): 53.
6. Berghaus LJ, Moore JN, Hurley DJ, Vandenplas ML, Fortes BP et al. Innate immune responses of primary murine macrophage-lineage cells and RAW 264.7 cells to ligands of Toll-like receptors 2, 3, and 4. Comparative Immunology, Microbiology and Infectious Diseases 2010; 33 (5): 443-454.
7. Schmitz F, Mages J, Heit A, Lang R, Wagner H. Transcriptional activation induced in macrophages by Toll-like receptor (TLR) ligands: from expression profiling to a model of TLR signaling. European Journal of Immunology 2004; 34 (10): 2863-2873.
8. Soromou LW, Zhang Z, Li R, Chen N, Guo W et al. Regulation of inflammatory cytokines in lipopolysaccharide-stimulated RAW 264.7 murine macrophage by 7-O-methyl-naringenin. Molecules 2012; 17 (3): 3574-3585.
9. Gasparini C, Foxwell BM, Feldmann M. RelB/p50 regulates TNF production in LPS-stimulated dendritic cells and macrophages. Cytokine 2013; 61 (3): 736-740.
10. Parameswaran N, Patial S. Tumor necrosis factor-α signaling in macrophages. Critical Reviews in Eukaryotic Gene Expression 2010; 20 (2): 87-103.
11. Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1β secretion. Cytokine and Growth Factor Reviews 2011; 22 (4): 189-195.
12. Manderson AP, Kay JG, Hammond LA, Brown DL, Stow JL. Subcompartments of the macrophage recycling endosome direct the differential secretion of IL-6 and TNFα. Journal of Cell Biology 2007; 178 (1): 57.
13. Grossi G, Di Braccio M, Roma G, Ballabeni V, Tognolini M et al. 1,8-Naphthyridines v. novel N-substituted 5-amino-N,N-diethyl-9-isopropyl [1,2,4]triazolo[4,3-a] [1,8]naphthyridine-6-carboxamides, as potent anti-inflammatory and/or analgesic agents completely devoid of acute gastrolesivity. European Journal of Medicinal Chemistry. 2005; 40 (1): 155-165.
14. Dianzani C, Collino M, Gallicchio M, Di Braccio M, Roma G et al. Effects of anti-inflammatory [1, 2, 4]triazolo[4, 3-a] [1, 8]naphthyridine derivatives on human stimulated PMN and endothelial cells: an in vitro study. Journal of Inflammation 2006; 3 (1): 4.
15. Broide DH. Immunomodulation of allergic disease. Annual Reviews of Medicine 2009; 60 (1): 279-291.
16. Iwalewa EO, McGaw LJ, Naidoo V, Eloff JN. Inflammation: the foundation of diseases and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory condition. African Journal of Biotechnology 2007; 6 (25): 2868-2885.
17. Ayaz, F. Ruthenium pyridyl thiocyanate complex increased the production of pro-inflammatory TNFα and IL1β cytokines by the LPS stimulated mammalian macrophages in vitro. Molecular Biology Reports 2018; 45 (6): 2307-2312.
18. Ayaz F, Yuzer A, Ince M. Immunostimulatory effect of zinc phthalocyanine derivatives on macrophages based on the pro-inflammatory TNFα and IL1β cytokine production levels. Toxicology in Vitro 2018; 53(1): 172-177. https://doi.org/10.1016/j.tiv.2018.08.011
19. Hancock REW, Nijnik A, Philpott DJ. Modulating immunity as a therapy for bacterial infections. Nature Reviews Microbiology 2012; 10 (1): 243-254.
20. Kaufmann T, Simon HU. Targeting disease by immunomodulation. Cell Death and Differentiation 2015; 22 (1): 185-186.
21. Julier Z, Park AJ, Briquez PS, Martino MM. Promoting tissue regeneration by modulating the immune system. Acta Biomaterialia 2017; 1 (1): 42.

22. Khalil DN, Smith EL, Brentjens RJ, Wolchok JD. The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. Nature Reviews Clinical Oncology 2016; 13 (5): 273-290.

23. Tan TT, Coussens LM. Humoral immunity, inflammation and cancer. Current Opinion in Immunology 2007; 19 (2): 209-216.

24. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity 2013; 39 (1): 1-10.

25. Guevara-Patiño JA, Turk MJ, Wolchok JD, Houghton AN. Immunity to cancer through immune recognition of altered self: studies with melanoma. Advances in Cancer Research 2003; 90 (1): 157-177.

26. Valdés-Ramos R, Benítez-Arciniega A. Nutrition and immunity in cancer. British Journal of Nutrition 2007; 98 (S1): S127-S132.

27. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010; 140 (6): 883-899.

28. Rakoff-Nahoum S. Why cancer and inflammation Yale Journal of Biology and Medicine 2006; 79 (3-4): 123-130.

29. Coussens LM, Werb Z. Inflammation and cancer. Nature 2002; 420 (6917): 860-867.

30. Algul O, Kaessler A, Apcin Y, Yilmaz A, Jose J. Comparative studies on conventional and microwave synthesis of some benzimidazole, benzothiazole and indole derivatives and testing on inhibition of hyaluronidase. Molecules 2008; 13 (4): 736-748.

31. Ayaz F, Kheeree R, Isse Q, Ersan R, Algul O. DNA base bioisosteres, bis-benzoxazoles, exert anti-proliferative effect on human prostate and breast cancer cells. Journal of the Turkish Chemical Society, Section A: Chemistry 2018; 5 (3): 1145-1152. 10.18596/jotcsa.429504

32. Kaessler A, Algul O, Jose J. A microplate based screening of benzimidazole derivatives on hyaluronidase inhibition at pH 7 and 3.5. Letters of Drug Design and Discovery 2007; 4 (8): 562-569.

33. Algul O, Duran N. Activity of bisbenzimidazole derivatives against *Staphylococcus epidermidis*. Asian Journal of Chemistry 2007; 19 (4): 3145-3151.

34. Kandeel MM, Abdelall EKA, Abd El Hamid MK, Abdelgawad MA, Philoppes JN. Synthesis and in vitro antitumor activity of new benzothiazole and benzoxazole derivatives. Journal of Chemical and Pharmaceutical Research 2013; 5 (8): 16-21.

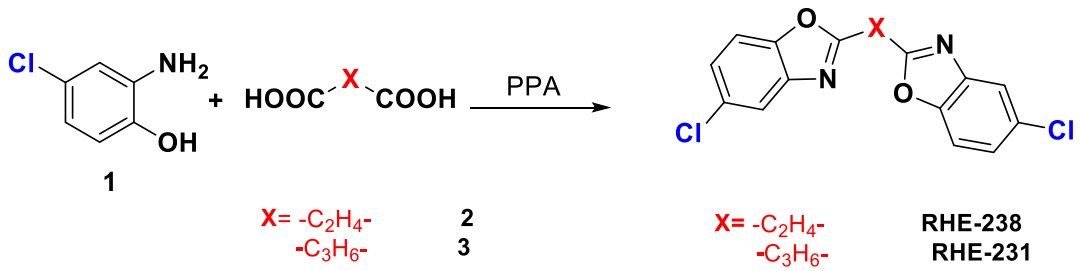
35. Shitha G, Amma VK, Kamala B, Babu G, Biju CR. In-silico docking investigation, synthesis and in vitro anticancer study of benzoxazole derivatives. Journal of Drug Delivery Technology 2014; 4 (6): 122-126.

36. Hedidi M, Bentabed-Ababsa G, Derdour A, Roisnel T, Dorcet V et al. Synthesis of C,N'-linked bis-heterocycles using a deprotometalation-iodination-N-arylation sequence and evaluation of their antiproliferative activity in melanoma cells. Bioorganic and Medicinal Chemistry 2014; 22 (13): 3498-3507.

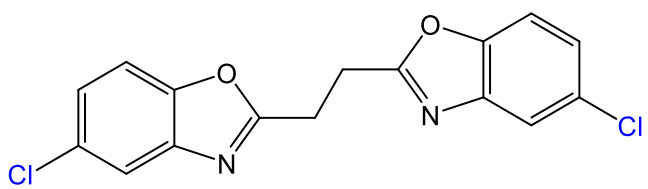
37. Kim D, Won HY, Hwang ES, Kim YK, Choo HP. Synthesis of benzoxazole derivatives as interleukin-6 antagonists. Bioorganic and Medicinal Chemistry 2017; 25 (12): 3127-3134.

Supplementary Information

General Procedure for Preparation of Compounds: 5 mmol of 4-chloro-2-aminophenol (**1**) and 2.5 mmol of the corresponding carboxylic acid derivatives (**2** and **3**) were refluxed in a reflux condenser with a magnetic stirrer for 13–15 h after being dissolved in PPA heated in an oil bath at 180 °C. The reaction was followed by thin layer chromatography (TLC). Ultraviolet light was used in the determination of stains in the TLC (Kieselgel 60 F254, ready-to-use aluminum plate coated with 0.2 mm thickness), which was conducted using ready-made plates. After cooling, the reaction mixture was poured onto ice water and neutralized by mixing with 5 N NaOH until slightly basic pH (8–9) to get the precipitate. The resulting precipitate was filtered off and washed with cold water. The resulting crystalline compounds were filtered and the vacuumed product was dried.



1,2-bis(5-chlorobenzo[d]oxazol-2-yl)ethane (RHE 238):



The above procedure was followed with **1** and **2** to yield **RHE 238** as a crystalline solid (41% yield). The crystallization solvent is ethanol/water. **Rf** (hexane:ethyl acetate 1:1) = 0.51; **mp** = 179 °C; **IR** (KBr, cm^{-1}) V_{max} 3090, 3066, 3048, 1562, 1344, 810, 777, 687; **^{1}H NMR** (400 MHz, $CDCl_3$) δ = 7.57 (d, J = 2.06 Hz, 2H, Ar-H), 7.33 (bs, 1H, Ar-H), 7.22 (dd, J = 2.06 Hz, J = 8.64 Hz, 2H, Ar-H), 7.19 (bs, 1H, Ar-H), 3.49 (s, 1H, $–CH_2$). **^{13}C NMR** (100 MHz, $CDCl_3$) δ = 165.2, 148.5, 141.3, 128.9, 124.2, 118.9, 110.1, 24.4.

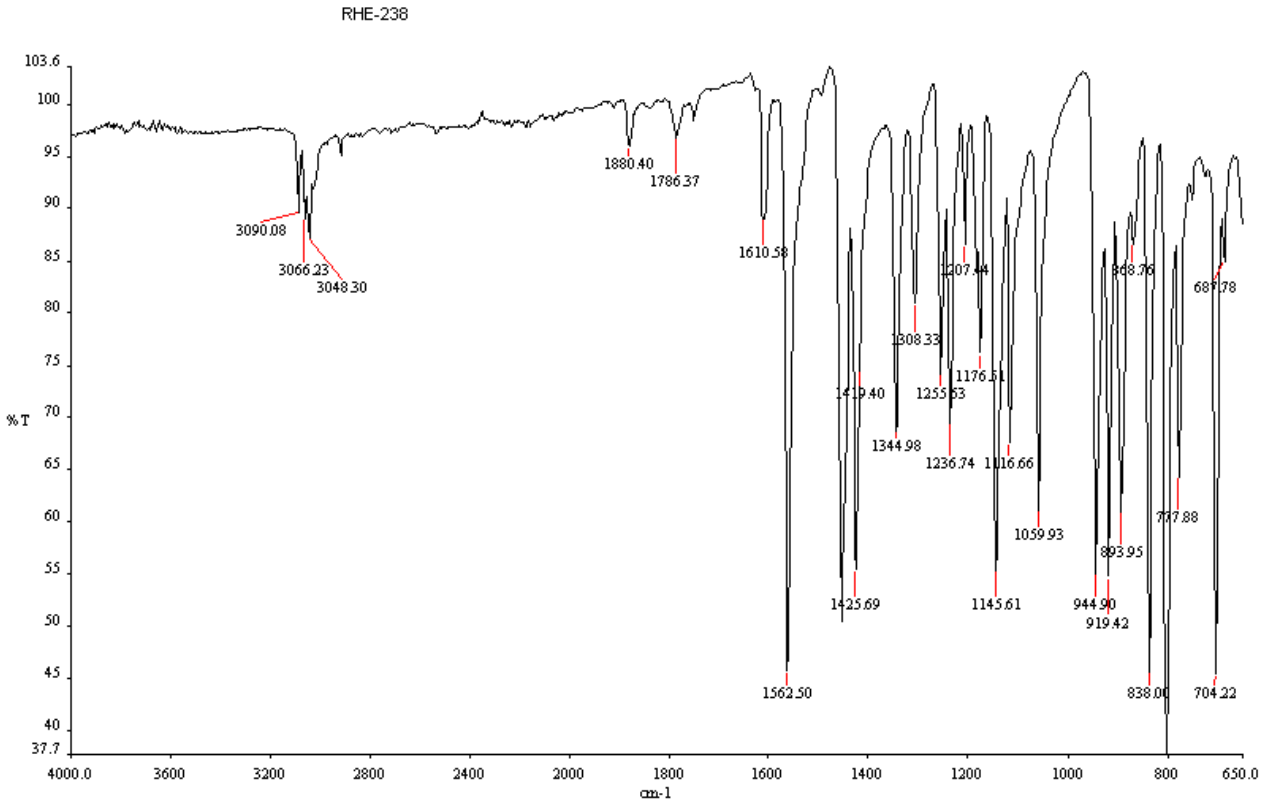


Figure S1. IR spectrum of 1,2-bis(5-chlorobenzo[d]oxazol-2-yl)ethane (**RHE 238**).

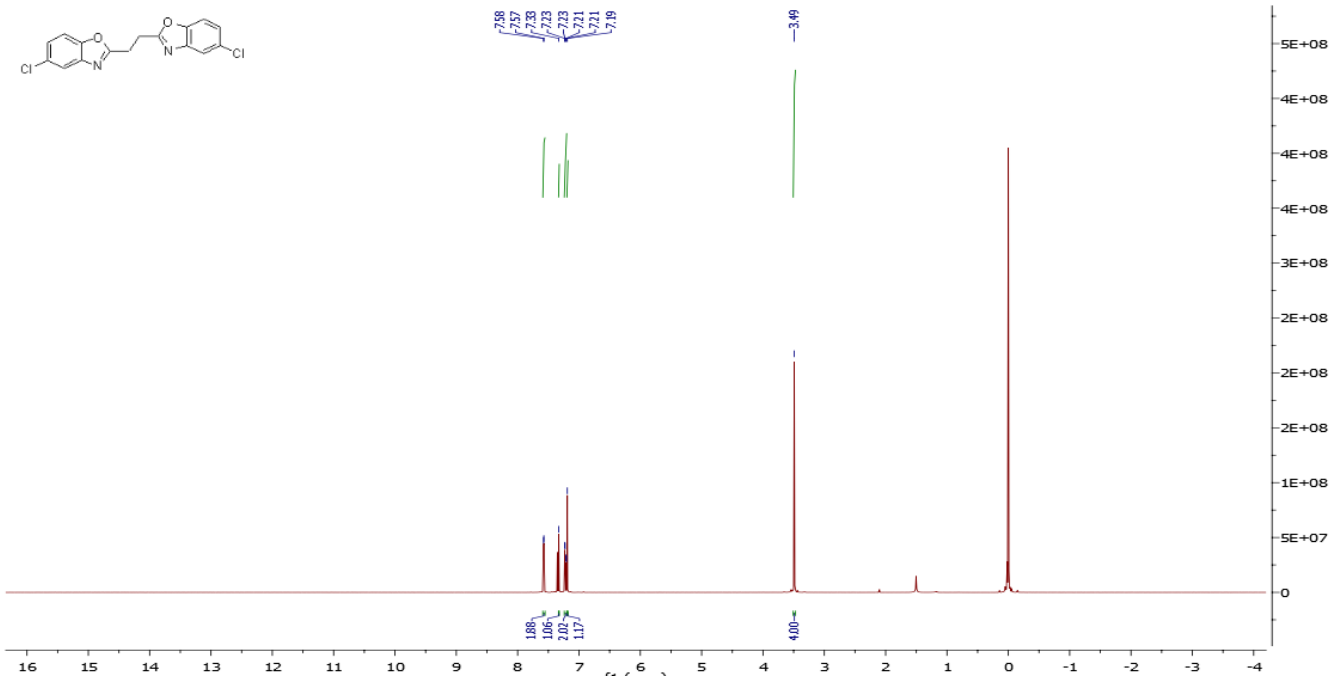


Figure S2. ^{1}H NMR spectrum of 1,2-bis(5-chlorobenzo[d]oxazol-2-yl)ethane (**RHE 238**).

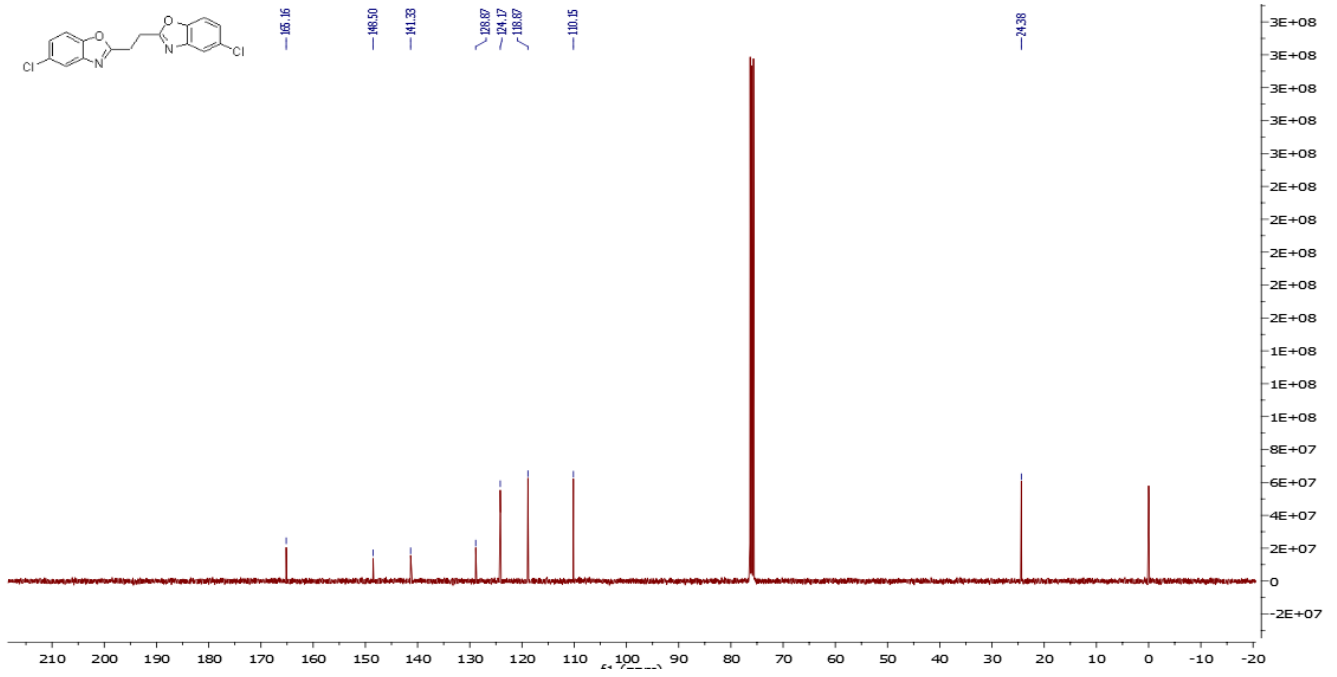
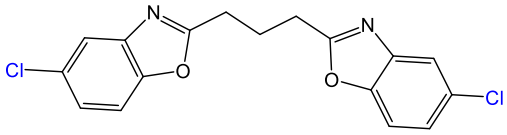


Figure S3. ^{13}C NMR spectrum of 1,2-bis(5-chlorobenzo[d]oxazol-2-yl)ethane (**RHE 238**).

1,3-bis(5-chlorobenzo[d]oxazol-2-yl)propane (RHE 231):



The above procedure was followed with **1** and **3** to yield **RHE 231** as a crystalline solid (47% yield). The crystallization solvent is ethanol/water. **Rf** (hexane:ethyl acetate 1:1) = 0.48; **mp** = 200 °C; **IR** (KBr, cm^{-1}) V_{max} 3065, 1567, 1447, 800, 753, 701; **^{1}H NMR** (400 MHz, $CDCl_3$) δ = 7.52 (d, J = 1.99 Hz, 2H, Ar-H), 7.32–7.29 (m, 1H, Ar-H), 7.20 (dd, J = 1.99 Hz, J = 8.25 Hz, 3H, Ar-H), 3.05 (t, J = 7.28 Hz, 4H, H-1), 2.43 (p, J = 2.48 Hz, 2H, H-2). **^{13}C NMR** (100 MHz, $CDCl_3$) δ = 167.3, 149.4, 142.4, 129.8, 125.0, 119.7, 111.0, 27.8, 23.4.

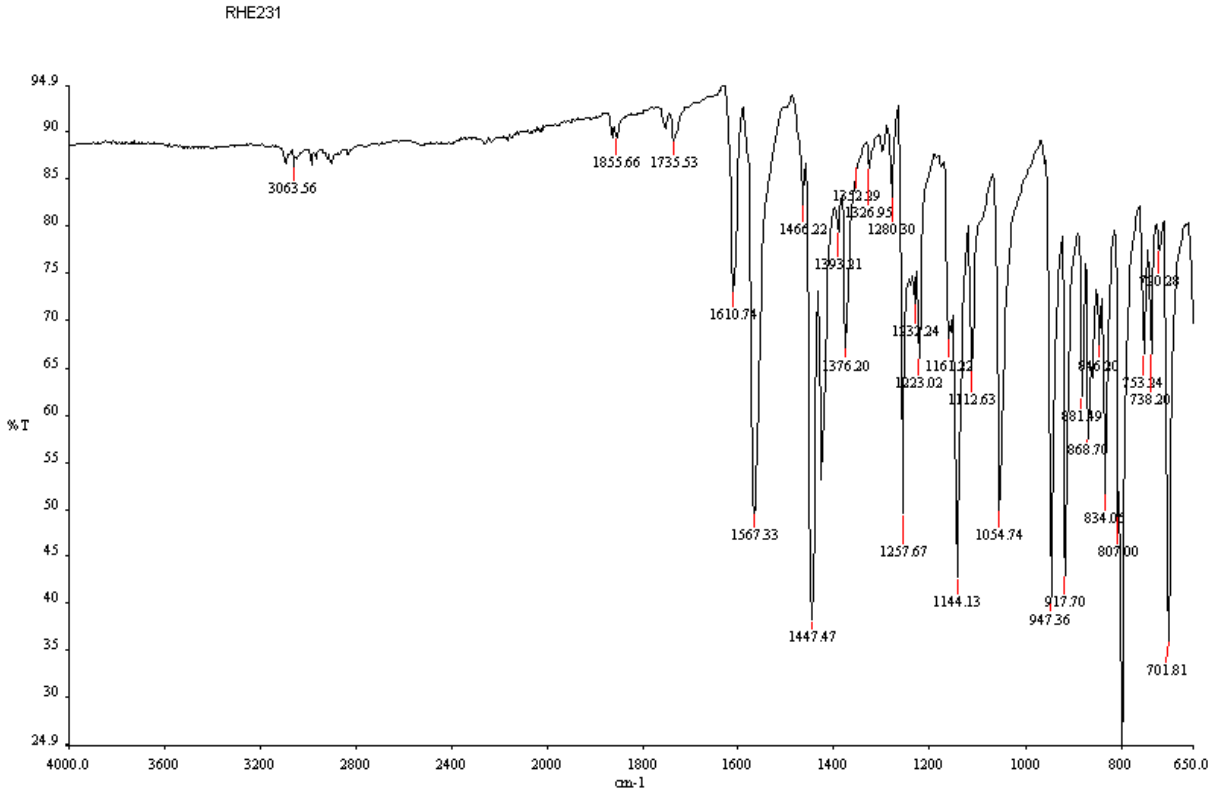


Figure S4. IR spectrum of 1,3-bis(5-chlorobenzo[d]oxazol-2-yl)propane (**RHE 231**).

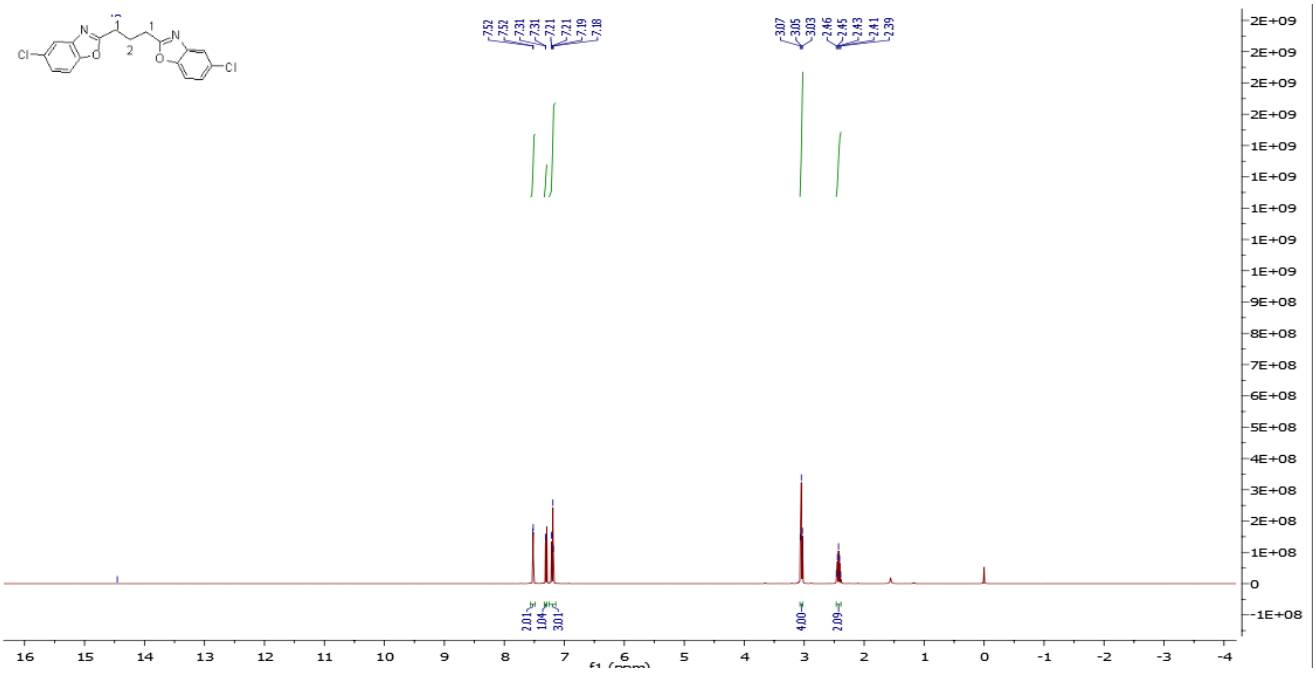


Figure S5. [1]H NMR spectrum of 1,3-bis(5-chlorobenzo[d]oxazol-2-yl)propane (**RHE 231**).

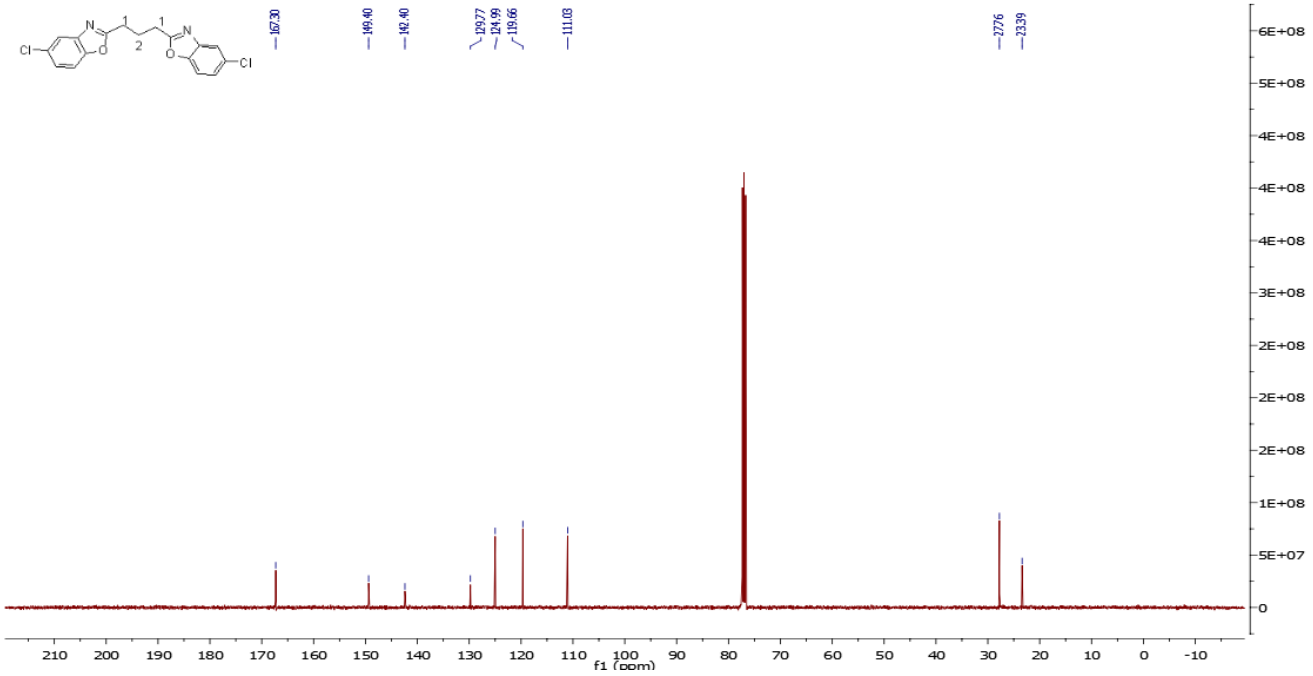


Figure S6. ^{13}C NMR spectrum of 1,3-bis(5-chlorobenzo[d]oxazol-2-yl)propane (**RHE 231**).