# Article

## Improved Synthesis of 1,3,4-Thiadiazolium-2-phenylamines using Microwave and Ultrasound Irradiation and Investigation of their Cytotoxic Activity

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Uma nova e eficiente síntese de oito derivados da classe 1,3,4-tiadiazolio-2-fenilaminas (**1-8**, sendo o derivado **8** inédito na literatura) foi realizada utilizando cloreto de tionila ou cloreto de trimetilsilano como catalisadores sob irradiação de microondas ou ultra-som. Os compostos alvos foram obtidos em bons rendimentos e em tempo extraordinariamente curtos, 5 min sob irradiação de microondas e 10 min sob irradiação de ultra-som, quando comparados com a metodologia tradicional (24 a 48 h em repouso a temperatura ambiente). Os melhores rendimentos foram obtidos usando irradiação de microondas e, de maneira geral, usando o cloreto de tionila ao invés de cloreto de trimetilsilano. A citotoxicidade foi avaliada frente à linhagem de leucemia humana K562 e do linfoma Daudi, apresentando resultados promissores para o derivado cloreto de 4-fenil-5-(4'-nitro-estiril)-1,3,4-tiadiazolio-2-fenilamina.

A new and efficient synthesis of eight 1,3,4-thiadiazolium-2-phenylamine derivatives (**1-8**, where **8** is novel in the literature) was performed using thionyl chloride or trimethylsilyl chloride as catalysts under microwave or ultrasound irradiation. The target compounds were obtained in good yields and remarkably short times, 5 min under microwave irradiation and 10 min under ultrasound irradiation, where compared to traditional methodology (24 to 48 h at room temperature standing). The best yields were obtained using the microwave irradiation and, in general way, using thionyl chloride instead trimethylsilyl chloride. The cytotoxicity against K562 human leukemia and Daudi lymphoma lines was evaluated and showed promising results from the 4-phenyl-5-(4'-nitro-styryl)-1,3,4-thiadiazolium-2-phenylamine chloride derivative.

**Keywords:** mesoionic heterocycle compounds, microwave, ultrasound, cytotoxic activity

## Introduction

Mesoionic compounds are a special class of heterocycles with potential therapeutic applications due to their unique chemical properties. They possess a betaine-like character with a partial positive charge on the heterocyclic ring that is balanced by a negative charge located on an exocyclic atom or group.[1,2] The large separation between the charged regions leads to large dipole moments of about 4-5 D.[3,4] These properties suggest the possibility of interacting with biomolecules such as proteins and DNA. Additionally, the overall neutral character of these compounds enables them to cross biological membranes. Different classes of mesoionic compounds have demonstrated a wide range of biological activities such as anti-inflammatory and analgesic,[5] antiparasitic,[6,7] antibacterial,[8,9] antiplatelet, fibrinolytic, thrombolytic and broncholytic effects,[10] as well as anticancer potency.[11-17] The promising therapeutic applications have led us to study these interesting compounds in our laboratory. We have previously described the survival enhancement of Ehrlich and Sarcoma 180 tumor-bearing mice treated with 4-phenyl-5-(4'-X-styryl)-1,3,5-thiadiazolium-2-phenylamine chlorides.[12] Respiratory chain inhibition, transmembrane potential collapse, and ATPase activity in intact rat liver mitochondria were observed when the 4-phenyl-5-(4'-X-styryl)-1,3,5-thiadiazolium-2-phenylamine chlorides

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were used.[13,14] In addition, Senff-Ribeiro *et al.*[15] showed that 4-phenyl-5-(4'-nitro-styryl)-1,3,5-thiadiazolium-2-phenylamine chloride displays an important antitumor activity against murine melanoma B16F10. This compound was cytotoxic *in vitro*, decreasing cell viability and proliferation, and was able to inhibit tumor growth by *ca.* 85% *in vivo* as well. Additionally, it decreased the viability and proliferation of cell lines of human melanoma (MEL-85, SK-MEL, A2058 and MEWO).[15,16] To contribute to the understanding of the molecular pathways involved in the pharmacological activities of mesoionic compounds, we have recently described the effects of 4-phenyl-5-(4'-nitro-styryl)-1,3,5-thiadiazolium-2-phenylamine chloride on lipid peroxidation in rat liver mitochondria, submitochondrial particles and phosphatidylcholine, as well as its ability to scavenge radicals.[17]

In addition, our group has investigated new, more efficient and cleaner synthetic methods for the preparation of these mesoionic compounds,[18] especially due to important environmental concerns with the current synthesis.[19,20] Therefore, the synthesis of the 1,3,4-thiadiazolium-2-phenylamine class of compounds was revisited using alternative energy sources to obtain better yields and lower reaction times.

Towards that end, this work presents the synthesis of 4-phenyl-5-(4'-X-phenyl)-1,3,5-thiadiazolium-2-phenylamine chlorides, where X = H (**1**), 4'−OCH₃ (**2**), 4'−NO₂ (**3**) and 3',4'−OCH₂O (**4**) (referred to as the benzaldehyde series), and 4-phenyl-5-(4'-X-styryl)-1,3,5-thiadiazolium-2-phenylamine chlorides, where X = H (**5**), 2'−OCH₃ (**6**), 4'−NO₂ (**7**) and 4'−N(CH₃)₂ (**8**) (the cinnamaldehyde series), by two new alternative methods: using microwave irradiation and an ultrasound bath. Furthermore, derivatives of **1**, **3** and **7** were assayed against K562 human leukemia and Daudi lymphoma lines.

## Experimental

### Chemistry

Melting points were determined with a Meltemp II apparatus and were uncorrected. Infrared spectra (KBr pellets) were recorded on a Perkin-Elmer 1605 FT-IR spectrophotometer. $^{1}$H and $^{13}$C NMR spectra were obtained on a Bruker AC200 (200 and 50.3 MHz) spectrometer with TMS as the internal reference and DMSO-$d_6$ as the solvent. The microwave and ultrasound bath assisted organic reactions were performed in a Panasonic Piccilo-NN-S45BH (a domestic oven) and an Ultra-cleaner 700, respectively.

### General procedure for preparation of mesoionic chlorides (*1-8*)

#### Method A

Using microwave irradiation. The aldehyde (0.35 mmol) and 1,4-diphenyl-thiosemicarbazide (0.35 mmol) were mixed in the presence of a Lewis acid (TMS-Cl or SOCl₂) and a few drops of 1,4-dioxane. The resulting mixture was submitted to microwave irradiation for full 5 min in open vessel. After this continuous time, the mixture was added to 1,4-dioxane and left to stand overnight at room temperature. The obtained solid was filtered, washed with ice-cold water and 1,4-dioxane, and recrystallized from chloroform:ethanol (60:40, v/v).

#### Method B

Using ultrasound bath irradiation. The aldehyde (0.35 mmol) and 1,4-diphenyl-thiosemicarbazide (0.35 mmol) were mixed in the presence of a Lewis acid (TMS-Cl or SOCl₂) and 1,4 dioxane as solvent. The resulting mixture was submitted to ultrasound bath irradiation for 10 min. The solution containing the reaction mixture was left to stand overnight. The formed precipitate was treated in a similar way as described above.

Table 1 shows the yields obtained for **1-8** using the method A and method B, and the characterization data[6,12,21] for all compounds are in Supplementary Information.

### Cell culture

The human erythroleukemia K562 and Burkett lymphoma Daudi cell lines were maintained in culture medium RPMI 1640 (Sigma) supplemented with β-mercaptoethanol 50 µmol L⁻¹, HEPES 25 mmol L⁻¹ (Sigma), penicillin 60 mg L⁻¹ (Sigma), streptomycin 100 mg L⁻¹ (Sigma) and 10% (v/v) fetal calf serum (Gibco, Life Technologies). They were incubated at 37 °C in a humidified atmosphere of air and 5% CO₂. The cell line was passaged twice a week at a concentration of $1 \times 10^4$ cell mL⁻¹.

### Cytotoxic assays

$1 \times 10^4$ cell mL⁻¹ were seeded on a 96-well cell culture plate. The cells were incubated with or without a mesoionic compound **1**, **3** or **7** at a concentration of 3.125-50 µmol L⁻¹ each. They were incubated for 24 h, 48 h, 72 h, 96 h and 7 days at 37 °C with 5% CO₂. After, cell viability was assessed by adding 20 µL cell⁻¹ of MTT (5 mg mL⁻¹; 3-[4,5-dimethylthiazol--2-yl]-2,5-diphenyl tetrazolium bromide, Sigma) as described earlier.[22] The plates were

read on a microplate reader (Sunrise-Basic TECAN) at a wavelength of 490 nm. The results are represented as the mean ± SD of 5 determinations.

## Results and Discussion

*Chemistry*

### Analysis of the cell cycle

$1 \times 10^4$ cell $mL^{-1}$ were seeded on a 96-well cell culture plate. The cells were incubated with or without mesoionic compound **7** at a concentration of 3.125-50 µmol $L^{-1}$ each. They were incubated for 24 h at 37 °C with 5% $CO_2$. After, the cells were collected and washed in Hank's buffer. The cell cycle was assessed by adding 200 µL of cell cycle solution (5 mg $mL^{-1}$). The cells were then analyzed by flow cytometry. The samples were excited at 488 nm with an argon laser; fluorescence emission was detected at 585 nm (FL-2). Ten thousand cells were acquired. All flow cytometry analyses were accomplished with WinMDI, version 2.9.

### Statistical analysis

The values are given as a mean ± SD. The statistical significance was calculated by one-way analysis of variance (ANOVA) followed by Bonferroni's t-test using Prism 5 software. P values of less than 0.05 were considered significant.

Eight mesoionic chloride salts were synthesized by two different methods, microwave irradiation and an ultrasound bath, aimed at reduction of the reaction time and adaptation to the principles of Green Chemistry.[23] The convergent strategy for mesoionic chloride salts synthesis included the reaction of 1,4-diphenyl-thiosemicarbazide with the appropriate aldehyde in the presence of a Lewis acid and 1,4-dioxane as solvent. In methods, two different Lewis acids, $SOCl_2$ and trimethylsilyl chloride (TMS-Cl), were investigated separately (Scheme 1). When compared to the traditional method,[7,24] the use of an aldehyde and a Lewis acid enables the elimination of a step involving a carboxylic acid and the subsequent acid chloride preparation, as well as a reduction of the time required to obtain the target compounds from 24-48 h standing at room temperature, after 8 h of stirring, to 5 min under microwave irradiation or 10 min under ultrasound irradiation.
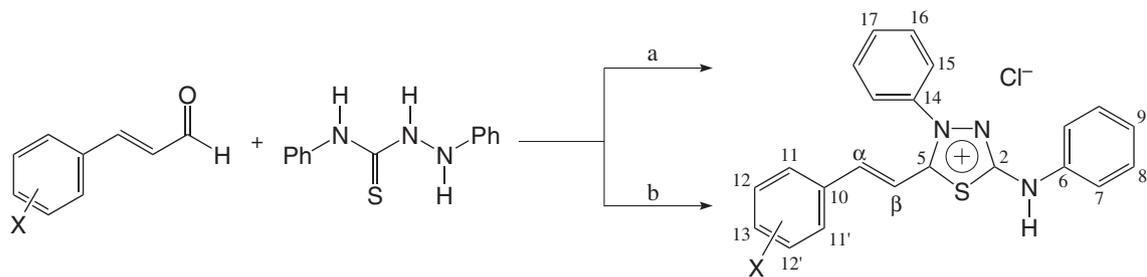
The first method consisted of submitting a mixture of 1,4-diphenyl-thiosemicarbazide, the appropriate aldehyde, $SOCl_2$ (three equivalents) or TMS-Cl (two equiv.) and a few drops of 1,4-dioxane to microwave irradiation (MW) for 5 min. Subsequently, an organic solvent was added, and the

Entry A (saturated series):
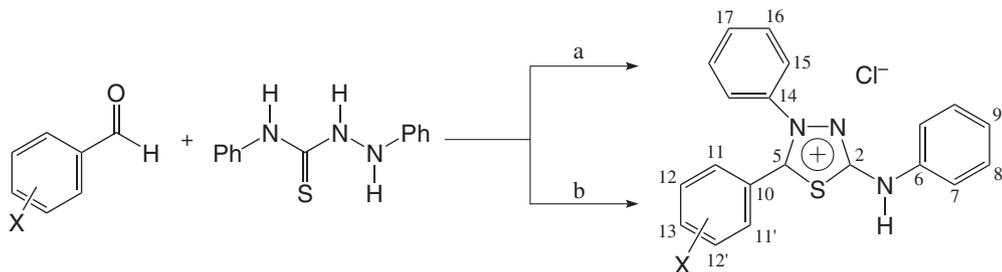


X = H (**1**); 4'-$OCH_3$ (**2**); 4'-$NO_2$ (**3**); 3',4'-$OCH_2O$ (**4**)

Entry B (unsaturated series):



X = H (**5**); 2'-$OCH_3$ (**6**); 4'-$NO_2$ (**7**); 4'-$N(CH_3)_2$ (**8**)

**Scheme 1.** Reagents and conditions: 1,4-dioxane, Lewis acid (SOCl2 or TMS-Cl), MW for 5 min (a) or ))) for 10 min (b).

mixture was allowed to stand overnight, after which time the precipitate was filtered and washed with ice-cold water to afford the target compounds. In the second method, using similar conditions as above, the reagents were dissolved in 1,4-dioxane and put in the ultrasound bath for 10 min, and the mixture was allowed to stand overnight. The isolation of the target compounds was performed in the same manner as for the microwave experiment. Scheme 1 shows the synthetic route of the two methods used in this work. In both methods, the mesoionic salts were obtained in good yields and high purity (Table 1). When microwave irradiation was employed, the yields were in the range of 41-95% when TMS-Cl was used and 90-98% when $SOCl_2$ was used. Using the ultrasound bath method, the respective yields for TMS-Cl and $SOCl_2$ were 66-98% and 72-87%. Thus, the best results were observed using $SOCl_2$ as the catalyst under microwave irradiation conditions.

The mesoionic chloride structures **1**-**8** were fully characterized by $^1H$ and $^{13}C$ NMR and IR spectroscopies. The $^1H$ and $^{13}C$ chemical shifts ($\delta$) were assigned based on literature data[6,12,21,25] and were consistent with the structures proposed.

**Table 1.** Mesoionic chloride yields obtained under microwave irradiation and ultrasound bath, using TMS-Cl and $SOCl_2$ as catalys

| Mesoionic chlorides | Traditional[a] Yield (%) | Microwave[b] Yield (%) | | Ultrasound[c] Yield (%) | |
|---|---|---|---|---|---|
| | | TMS-Cl | $SOCl_2$ | TMS-Cl | $SOCl_2$ |
| **1** | 87 | 68 | 92 | 89 | 83 |
| **2** | 52 | 41 | 96 | 66 | 74 |
| **3** | 78 | 95 | 98 | 98 | 83 |
| **4** | 68 | 74 | 92 | 87 | 87 |
| **5** | 58 | 87 | 90 | 73 | 86 |
| **6** | 73 | 87 | 95 | 83 | 84 |
| **7** | 89 | 92 | 93 | 90 | 91 |
| **8** | no[d] | 93 | 91 | 68 | 72 |

[a]24-48 h at room temperature standing; [b]5 min of microwave irradiation and stand overnight; [c]10 min on ultrasound bath and stand overnight; [d] no product obtained.

## Cytotoxicity activity

In Brazil, leukemia represents about 2% of the total cancer cases and affects adults, the elderly and young individuals.[26]
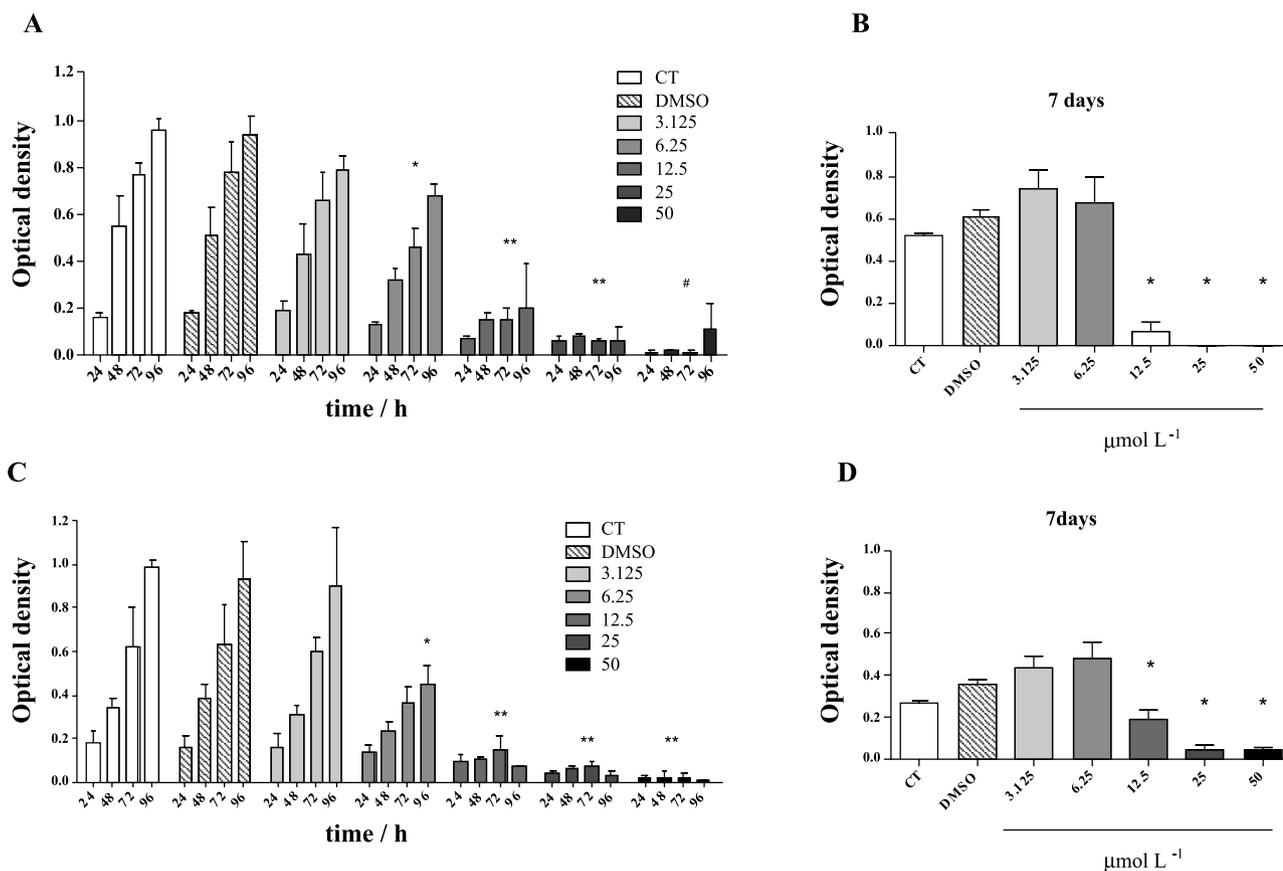


**Figure 1.** Effects of mesoionic compound **7** on Daudi and K562 cell viability. $1 \times 10^4$ cell $mL^{-1}$ were incubated with or without **7** at 37 °C and $CO_2$ 5%. After 24, 48, 72, 96 h and 7 days, Daudi cell line (A and B) or K562 cell line (C and D) were incubated with MTT for 3 h at the same conditions. The optical density was acquired at 490 nm wavelength. The graphs above represent mean ± standard deviation values of the optical density. A p < 0.05 was considered significant when compared to control (*), or to control and 6.25 $\mu mol\ L^{-1}$ (**), or when compared to control, 12.5 and 25 $\mu mol\ L^{-1}$ (#).

Cases of leukemia are classified as acute or chronic and, for lymphoid or myeloid, according to the origin of the cell type. The treatment of leukemia is performed using different combinations of chemotherapy drugs; however, the index of remission remains unsatisfactory. The lymphoma cell line Daudi and myeloid cell line K562 were used for studying the effects of the synthesized mesoionic compounds on leukemic cells. The cytotoxic activities of the **1**, **3** and **7** were assayed *in vitro* using MTT.[22] Compounds **1** and **3** did not alter the viability of the lymphoma cell line Daudi in 72 h of culture.

The positive results obtained with **7** (the unsaturated series compound with an $NO_2$ moiety), led us to assay it in 24 h, 48 h, 72 h, 96 h and 7 days incubations with Daudi cell cultures. The compound showed significant cytotoxic activity after 24 h. Moreover, this mesoionic derivative decreased the cellular viability in a concentration-dependent manner (Figure 1A and B). Similar results were obtained in the human erythroleukemia cell line K562. We observed that the mesoionic compound **7** reduced the cellular viability in a concentration-dependent manner for this cell line as well. The compound showed significant cytotoxic activity beginning at 24 h and continuously growing over time (Figure 1C and D).

The $IC_{50}$ values were determined in $\mu mol\ L^{-1}$ in at least three independent experiments using **7** at concentrations in the range of 5-25 $\mu mol\ L^{-1}$. The compound **7** was incubated for 24 h, 48 h, 72 h, 96 h, and 7 days with cultures of Daudi (lymphoid origin) and K562 cells (myeloid cells). When the results with the different cell lines were compared, they showed similar sensitivities for the first 72 h; however, the myeloid cells demonstrated more sensitivity after a long exposure time (7 days), as shown in Table 2.

**Table 2.** $IC_{50}$ ($\mu mol\ L^{-1}$) values of compound **7** against Daudi lymphoma and K562 leukemia cells

| time of culture | Daudi $IC_{50}$ ($\mu mol\ L^{-1} \pm SD^a$) | K562 $IC_{50}$ ($\mu mol\ L^{-1} \pm SD$) |
|---|---|---|
| 24 h | $10.76 \pm 0.09$ | $12.12 \pm 0.10$ |
| 48 h | $8.62 \pm 0.07$ | $7.63 \pm 0.06$ |
| 72 h | $7.93 \pm 0.08$ | $7.08 \pm 0.08$ |
| 96 h | $10.17 \pm 0.11$ | $6.03 \pm 0.07$ |
| 7 days | $11.55 \pm 0.07$ | $8.76 \pm 0.09$ |

[a]SD: standard deviation.

To understand the mechanism of the cytotoxicity exhibited by the mesoionic compound **7** on the Daudi and K562 cells, they were incubated for 24 h with **7** at a concentration of 3.25-50 $\mu mol\ L^{-1}$. The capacity of this compound to induce apoptosis in Daudi and K562 cells was analyzed with a cell cycle assay. The results showed an increase in hypodiploid cells, which was correlated with the cytotoxicity results
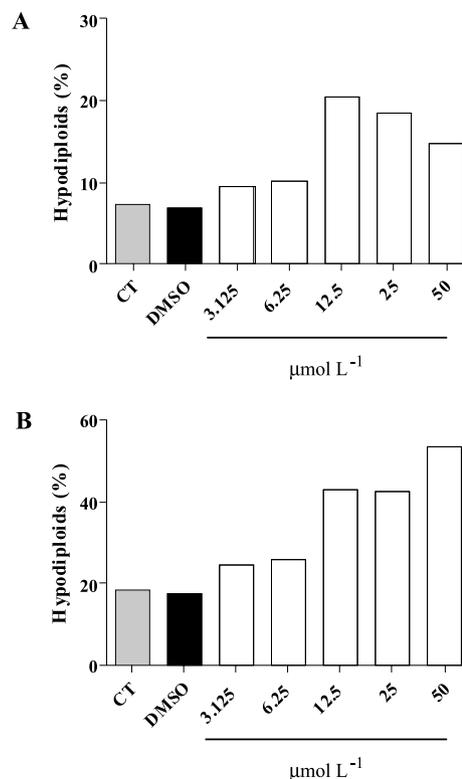


**Figure 2.** Mesoionic compound **7** induces apoptosis on Daudi and K562. $1 \times 10^4$ cell $mL^{-1}$ of Daudi (A) and K562 (B) were incubated with or without mesoionic compound **7** at 37 °C and $CO_2$ 5%. After 24 h, cells were incubated with cell cycle solution at room temperature for 15 min. Following, cells were acquired by flow cytometry and analyzed on WinMDI 2.9. The graphs above represent mean values of hyplodiploid cells percentage.

(Figure 2). This suggests that the mesoionic **7** induces cell death by apoptosis.

## Conclusions

In summary, the new synthetic methods using microwave irradiation and an ultrasound bath provided the products **1**-**8**, where **8** is novel in the literature, in shorter reaction times and higher yields when compared to the traditional method. $SOCl_2$ proved to be a better Lewis acid than TMS-Cl, and microwave irradiation led to a more efficient reaction when compared to the ultrasound bath. In addition to improving the synthetic route, these methods adhere to the principles of Green Chemistry. Furthermore, the 4'-nitro-styryl derivative has shown to be cytotoxic against Daudi lymphoma and K562 leukemic myeloid cells in preliminary *in vitro* studies of cytotoxicity.

## Supplementary Information

Supplementary information with characterization data for all compounds (IR, ^{1}H and ^{13}C NMR data) and the

corresponding spectra for compound **8** are available free of charge at http://jbcs.sbq.org.br as PDF file.

## Acknowledgments

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