PRÁCTICA I.5

ATIVIDADE SEQUESTRADORA DE RADICAL LIVRE DETERMINAÇAO DO POTENCIAL ANTIOXIDANTE DE SUBSTÂNCIAS BIOATIVAS

FREE RADICAL SCAVENGER ACTIVITY: DETERMINATION OF THE ANTIOXIDANT PROFILE OF BIOACTIVE SUBSTANCES

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INTRODUÇÃO

Substâncias antioxidantes desempenham um papel importante na saúde através de seus efeitos na modulação dos processos oxidativos que ocorrem no organismo. A formação de espécies reativas de oxigênio e subsequente oxidação de moléculas biológicas constitui um mecanismo de dano tecidual presente em vários processos patológicos como inflamação, derrame, infarto do miocárdio, arteroesclerose, doença de Alszheimer e Parkinson e em alguns tipos de cancer [1,2]. Os mecanismos biológicos responsáveis pela formação de espécies reativas de oxigênio são: perixidação lipídica, dano oxidativo do DNA, oxidação proteica. Vários ensaios para avaliação de atividade sequestradora de radical livre e atividade antioxidante estão descritos na literatura. O ensaio espectrofotométrico do radical 1,1-difenil-2-picrilhidrazil (DPPH) é um teste simples e amplamente empregado [3]. DPPH é um radical livre estável, não natural, cujas propriedades diferem dos radicais de oxigênio altamente reativos como os radicais

hidroxil, alcoxil, superóxido, etc., que apresentam um importante papel nos processos oxidativos biológicos. Várias séries de compostos químicos tem apresentado estreita correlação entre as atividades sequestradora de DPPH e antioxidante determinada em modelos biológicos e outros não-biológicos [4–6]. Compostos como polifenois, hetero-aril-pirrol, acetofenonas, etc. apresentam capacidade de deslocar életrons, conferindo à estes compostos propriedades oxidáveis. Logo, o ensaio de atividade sequetradora do radical livre DPPH se apresenta como um teste de predicção de uma potencial atividade antioxidante e pode ser empregado para screening de compostos químicos sintéticos e produtos naturais.

PRÍNCIPIO

O ensaio fundamenta-se na propriedade do DPPH apresentar uma forte absorção no espectro visível, comprimento de onda de 515 nm, caracterizado por uma coloração violácea intensa, devido à presença de elétrons livres. Quando o DPPH é colocado em presença de substâncias capazes de sequestrar radicais livres, a absorção é inibida, resultando em uma descoloração estequiométrica em relação ao número de életrons retirados e independente de qualquer atividade enzimática. O grau de descoloração indica a capacidade sequestradora de radical livre [7,8].

OBJETIVO

Avaliar a capacidade sequestradora de radical livre de fármacos planejados ou produtos naturais com possíveis propriedades antinflamatórias, anticancer, etc. A partir dos resultados obtidos avaliar as propriedades químicas (estruturais e eletrônicas) responsável pela atividade observada.

METODOLOGIA

Preparar uma solução etanólica de DPPH (0,1 mM) e misturar com solução do composto teste à diferentes concentrações ou à uma concentração fixa de “screening” de 0,1 mM. A mistura reacional deve ser agitada vigorosamente. As medidas de absorbância são efetuadas 10 min após em um espectrofotômetro UV–vis no comprimento de onda de 515 nm. A inibição da coloração é expressa em % de atividade sequestradora, através da fórmula:

$$\% \text{ AS} = 100 \times \frac{\Delta h_x}{h_c}$$

h_c = absorbância controle (DPPH + EtOH)

h_x = absorbância teste (DPPH + subst. teste)

$\Delta h_x = h_c - h_x$

O BHT (2,6-di-*terc*-butil-4-metil-fenol) será utilizado como substância de refrência da atividade sequestradora de radical livre (controle positivo). Podem ser empregados também como substâncias de refrência o α-tocoferol ou o Trolox (análogo estável da vitamina E). Todos devem apresentar 90–100 % de AS [7–9].

A potência da atividade sequestradora pode ser obtida através da construção de uma curva concentração/efeito e determinação da EC50, quamdo o ensaio for realizado para diferentes concentrações do composto teste.

REFERÊNCIAS BIBLIOGRÁFICAS

1. T. Ozben. *Free Radical, Oxidative Stress and Antioxidants. Pathological and Physiological Significance*, Plenum Press, New York (1998).
2. C. A. Rice-Evans, A. T. Diplock. *Free Radical Biol. Med.* **15**, 77 (1992).
3. H. S. Blois. *Nature* **181**, 1199 (1958).
4. K. E. Malterud, T. L. Farbrot, A. E. Huse, R. B. Sund. *Pharmacology* **47** (suppl. 1), 77 (1993).
5. M. Joyeux, F. Mortier, J. Fleurentin. *Phytother. Res.* **9**, 228 (1995).
6. K. Yamasaki, A. Hashimoto, Y. Kokusenya, T. Miyamoto, T. Sato. *Chem. Pharm. Bull.* **42**, 351 (1994).
7. J. Lehuédé, B. Faucounneau, L. Barrier, M. Ourakow, A. Piriou, J. M. Vierfond. *Eur. J. Med. Chem.* **34**, 991 (1999).
8. L. Mathiesen, K. E. Malterud, R. B. Sund. *Free Radical Biol. Med.* **22**, 307 (1997).
9. A. Tait, S. Ganzerli, M. Di Bella. *Tetrahedron* **52**, 12587 (1996).

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O trabalho em laboratórios de Química Medicinal requer cuidado e uso de boas práticas de laboratório. O manuseio de instrumentos eletrônicos, a utilização de calor, o uso de vidrarias e de solventes não representam problemas

especiais, desde que sejam seguidas, de forma cuidadosa, as instruções do supervisor.

Este documento foi supervisionado pelo Prof. CARLOS ALBERTO MANSSOUR FRAGA (cmfraga@pharma.ufrj.br) que informou sobre a inexistência de riscos específicos na realização deste exercício (*e.g.*, toxicidade, inflamabilidade, riscos de explosão, etc.), fora aqueles comuns a execução de toda e qualquer prática em laboratórios de Química Medicinal.

Se seu exercício ou prática envolver qualquer risco específico, favor informar ao Editor.

EXERCISE I.5

FREE RADICAL SCAVENGER ACTIVITY: DETERMINATION OF THE ANTIOXIDANT PROFILE OF BIOACTIVE SUBSTANCES

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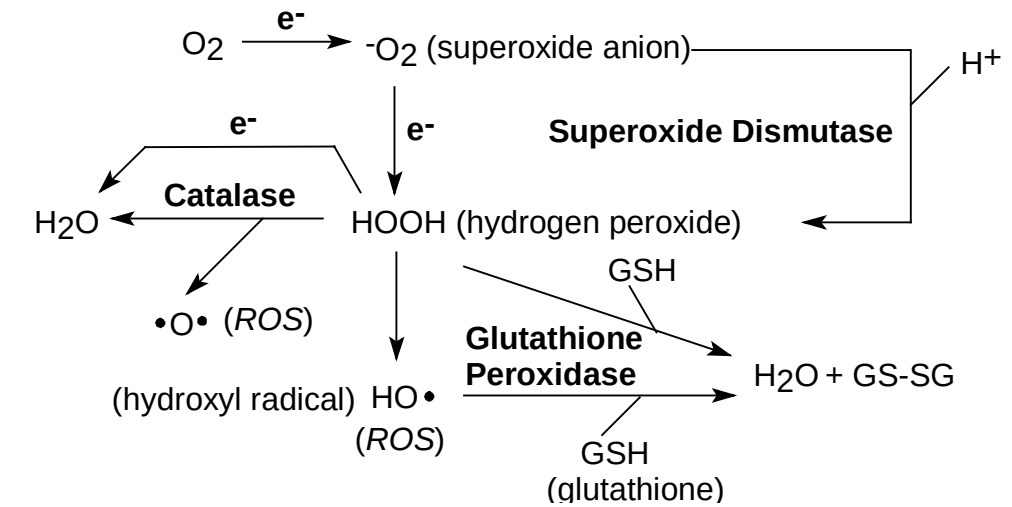
INTRODUCTION

Reactive oxygen (ROS) and nitrogen (RNS) species are normally produced in every cell of the human body, as subproducts from the reduction of oxygen into water at the level of the mitochondrial respiratory chain. Enzymatic antioxidants, e.g., superoxide dismutase, catalase and glutathione peroxidase, and non-enzymatic ones, e.g., glutathione (**1**), vitamin C (**2**), vitamin E (**3**), ß-carotene (**4**), detoxify RSO and RSN and minimize the damage caused to the biomolecules (Fig. 1). The disequilibrium between the production of RSO and RSN and the antioxidant capacity leads to the state of oxidative stress, which contributes to the genesis of a large number of pathological frames, resultant from structural damages provoked by the oxidation of proteins, DNA, and lipids peroxidation. Environmental and nutritional factors, such as irradiation (X-rays, γ-rays, ultraviolet light), pollutants in the atmosphere (ozone, N_2O_2, NO_2, cigarette smoke), diet poor in carotenoids, polyunsaturated fatty acids and antioxidant vitamins, can increase the activity of the generating systems of RSO, inducing the oxidative stress [1].



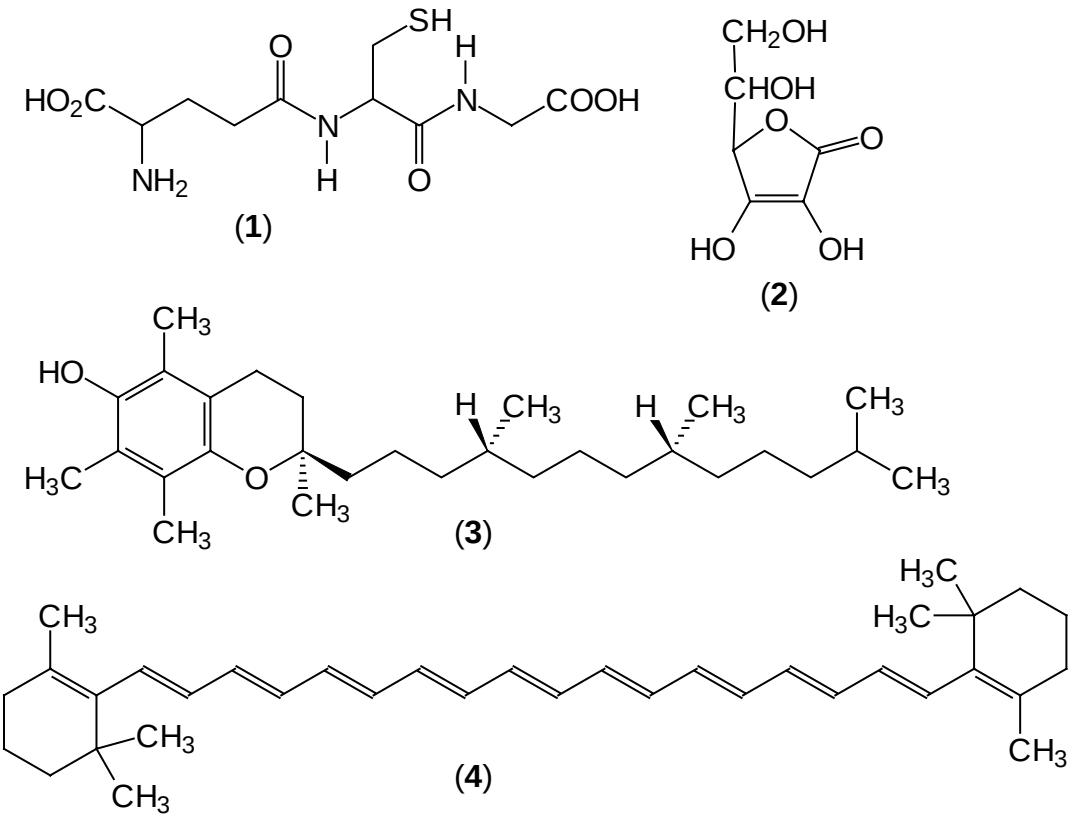


Fig. 1 Mechanisms of RSO formation and detoxification.

Antioxidants are substances that easily oxidize. Therefore, they promptly react with ROS and RNS forming stable radicals, avoiding the cellular damages caused by the action of these free radicals that will eventually culminate in a pathological process,

e.g., inflammation, stroke, acute myocardial infarction, atherosclerosis, kidney failure, Alzheimer's disease, Parkinson's disease, Crohn's disease, early aging, skin cancer [1–7].

Flavonoids are a group of natural polyphenolic antioxidants found in several fruits and vegetables and in some beverages like tea and wine (Fig. 2), whose consumption has been related to a low incidence of coronary diseases [8].

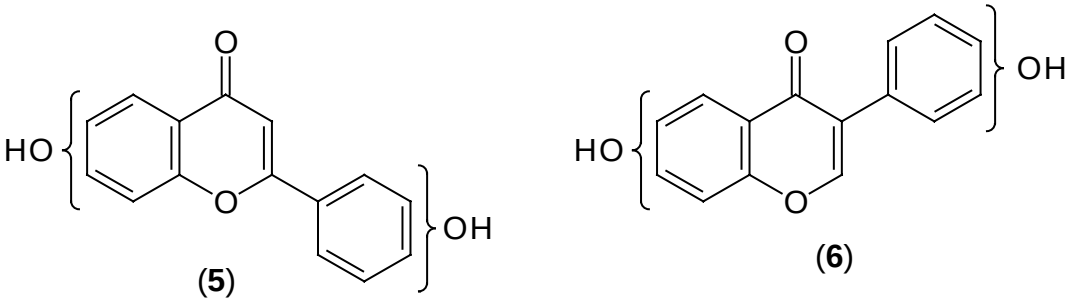


Fig. 2 Polyhydroxylated flavonoids—flavons (**5**) and isoflavons (**6**).

Inflammation is a degenerative disorder mainly modulated by prostaglandins and leukotrienes produced respectively by the action of the cyclooxygenase and 5-lipoxygenase enzymes over the arachidonic acid (AA) [9]. The key step in the biosynthesis of these autacoids involves the Fe^{+3}-catalyzed enzymatic peroxidation reaction of AA with the formation of endoperoxide intermediates and unstable hydroperoxides. During this process, ROS can be formed, aggravating the patient's inflammatory process. In this context, arachidonic acid cascade enzyme inhibitors that present structural characteristics favorable to oxidant activity, e.g., nimesulid [9] (**7**, Scaflam^{R}), BF-389 (**8**), curcumine [10] (**9**, *Curcuma longa*), have their anti-inflammatory profile improved by the interruption of the AA enzymatic peroxidation associated with the capacity for trapping oxygen free radicals that are abundant in the inflamed tissue [9]. Owing to the fact that the electrophilic features are similar to the carbocations ones, the free radicals are predominantly stabilized by electronic delocalization involving π-electrons (resonance effect) or σ-electrons (hyperconjugation effect); the conjunction of these two effects lead phenol compounds that were *ortho*-substituted with *tert*-butyl groups, e.g., di-*tert*-butylhydroxyltoluene (**10**, BHT), to present great antioxidant potential due to the capacity for trapping free radicals (Fig. 3).

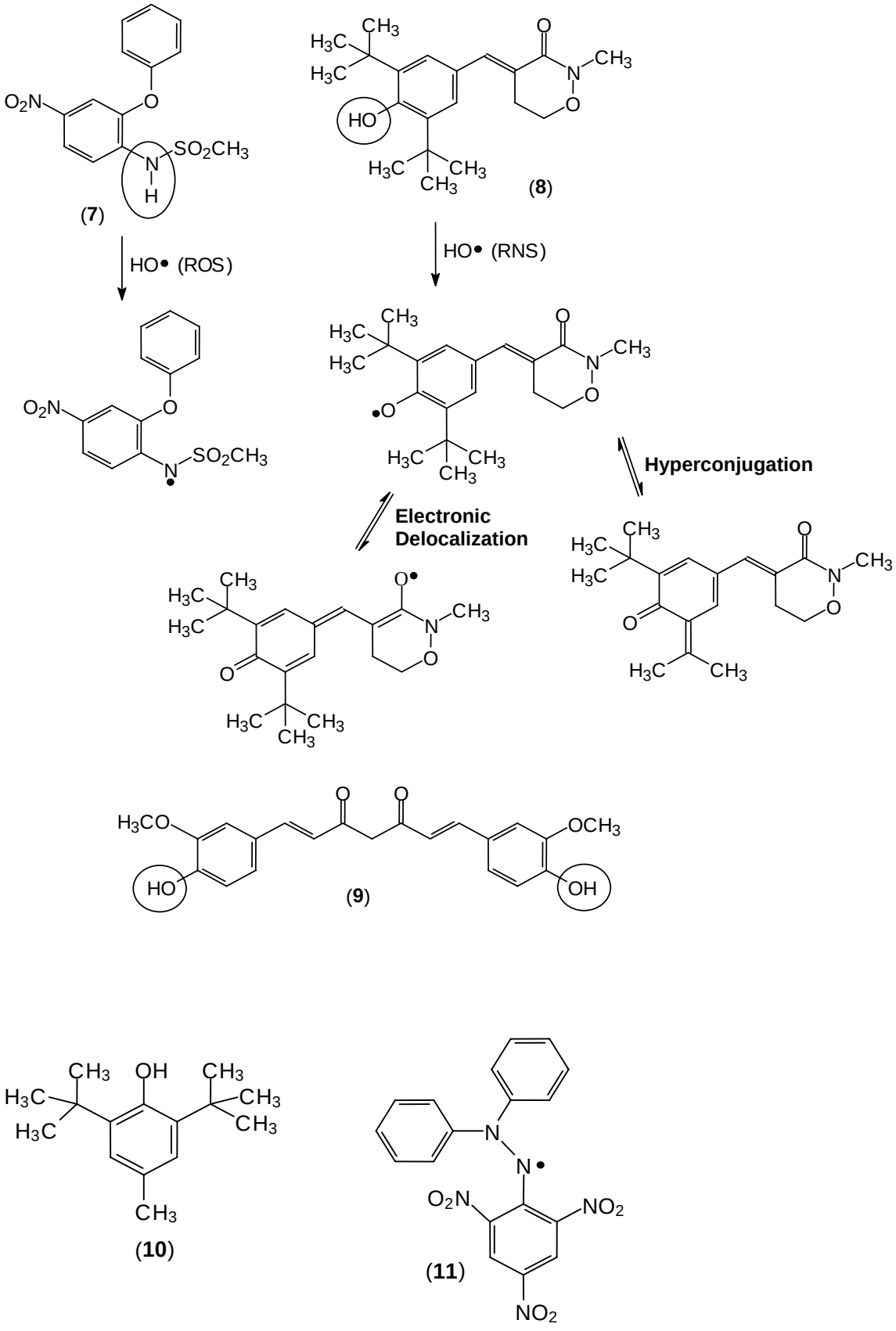


Fig. 3 Antioxidant mechanisms of action of the cyclooxygenase and/or 5-lipoxygenase anti-inflammatory inhibitors.

Several assays to evaluate the redox potential and free radical scavenger activity are described in the literature, including the use of more advanced techniques, such as the cyclic voltammetry [11], electronic paramagnetic resonance (EPR) [10], and electronic spin resonance (ESR) [12]. The spectrophotometric assay of the 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazyl radical (DPPH, **11**) is a simple and widely employed test [13]. DPPH is a stable free radical, non-natural, whose properties differ from the highly reactive oxygen radicals such as the hydroxyl, alkoxyl, and superoxide, which play an important role on the biological oxidative processes. Innumerous series of chemical compounds have presented a narrow correlation between the DPPH scavenger activity and the antioxidant one determined on both biological and nonbiological models [14–16]. Chemical compounds like polyphenols, heteroarylpyrroles, acetophenones, among others have the capacity for trapping and dislocating electrons, providing these substances with oxidative properties. Thus, the DPPH free radical scavenger assay presents itself as a test of prediction of the antioxidant potential activity and may be used for screening of synthetic chemical substances as well as natural products.

PRINCIPLE

The assay is grounded on the DPPH (**11**) property of presenting a strong absorption at the visible spectrum, wave length of 515 nm, characterized by an intense violet coloration (Fig. 4), due to the presence of free electrons. When the DPPH is in the presence of substances able to scavenge free radicals, the absorption is inhibited, leading to a stoichiometric discoloration in relation to the number of reduced molecules of DPPH, independently of any enzymatic activity. The degree of discoloration is directly correlated with the free radical scavenger activity of the evaluated substance [17,18].

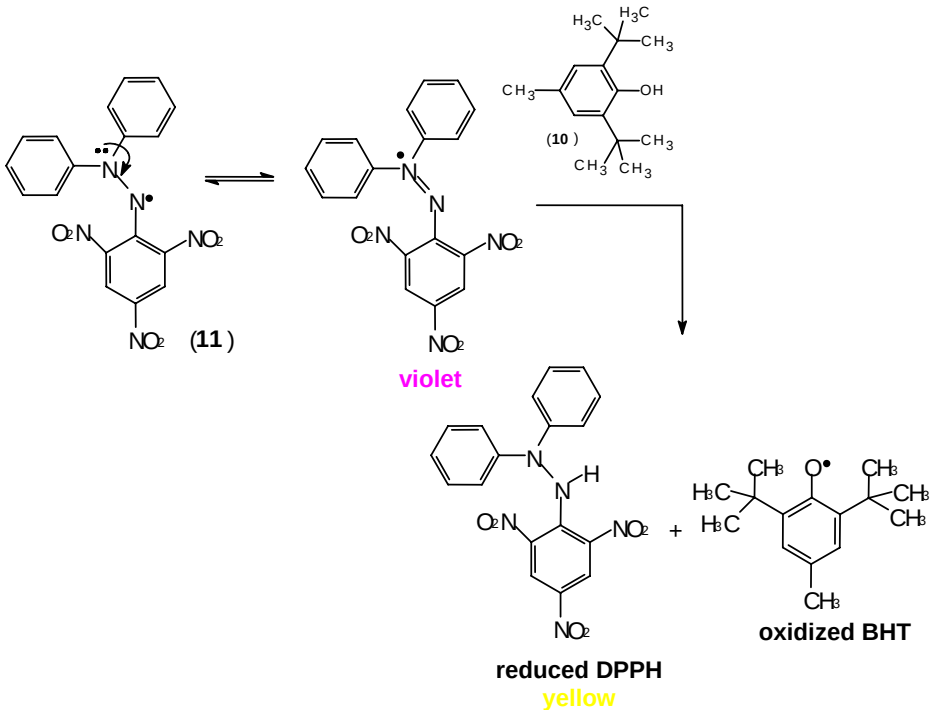


Fig. 4 Colorimetric reaction between the DPPH (**11**) and the BHT (**10**).

OBJECTIVE

To evaluate the scavenger activity of free radicals of planned compounds or natural products with possible anti-inflammatory, anti-cancer, etc. properties.

To analyze the chemical properties (structural and electronic) responsible for the observed free radical scavenger activity.

METHODOLOGY

To prepare a DPPH ethanolic solution (0.1 mM) and mix with a solution of the test compound in different concentrations or in a 0.1 mM fixed screening concentration. The reactional mixture must be vigorously agitated. The absorbance measures are made 30 min afterward in a UV–vis spectrophotometer at a wave length of 515 nm. The change of the coloration (violet to slight yellow) is accompanied by an absorbance decrease, and the scavenger activity (SA) is expressed in % through the formula:

$$\% \text{ SA} = 100 \times \frac{\Delta h_x}{h_c}$$

h_c = control absorbance (DPPH + EtOH)

h_x = test absorbance (DPPH + subst. test)

$\Delta h_x = h_c - h_x$

The BHT (**10**) will be utilized as reference substance of the scavenger activity of free radicals (positive control). The α-tocopherol or the Trolox (stable analogous of the vitamin E) may also be employed as reference substances. All must present 90–100 % of SA [17–19].

The potency of the scavenger activity may be obtained through the construction of a concentration-effect curve for the determination of the EC_{50}, when the assay is done for different concentrations of the test compound.

An application of the assay: Evaluation of the antioxidant profile of new prototypes of anti-inflammatory drugs.

In our research group, we have described a new series of 1,2-benzothiazine derivatives [20] (**13**) structurally planned by the molecular hybridization of the nonsteroidal anti-inflammatory drug meloxicam (**12**, Movatec^R) with the BF-389 (**8**), aiming at incorporating structural requisites that aggregate a free radical scavenger profile into the classical anti-inflammatory profile described by (**12**) (Fig. 5).

The evaluation of the antioxidant potential of this series of compounds in the spectrophotometric assay of the DPPH (**11**) allowed us to evidence a very important activity for the phenolic derivative (**13a**), i.e., SA = 157 %, superior to that of the BHT (**10**) used as standard (SA = 100 %) [21]. The investigation of the anti-inflammatory profile of this series of derivatives (**13**) on the carrageenan-induced rat paw edema model permitted us to characterize an anti-inflammatory activity for (**13a**), i.e., 33 % of inhibition of the formation of the edema, correlating with its accentuated capacity of trapping free radicals [21].

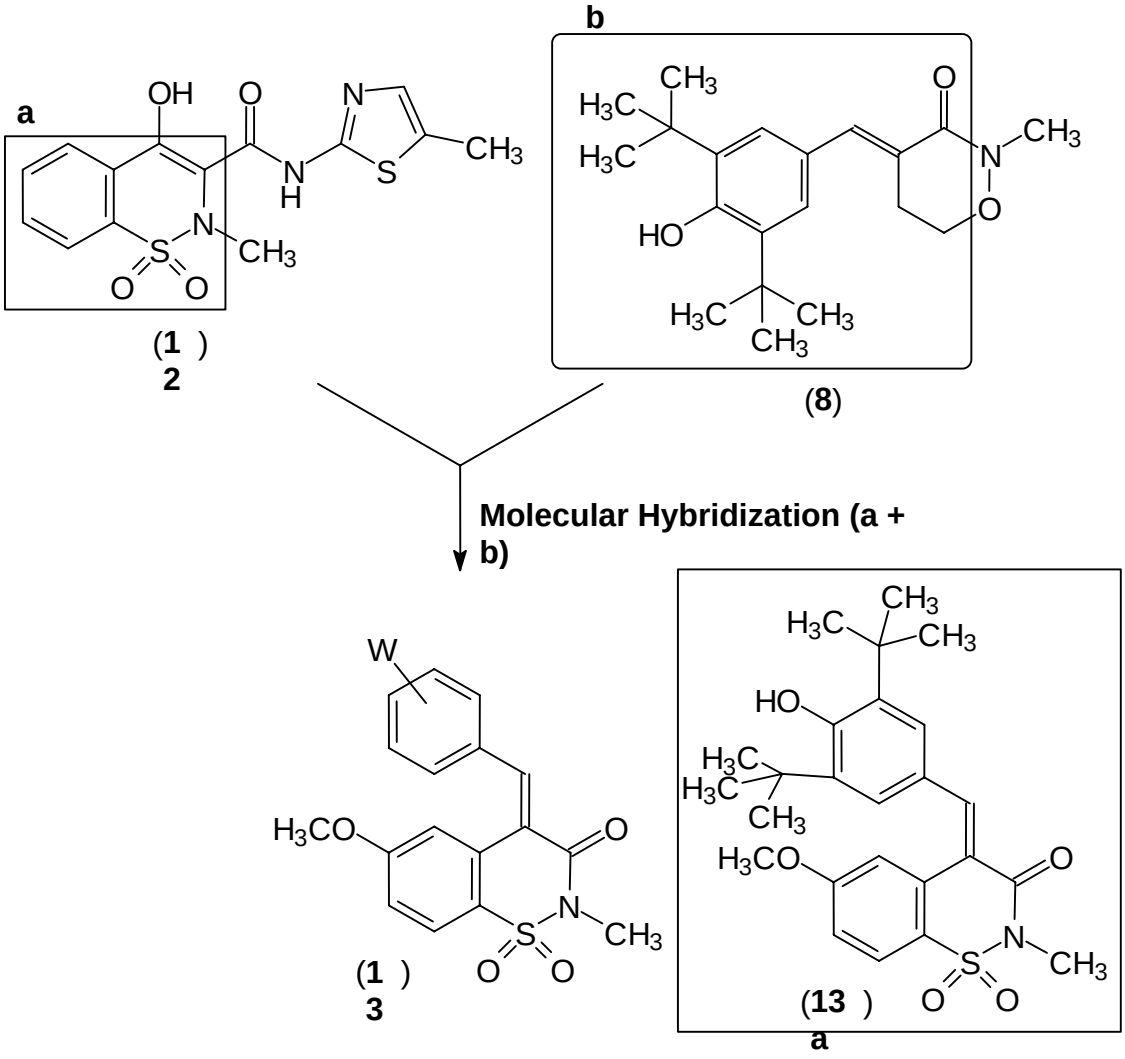


Fig. 5 Structural planning of a new nonsteroidal anti-inflammatory prototype 1,2-benzothiazine derivative (**13a**).

BIBLIOGRAPHY

1. Y. S. Young, J. V. Wooside. *J. Clin. Pathol*. **54**, 176 (2001).
2. T. Ozben. *Free Radical, Oxidative Stress and Antioxidants. Pathological and Physiological Significance*, Plenum Press, New York (1998).
3. C. A. Rice-Evans, A. T. Diplock. *Free Radical Biol. Med.* **15**, 77 (1992).
4. S. A. A. Comhair, S. C. Erzurum. *Am. J. Physiol. (Lung Cel. Mol. Physiol.)* **283**, 246 (2002).
5. G. J. Kelloff, J. A. Crowell, V. E. Steele, R. A. Lubet, C. W. Boone, W. A. Malone, E. T. Hawk, R. Lieberman, J. A. Lawrence, L. Kopelovich, I. Ali, J. L. Viner, C. C. Sigman. *Ann. N.Y. Acad. Sci.* **889**, 1 (1999).
6. M. Grundman, P. Delaney. *Proc. Nutrition Soc.* **61**, 191 (2002).

7. T. R. Koch, S. J. Stryker, G. L. Telford, E. C. Opara, T. Ozben. *Nutrition Res.* 22, 825 (2002).
8. P. Cos, M. Calomme, M. Pieters, A. J. Vlietinck, D. Vanden Berghe. In *Studies in Natural Products Chemistry*, Atta-Ur-Rahman (Ed.), Elsevier Science, Amsterdam (2000).
9. A. S. Lages, N. C. Romeiro, C. A. M. Fraga, E. J. Barreiro. *Química Nova* **21**, 761 (1998).
10. K. C. Das, C. K. Das. *Biochem. Biophys. Res. Comm.* **295**, 62 (2002).
11. S. Bollo, L. J. Núñez-Vergara, M. Bontá, G. Chauviere, J. Perié, J. Squella. *J. Electroanal. Chem.* **511**, 46 (2001).
12. Y. Okada, H. Okajima Y. Shima, H. Ohta. *Redox Report* **7**, 153 (2002).
13. H. S. Blois. *Nature* **181**, 1199 (1958).
14. K. E. Malterud, T. L. Farbrot, A. E. Huse, R. B. Sund. *Pharmacology* **47** (suppl. 1), 77 (1993).
15. M. Joyeux, F. Mortier, J. Fleurentin. *Phytother. Res.* **9**, 228 (1995).
16. K. Yamasaki, A. Hashimoto, Y. Kokusenya T. Miyamoto, T. Sato. *Chem. Pharm. Bull.* **42**, 351 (1994).
17. J. Lehuédé, B. Faucounneau, L. Barrier, M. Ourakow, A. Piriou, J. M. Vierfond. *Eur. J. Med. Chem.* **34**, 991 (1999).
18. L. Mathiesen, K. E. Malterud, R. B. Sund. *Free Radical Biol. Med.* **22**, 307 (1997).
19. A. Tait, S. Ganzerli, M. Di Bella. *Tetrahedron* **52**, 12587 (1996).
20. L. H. P. Teixeira, C. A. M. Fraga. *J. Braz. Chem. Soc.* **9**, 119 (1998).
21. L. H. P. Teixeira. Síntese de Novos Derivados Benzotiazínicos Planejados Racionalmente como Inibidores Seletivos de PGHS-2 e 5-LO. M.Sc. thesis, Instituto de Química, UFRJ, Rio de Janeiro, Brazil, 1998.

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