

Novel antimicrobial and anti-acetylcholinesterase dihydroisoxazoles from (R)-limonene

Enis Ben Bnina ¹, Anis Romdhane ¹, Majda Daami-Remadi ² and Hichem Ben Jannet ^{1,*}

¹ Laboratoire de Chimie Hétérocyclique, Produits Naturels et Réactivité, Equipe: Chimie Médicinale et Produits Naturels, Faculté des Sciences de Monastir, Université de Monastir, Avenue de l'Environnement, 5019 Monastir, Tunisie

² UR13AGR09, Production Horticole Intégrée au Centre Est Tunisien, Centre Régional des Recherches en Horticulture et Agriculture Biologique de Chott-Mariem, Université de Sousse, 4042, Chott-Mariem, Tunisie

* Corresponding author at: Laboratoire de Chimie Hétérocyclique, Produits Naturels et Réactivité, Equipe: Chimie Médicinale et Produits Naturels, Faculté des Sciences de Monastir, Université de Monastir, Avenue de l'Environnement, 5019 Monastir, Tunisie.
 Tel.: +21.67.3500279. Fax: +21.67.3500278. E-mail address: hich.benjanjet@yahoo.fr (H.B. Jannet).

ARTICLE INFORMATION



DOI: 10.5155/eurjchem.6.1.21-30.1137

Received: 22 August 2014

Received in revised form: 19 September 2014

Accepted: 19 September 2014

Published online: 31 March 2015

Printed: 31 March 2015

KEYWORDS

Lactam
 (R)-Limonene
 Antimicrobial
 Cycloaddition
 Dihydroisoxazoles
 Anti-acetylcholinesterase

ABSTRACT

We report herein the convenient procedures for the efficient and easy synthesis, and the antimicrobial and the anti-acetylcholinesterase evaluation of two new series of (R)-limonene derivatives. A substantial modification aimed at targeting to discover novel structures with a better antimicrobial and anti-acetylcholinesterase (anti-AChE) activities. The condensation of (R)-limonene (**1**) with various aryl nitrile oxides led, via the 1,3-dipolar cycloaddition reaction, conducted with complete region-specificity, to a series of new limonene-dihydroisoxazoles, **2a-h**. On the other hand, N-alkylation of the previously prepared limonene-lactam derivative (**3**) yielded the corresponding dipolarophile (**4**), which affords by condensation with aryl nitrile oxides the expected new dihydroisoxazoles, **5a-h**. The target compounds were completely characterized by ¹H NMR, ¹³C NMR and MS. All the synthesized heterocyclic compounds were tested for their antimicrobial and anti-acetylcholinesterase activities. The dihydroisoxazoles **2a** (IZ = 13.25 mm, cc = 1 mg/mL) and **5b** (IZ = 13.75 mm, cc = 1 mg/mL) exhibited the highest antifungal activity. The greatest anti-acetylcholinesterase activity was exhibited by **2f** (IC₅₀ = 82±3 µg/mL) and by **5a** (IC₅₀ = 99±1 µg/mL).

Cite this: *Eur. J. Chem.* 2015, 6(1), 21-30

1. Introduction

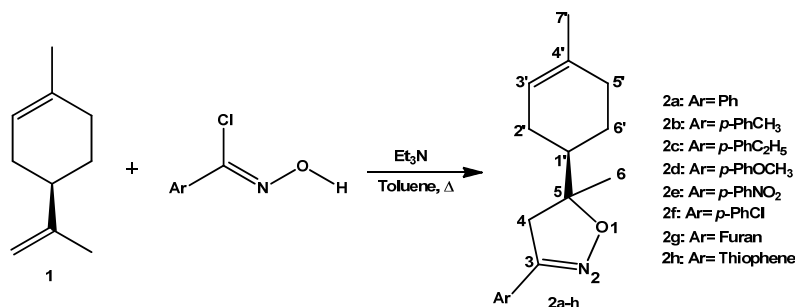
Limonene is the most abundant monoterpene, which has both an endocyclic and an exocyclic double bond [1]. It is found in several aromatic plants and particularly in peels and flowers of citrus species [2]. Several studies carried out proved that limonene presents various biological activities such as antifungal, anti-aflatoxigenic, antioxidant [3] and anti-acaricide effects [4] it prevents also tumor development induced in the mammary glands [5] the skin [6] the liver [7] the lungs, the forestomach [8] and the pancreas [9]. Moreover, it has been reported that limonene has an antiproliferative effect in a variety of cell types, such as melanoma, gastric and prostate cancer cells [10]. Thus far, most efforts have been directed in characterizing its ability to prevent carcinogen-induced cancer [11].

This monocyclic monoterpene drew the attention of the researchers to be able to develop it as being a precursor in several synthesis reactions leading important molecules that find application in several fields [12] such as chiral vicinal diamines, amino alcohols and aminophosphines [13]. The alkoxylation of limonene furnished 1-methyl-4-(α-alkoxy-

isopropyl)-cyclohexenes [14] which are used as flavors and fragrances for perfume and cosmetic products, as additives for pharmaceuticals and agricultural chemicals, as well as in the food industry [15].

In the other hand, heterocyclic compounds have so far been synthesized mainly due to their wide range of biological activities. In fact, much attention has been paid to the synthesis of heterocyclic compounds bearing nitrogen and oxygen containing ring system dihydroisoxazoles (also named isoxazolines) are of great interest because they have proven potential pharmaceutical leads [16,17] and versatile intermediates for the synthesis of different classes of functionalized molecules and a variety of bioactive compounds [18]. They are associated with diverse pharmacological activities such as human influenza A virus [19], anti-tuberculosis [20], antimicrobial [21,22] antidepressant [23], antimuscarinic [24], anti-inflammatory [25] and herbicidal effects [26].

Several methods for the preparation of dihydroisoxazoles have been reported [27,28]. Particularly, 1,3-dipolar cycloaddition reaction represents a useful synthetic method for the preparation of five membered-ring heterocyclic compounds.



Scheme 1

In recent work, the fully isoxazolines were prepared by the reaction of aryl nitrile oxides with alkenes [29,30].

In continuation of our research directed towards the study of the reactivity of some natural products, in the present work, (*R*)-limonene was our starting material. To enrich the previously series of dihydroisoxazoles prepared from limonene [31], we have treated it with another series of aryl nitrile oxides leading to new region-specific limonene-dihydroisoxazoles, **2a-h**. In the other hand, in order to continue the exploration of the exocyclic double bond in limonene, a lactamation reaction was carried out regioselectively affording an original limonene-lactam derivative (**3**) which was the subject to an *N*-allylation reaction yielding a novel *N*-allyl limonene-lactame (**4**) used as a second precursor to access to a new series of dihydroisoxazoles, **5a-h**. Considering the potential interest of antibacterial [32-34] and antifungal [35,36] effects of dihydroisoxazoles, we focused our biological valorization on the study of these activities for all the synthesized compounds. The anti-acetylcholinesterase activity of compounds **2a-h** and **5a-h** was also evaluated and discussed in this work.

2. Experimental

2.1. Chemistry

Mass spectra were obtained with ESI-TOF (LCT Premier XE, Waters) using the reflectron mode in the positive ion mode. Leucine-enkephaline peptide was employed as the Lock Spray lockmass. ¹H (300 MHz) and ¹³C (75 MHz) NMR spectra were recorded on a Bruker AM-300 spectrometer, using CDCl₃ as solvent and non deuterated residual solvent was used as internal standard. Chemical shifts (δ) are given in parts per million (ppm) and coupling constants (J) in Hertz.

2.1.1. General method for the preparation of compounds **2a-h** [30]

In a typical procedure, to a stirred solution of chlorinated oximes (1.1 mmol) and limonene **1** (Aldrich Chemical Co., 90% purity, 98%, enantiomeric excess, ee) (1 mmol in dry toluene 30 mL), was added dropwise a solution of triethylamine (1.1 mmol) in 5 mL of toluene. The mixture was refluxed for 2-4 h under nitrogen. The reaction evolution was monitored by TLC. When all the starting materials were consumed, the mixture was cooled to room temperature, the solvent was evaporated off and the crude was purified by chromatography on a silica gel column using petroleum ether: chloroform (6:4, v:v) to give the desired isoxazolines, **2a-h** (Scheme 1).

(*R*)-5-methyl-5-((*R*)-4-methylcyclohex-3-enyl)-3-phenyl-4,5-dihydroisoxazole (**2a**): Color: White solid. Yield: 67%. ¹H NMR (300 MHz, CDCl₃, δ, ppm): 1.40 (s, 3H, H-6), 1.65 (s, 3H, H-7'), 1.84-2.17 (m, 7H, H-1', H-2', H-5', H-6'), 2.90 (d, 1H, J = 16.5 Hz, H-4a), 3.22 (d, 1H, J = 16.5 Hz, H-4b), 5.39 (s, 1H, H-3'), 7.36-

7.71 (m, 5H, Ar-H). ¹³C NMR (75 MHz, CDCl₃, δ, ppm): 23.3-30.5, 42.4, 43.6, 89.9, 120.0, 126.0-134.3, 134.1, 155.8. HRMS (ESI⁺): calcd. for (C₁₇H₂₁NO)⁺ [M+Na]⁺ 278.1623, found: 278.1638.

(*R*)-5-methyl-5-((*R*)-4-methylcyclohex-3-enyl)-3-*p*-tolyl-4,5-dihydroisoxazole (**2b**): Color: White solid. Yield: 73%. ¹H NMR (300 MHz, CDCl₃, δ, ppm): 1.29 (s, 3H, H-6), 1.55 (s, 3H, H-7'), 1.73-2.06 (m, 7H, H-1', H-2', H-5', H-6'), 2.41 (s, 3H, H-7''), 2.79 (d, 1H, J = 16.5 Hz, H-4a), 3.11 (d, 1H, J = 16.5 Hz, H-4b), 5.30 (s, 1H, H-3'), 7.09 (d, 2H, J = 8.1 Hz, Ar-H), 7.45 (d, 2H, J = 8.1 Hz, Ar-H). ¹³C NMR (75 MHz, CDCl₃, δ, ppm): 23.6-30.9, 24.3, 42.9, 43.0, 89.9, 120.4, 126.3-140.2, 134.4, 156.0. HRMS (ESI⁺): calcd. for (C₁₈H₂₃NO)⁺ [M+Na]⁺ 292.1780, found: 292.1792.

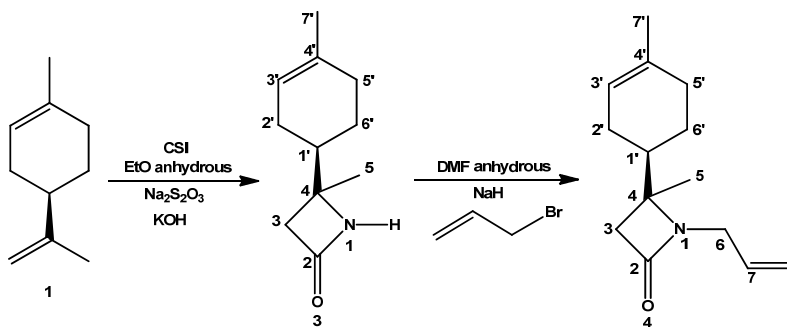
(*R*)-3-(4-ethylphenyl)-5-methyl-5-((*R*)-4-methylcyclohex-3-enyl)-4,5-dihydroisoxazole (**2c**): Color: White solid. Yield: 76%. ¹H NMR (300 MHz, CDCl₃, δ, ppm): 1.24 (t, 3H, J = 7.5 Hz, H-8''), 1.39 (s, 3H, H-6), 1.65 (s, 3H, H-7'), 1.83-2.12 (m, 7H, H-1', H-2', H-5', H-6'), 2.66 (q, 2H, J = 7.5 Hz, H-7''), 2.90 (d, 1H, J = 16.5 Hz, H-4a), 3.21 (d, 1H, J = 16.5 Hz, H-4b), 5.39 (s, 1H, H-3'), 7.21 (d, 2H, J = 8.1 Hz, Ar-H), 7.58 (d, 2H, J = 8.1 Hz, Ar-H). ¹³C NMR (75 MHz, CDCl₃, δ, ppm): 15.5, 23.3-30.6, 42.6, 43.7, 89.6, 120.1, 126.5-146.2, 134.1, 155.7. HRMS (ESI⁺): calcd. for (C₁₉H₂₅NO)⁺ [M+Na]⁺ 306.1936, found: 306.1938.

(*R*)-3-(4-methoxyphenyl)-5-methyl-5-((*R*)-4-methylcyclohex-3-enyl)-4,5-dihydroisoxazole (**2d**): Color: White solid. Yield: 80%. ¹H NMR (300 MHz, CDCl₃, δ, ppm): 1.43 (s, 3H, H-6), 1.63 (s, 3H, H-7'), 1.87-2.16 (m, 7H, H-1', H-2', H-5', H-6'), 2.94 (d, 1H, J = 16.5 Hz, H-4a), 3.25 (d, 1H, J = 16.5 Hz, H-4b), 3.83 (s, 3H, H-7''), 5.39 (s, 1H, H-3'), 6.89 (d, 2H, J = 8.7 Hz, Ar-H), 7.61 (d, 2H, J = 8.7 Hz, Ar-H). ¹³C NMR (75 MHz, CDCl₃, δ, ppm): 23.2-30.5, 42.4, 43.8, 55.4, 114.1-128.0, 120.0, 134.3, 160.8. HRMS (ESI⁺): calcd. for (C₁₈H₂₃NO₂)⁺ [M+Na]⁺ 308.1619, found: 308.1630.

(*R*)-5-methyl-5-((*R*)-4-methylcyclohex-3-enyl)-3-(4-nitrophenyl)-4,5-dihydroisoxazole (**2e**): Color: White solid. Yield: 82%. ¹H NMR (300 MHz, CDCl₃, δ, ppm): 1.38 (s, 3H, H-6), 1.64 (s, 3H, H-7'), 1.73-2.17 (m, 7H, H-1', H-2', H-5', H-6'), 2.91 (d, 1H, J = 16.8 Hz, H-4a), 3.25 (d, 1H, J = 16.8 Hz, H-4b), 5.39 (s, 1H, H-3'), 7.79 (d, 2H, J = 9 Hz, Ar-H), 8.24 (d, 2H, J = 9 Hz, Ar-H). ¹³C NMR (75 MHz, CDCl₃, δ, ppm): 23.4-30.4, 42.0, 42.7, 91.6, 119.7, 123.4-148.3, 134.3, 154.4. HRMS (ESI⁺): calcd. for (C₁₇H₂₀N₂O₃)⁺ [M+Na]⁺ 323.1474, found: 323.1486.

(*R*)-3-(4-chlorophenyl)-5-methyl-5-((*R*)-4-methylcyclohex-3-enyl)-4,5-dihydroisoxazole (**2f**): Color: White solid. Yield: 87%. ¹H NMR (300 MHz, CDCl₃, δ, ppm): 1.39 (s, 3H, H-6), 1.65 (s, 3H, H-7'), 1.93-2.11 (m, 7H, H-1', H-2', H-5', H-6'), 2.89 (d, 1H, J = 16.5 Hz, H-4a), 3.19 (d, 1H, J = 16.5 Hz, H-4), 5.38 (s, 1H, H-3'), 7.35 (d, 2H, J = 9 Hz, Ar-H), 7.58 (d, 2H, J = 9 Hz, Ar-H). ¹³C NMR (75 MHz, CDCl₃, δ, ppm): 23.0-30.8, 42.8, 43.7, 90.6, 120.2, 128.0-136.0, 134.6, 155.2. HRMS (ESI⁺): calcd. for (C₁₇H₂₀ClNO)⁺ [M+Na]⁺ 312.1233, found: 312.1242.

(*R*)-3-(furan-2-yl)-5-methyl-5-((*R*)-4-methylcyclohex-3-enyl)-4,5-dihydroisoxazole (**2g**): Color: Gray solid. Yield: 70%.



Scheme 2

^1H NMR (300 MHz, CDCl_3 , δ , ppm): 1.38 (s, 3H, H-6), 1.65 (s, 3H, H-7'), 1.82-2.16 (m, 7H, H-1', H-2', H-5', H-6'), 2.86 (d, 1H, $J = 16.8$ Hz, H-4a), 3.18 (d, 1H, $J = 16.8$ Hz, H-4b), 5.39 (s, 1H, H-3'), 6.47 (dd, 1H, $J_1 = 3.3$ Hz, $J_2 = 1.8$ Hz, Ar-H), 6.65 (d, 1H, $J = 3.3$ Hz, Ar-H), 7.49 (d, 1H, $J = 1.8$ Hz, Ar-H). ^{13}C NMR (75 MHz, CDCl_3 , δ , ppm): 23.1-30.8, 42.7, 43.7, δ 90.2 (C-5), 111.3-112.0, 120.3, 134.5, 144.3-146.0, 153.7. HRMS (ESI $^+$): calcd. for $(\text{C}_{15}\text{H}_{19}\text{NO}_2)^+$ $[\text{M}+\text{Na}]^+$ 268.1416, found: 268.1427.

(*R*)-5-methyl-5-((*R*)-4-methylcyclohex-3-enyl)-3-(thiophen-2-yl)-4,5-dihydroisoxazole (**2h**): Color: Gray solid. Yield: 68%. ^1H NMR (300 MHz, CDCl_3 , δ , ppm): 1.39 (s, 3H, H-6), 1.64 (s, 3H, H-7'), 1.87-2.17 (m, 7H, H-1', H-2', H-5', H-6'), 2.90 (d, 1H, $J = 16.8$ Hz, H-4a), 3.22 (d, 1H, $J = 16.8$ Hz, H-4b), 5.38 (s, 1H, H-3'), 7.03 (dd, 1H, $J_1 = 5.7$ Hz, $J_2 = 4.5$ Hz, Ar-H), 7.14 (d, 1H, $J = 4.5$ Hz, Ar-H), 7.34 (d, 1H, $J = 5.7$ Hz, Ar-H). ^{13}C NMR (75 MHz, CDCl_3 , δ , ppm): 24.5-31.4, 42.6, 43.7, 90.7, 116.1-117.6, 120.3, 133.9, 151.9-152.1, 154.0. HRMS (ESI $^+$): calcd. for $(\text{C}_{15}\text{H}_{19}\text{NOS})^+$ $[\text{M}+\text{Na}]^+$ 284.1187, found: 284.1199.

2.1.2. General method for the preparation of (*R*)-4-methyl-4-((*R*)-4-methylcyclohex-3-enyl)azetididin-2-one (**3**) [37]

A mixture of 2.72 g (20.0 mmol) of (*R*)-limonene (**1**) and 2.84 g (20.0 mmol) of CSI was stirred in dry diethyl ether (50 mL) at room temperature for 9 h. Na_2SO_3 (3.78 g) in water (50 mL) was then cautiously added dropwise to the solution. The pH was held at 7-8 by the addition of 20% aqueous KOH. After separation of the organic layer, the aqueous one was extracted with diethyl ether (2 \times 50 mL). The combined organic layers were dried (Na_2SO_4), evaporated and the resulting residue was purified by chromatography on silica gel using chloroform: ethyl acetate (6:4, v:v) to yield compound **3** (Scheme 2).

(*R*)-4-methyl-4-((*R*)-4-methylcyclohex-3-enyl)azetididin-2-one (**3**): Color: White solid. Yield: 78%. ^1H NMR (300 MHz, CDCl_3 , δ , ppm): 1.52 (s, 3H, s, H-5'), 1.56-1.94 (m, 7H, H-1', H-2', H-5', H-6'), 1.71 (s, 3H, H-7'), 2.42 (d, 1H, $J = 14.4$ Hz, H-3a), 2.66 (d, 1H, $J = 14.4$ Hz, H-3a), 5.37 (s, 1H, H-3'), 8.08 (s, 1H, NH). ^{13}C NMR (75 MHz, CDCl_3 , δ , ppm): 21.3-31.0, 36.3, 39.9, 54.0, 119.2, 133.6, 167.7. HRMS (ESI $^+$): calcd. for $(\text{C}_{11}\text{H}_{17}\text{NO})^+$ $[\text{M}+\text{H}]^+$ 180.1368, found: 180.1380.

2.1.3. General method for the preparation of (*R*)-1-allyl-4-methyl-4-((*R*)-4-methylcyclohex-3-enyl)azetididin-2-one (**4**) [38]

A mixture of lactam **3** (0.70 mmol), anhydrous DMF (12 mL) and sodium hydride (1.3 mmol) was stirred at room temperature in the argon atmosphere until evolution of hydrogen had ceased. Then the allyl bromide (3.45 mmol) was dropped into the mixture and the stirring was continued for 1 h. The mixture was poured into water and extracted with diethyl ether. The organic layer was dried over MgSO_4 . Then the solvent was removed and the residue was purified by

chromatography on silica gel using chloroform-ethyl acetate (9:1, v:v) to produce **4** (Scheme 2).

(*R*)-1-allyl-4-methyl-4-((*R*)-4-methylcyclohex-3-enyl)azetididin-2-one (**4**): Color: White solid. Yield: 82%. ^1H NMR (300 MHz, CDCl_3 , δ , ppm): 1.35 (s, 3H, H-5), 1.62-1.90 (m, 7H, H-1', H-2', H-5', H-6'), 1.71 (s, 3H, H-7'), 2.42 (d, 1H, $J = 14.4$ Hz, H-3a), 2.66 (d, 1H, $J = 14.4$ Hz, H-3b), 3.68-3.72 (m, 2H, H-6), 5.12 (dd, 1H, $J_{\text{cis}} = 10.2$ Hz, $J_2 = 1.2$ Hz, H-8), 5.22 (dd, 1H, $J_{\text{trans}} = 17.1$ Hz, $J_2 = 1.8$ Hz, H-8), 5.37 (s, 1H, H-3'), 5.77 (m, 1H, H-7). ^{13}C NMR (75 MHz, CDCl_3 , δ , ppm): 21.4-30.2, 38.6, 46.1, 116.2, 120.3, 133.4, 134.2, 167.2. HRMS (ESI $^+$): calcd. for $(\text{C}_{14}\text{H}_{21}\text{NO})^+$ $[\text{M}+\text{H}]^+$ 220.1682, found: 220.1693.

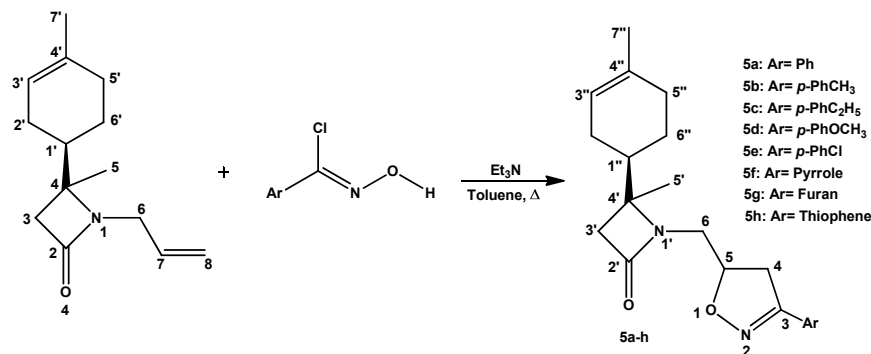
2.1.4. General method for the preparation of compounds **5a-h** [30]

In a typical procedure, to a stirred solution of aryl nitrile oxide (1.2 mmol) and compound **4** (1 mmol) in dry toluene (30 mL), was added dropwise a solution of triethylamine (1.2 mmol) in 5 mL of toluene. The mixture was refluxed for 2-4 hours under nitrogen. The reaction evolution was checked by TLC. When all the starting materials were consumed, the mixture was cooled to room temperature, the solvent was evaporated off and the crude was purified by chromatography on a silica gel column using chloroform-ethyl acetate (9:1, v:v) to yield the desired cycloadducts, **5a-h** (Scheme 3).

(*R*)-4-methyl-4-((*R*)-4-methylcyclohex-3-enyl)-1-((3-phenyl-4,5-dihydroisoxazol-5-yl)methyl)azetididin-2-one (**5a**): Color: White solid. Yield: 70%. ^1H NMR (300 MHz, CDCl_3 , δ , ppm): 1.40 (s, 3H, H-5'), 1.70 (s, 3H, H-7'), 1.79-1.99 (m, 7H, H-1', H-2', H-5', H-6'), 2.38 (d, 1H, $J = 14.7$ Hz, H-3'a), 2.73-3.07 (m, 2H, H-4), 2.75 (d, 1H, $J = 14.7$ Hz, H-3'b), 3.22-3.52 (m, 2H, H-6), 5.30 (s, 1H, H-3'), 5.71-5.84 (m, 1H, H-5), 7.33-7.61 (m, 5H, Ar-H). ^{13}C NMR (75 MHz, CDCl_3 , δ , ppm): 21.4-30.2, 23.0, 23.8, 39.1, 39.5, 54.5, 55.6, 61.8, 120.5, 127.8-132.3, 134.2, 159.8, 166.5. HRMS (ESI $^+$): calcd. for $(\text{C}_{21}\text{H}_{26}\text{N}_2\text{O}_2)^+$ $[\text{M}+\text{H}]^+$ 339.1994, found: 339.1999.

(*R*)-4-methyl-4-((*R*)-4-methylcyclohex-3-enyl)-1-((3-*p*-tolyl-4,5-dihydroisoxazol-5-yl)methyl)azetididin-2-one (**5b**): Color: White solid. Yield: 77%. ^1H NMR (300 MHz, CDCl_3 , δ , ppm): 1.41 (s, 3H, H-5'), 1.71 (s, 3H, H-7'), 1.79-1.91 (m, 7H, H-1', H-2', H-5', H-6'), 2.39 (s, 3H, H-7''), 2.42 (d, 1H, $J = 14.4$ Hz, H-3'a), 2.76 (d, 1H, $J = 14.4$ Hz, H-3'b), 2.77-3.10 (m, 2H, H-4), 3.18-3.60 (m, 2H, H-6), 5.30 (s, 1H, H-3'), 5.70-5.84 (m, 1H, H-5), 7.14 (d, 2H, $J = 8.1$ Hz, Ar-H), 7.48 (d, 2H, $J = 8.1$ Hz, Ar-H). ^{13}C NMR (75 MHz, CDCl_3 , δ , ppm): 21.6-30.3, 22.9, 23.9, 24.2, 39.1, 39.6, 54.4, 55.9, 62.4, 120.1, 128.6-130.8, 134.3, 139.8, 160.6, 166.3. HRMS (ESI $^+$): calcd. for $(\text{C}_{22}\text{H}_{28}\text{N}_2\text{O}_2)^+$ $[\text{M}+\text{H}]^+$ 353.2151, found: 353.2162.

(*R*)-1-((3-(4-ethylphenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-methyl-4-((*R*)-4-methylcyclohex-3-enyl)azetididin-2-one (**5c**): Color: White solid. Yield: 75%.



Scheme 3

^1H NMR (300 MHz, CDCl_3 , δ , ppm): 1.24 (t, 3H, $J = 7.2$ Hz, H-8'''), 1.40 (s, 3H, H-5''), 1.57-1.89 (m, 7H, H-1'', H-2'', H-5'', H-6''), 1.71 (s, 3H, H-7''), 2.44 (d, 1H, $J = 15.3$ Hz, H-3'a), 2.65 (q, 2H, $J = 7.2$ Hz, H-7''), 2.73 (d, 1H, $J = 15.3$ Hz, H-3'b), 2.75-3.11 (m, 2H, H-4), 3.81-3.57 (m, 2H, H-6), 5.28 (s, 1H, H-3''), 5.68-5.82 (m, 1H, H-5), 6.96 (d, 2H, $J = 8.1$ Hz, Ar-H), 7.48 (d, 2H, $J = 8.1$ Hz, Ar-H). ^{13}C NMR (75 MHz, CDCl_3 , δ , ppm): 14.2, 21.4-30.6, 23.1, 24.0, 31.8, 39.2, 39.6, 54.8, 55.8, 62.0, 119.8, 127.3-130.9, 134.2, 160.7, 167.3. HRMS (ESI⁺) calcd. for $(\text{C}_{23}\text{H}_{30}\text{N}_2\text{O}_2)^+$ [M+H]⁺ 367.2307, found: 367.2321.

(*R*)-1-((3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-methyl-4-((*R*)-4-methylcyclohex-3-enyl)azetidin-2-one (**5d**): Color: White solid. Yield: 87%. ^1H NMR (300 MHz, CDCl_3 , δ , ppm): 1.41 (s, 3H, H-5''), 1.63-1.98 (m, 7H, H-1'', H-2'', H-5'', H-6''), 1.75 (s, 3H, H-7''), 2.52 (d, 1H, $J = 14.7$ Hz, H-3'a), 2.84 (d, 1H, $J = 14.7$ Hz, H-3'b), 2.85-3.38 (m, 2H, H-4), 3.66-3.70 (m, 2H, H-6), 3.83 (s, 3H, H-7''), 5.36 (s, 1H, H-3''), 5.72-5.83 (m, 1H, H-5), 6.90 (d, 2H, $J = 8.7$ Hz, Ar-H), 7.60 (d, 2H, $J = 8.7$ Hz, Ar-H). ^{13}C NMR (75 MHz, CDCl_3 , δ , ppm): 21.9-30.9, 22.8, 23.5, 39.2, 39.5, 55.4, 56.3, 58.2, 61.8, 114.1, 121.7, 127.3-129.7, 134.3, 160.4, 161.1, 168.7. HRMS (ESI⁺): calcd. for $(\text{C}_{22}\text{H}_{28}\text{N}_2\text{O}_3)^+$ [M+H]⁺ 369.2100, found: 369.2108.

(*R*)-1-((3-(4-chlorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)-4-methyl-4-((*R*)-4-methylcyclohex-3-enyl)azetidin-2-one (**5e**): Color: Yellow solid. Yield: 85%. ^1H NMR (300 MHz, CDCl_3 , δ , ppm): 1.41 (s, 3H, H-5''), 1.61 (s, 3H, H-7''), 1.77-1.99 (m, 7H, H-1'', H-2'', H-5'', H-6''), 2.50 (d, 1H, $J = 14.1$ Hz, H-3'a), 2.75 (d, 1H, $J = 14.1$ Hz, H-3'b), 2.84-3.38 (m, 2H, H-4), 3.64-3.78 (m, 2H, H-6), 5.34 (s, 1H, H-3''), 5.72-5.83 (m, 1H, H-5), 7.54 (d, 2H, $J = 8.4$ Hz, Ar-H), 7.67 (d, 2H, $J = 8.4$ Hz, Ar-H). ^{13}C NMR (75 MHz, CDCl_3 , δ , ppm): 21.5-30.4, 23.2, 23.8, 39.3, 39.7, 55.7, 59.6, 62.4, 121.3, δ 127.8-131.7, 134.2, 135.3, 161.7, 167.8. HRMS (ESI⁺): calcd. for $(\text{C}_{21}\text{H}_{25}\text{ClN}_2\text{O}_2)^+$ [M+H]⁺ 373.1605, found: 373.1613.

(*R*)-1-((3-(1H-pyrrol-2-yl)-4,5-dihydroisoxazol-5-yl)methyl)-4-methyl-4-((*R*)-4-methylcyclohex-3-enyl)azetidin-2-one (**5f**): Color: Crimson solid. Yield: 72%. ^1H NMR (300 MHz, CDCl_3 , δ , ppm): 1.40 (s, 3H, H-5''), 1.62-2.01 (m, 7H, H-1'', H-2'', H-5'', H-6''), 1.73 (s, 3H, H-7''), 2.49 (d, 1H, $J = 11.4$ Hz, H-3'a), 2.77-3.08 (m, 2H, H-4), 2.84 (d, 1H, $J = 11.4$ Hz, H-3'b), 3.16-3.52 (m, 2H, H-6), 5.35 (s, 1H, H-3''), 5.77-5.86 (m, 1H, H-5), 7.04 (dd, 1H, $J_1 = 3.6$ Hz, $J_2 = 2.4$ Hz, Ar-H), 7.18 (d, 1H, $J = 3.6$ Hz, Ar-H), 7.36 (d, 1H, $J = 2.4$ Hz, Ar-H), 11.18 (s, 1H, NH). ^{13}C NMR (75 MHz, CDCl_3 , δ , ppm): 21.8-30.3, 23.3, 24.1, 39.3, 40.0, 61.4, 61.6, 79.4, 117.8-119.7, 119.8, 134.2, 167.0, 168.7. HRMS (ESI⁺): calcd. for $(\text{C}_{19}\text{H}_{25}\text{N}_3\text{O}_2)^+$ [M+H]⁺ 328.1947, found: 328.1959.

(*R*)-1-((3-(furan-2-yl)-4,5-dihydroisoxazol-5-yl)methyl)-4-methyl-4-((*R*)-4-methylcyclohex-3-enyl)azetidin-2-one (**5g**): Color: White solid. Yield: 68%. ^1H NMR (300 MHz, CDCl_3 , δ , ppm): 1.39 (s, 3H, H-5''), 1.57-1.92 (m, 7H, H-1'', H-2'', H-5'', H-6''), 1.69 (s, 3H, H-7''), 2.41 (d, 1H, $J = 11.1$ Hz, H-3'a), 2.73-3.07

(m, 2H, H-4), 2.76 (d, 1H, $J = 11.1$ Hz, H-3'b), 3.22-3.52 (m, 2H, H-6), 5.31 (s, 1H, H-3''), 5.71-5.84 (m, 1H, H-5), 7.18 (dd, 1H, $J = 3.6$ Hz, $J = 1.5$ Hz, Ar-H), 7.18 (d, 1H, $J = 3.6$ Hz, Ar-H), 7.36 (d, 1H, $J = 1.5$ Hz, Ar-H). ^{13}C -NMR (75 MHz, CDCl_3 , δ , ppm): 21.8-30.3, 23.1, 24.0, 39.3, 39.9, 61.5, 61.7, 79.2, 108.6-109.7, 122.1, 134.4, 139.2-143.9, 165.7, 167.3. HRMS (ESI⁺): calcd. for $(\text{C}_{19}\text{H}_{24}\text{N}_2\text{O}_3)^+$ [M+H]⁺ 329.1787, found: 329.1799.

(*R*)-4-methyl-4-((*R*)-4-methylcyclohex-3-enyl)-1-((3-(thiophen-2-yl)-4,5-dihydroisoxazol-5-yl)methyl)azetidin-2-one (**5h**): Color: Gray solid. Yield: 74%. ^1H NMR (300 MHz, CDCl_3 , δ , ppm): 1.41 (s, 3H, H-5''), 1.63-1.97 (m, 7H, H-1'', H-2'', H-5'', H-6''), 1.69 (s, 3H, H-7''), 2.51 (d, 1H, $J = 13.5$ Hz, H-3'a), 2.75-2.86 (m, 2H, H-4), 2.80 (d, 1H, $J = 13.5$ Hz, H-3'b), 3.38-3.73 (m, 2H, H-6), 5.35 (s, 1H, H-3''), 5.83 (m, 1H, H-5), 7.05 (dd, 1H, $J = 6$ Hz, $J = 4.8$ Hz, Ar-H), 7.19 (d, 1H, $J = 4.8$ Hz, Ar-H), 7.39 (d, 1H, $J = 6$ Hz, Ar-H). ^{13}C NMR (75 MHz, CDCl_3 , δ , ppm): 21.8-30.2, 23.2, 23.9, 39.3, 39.9, 61.4, 61.6, 79.4, 119.8, 127.4-128.7, 134.0, 167.0, 168.1. HRMS (ESI⁺): calcd. for $(\text{C}_{19}\text{H}_{24}\text{N}_2\text{O}_2\text{S})^+$ [M+H]⁺ 345.1635, found: 345.1649.

2.2. Biological activities

2.2.1. Antimicrobial evaluation

For the assessment of the antibacterial activity of (*R*)-Limonene **1** and the synthesized compounds, *Burkholderia glathei* 153 and *Bacillus pumilus* 420 *Pseudomonas aureofaciens* 499 were used as bacterial organisms. They were cultured at 25 °C on Nutrient Agar (NA) medium for 48 h before use.

For the antifungal test, five fungal species were used namely *Aspergillus niger*, *A. flavus*, *Penicillium digitatum*, *Trichoderma harzianum* and *Fusarium solani*. They were cultured at 25 °C during 7 days on Potato Dextrose Agar (PDA) medium before use.

These microorganisms were obtained from the Laboratory of Phytopathology of the Regional Center of Research on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia.

2.2.2. Antibacterial activity

(*R*)-Limonene **1** and the synthesized products were screened for their antibacterial activity using the agar disc diffusion method [39]. Nutrient Agar (NA) medium cooled at 45 °C was supplemented with a bacterial suspension (10^6 CFU/mL) and poured into Petri plates. After solidification, sterile Whatman paper discs (diameter 6 mm) were placed at the surface of the culture medium and 20 μL of the product dissolved in DMSO at different concentrations (250, 500 and 1000 $\mu\text{g/mL}$) were dropped onto each disc. The negative control plates had no product added to the filter paper whereas in the positive control plates, discs were impregnated

with the same volume of Ampicillin solution (5 mg/mL). The treated Petri dishes were incubated at 25 °C for 48 h. The antibacterial activity was evaluated by measuring the diameter of the inhibitory zones formed around the discs. The experiment was performed in triplicate.

2.2.3. Antifungal activity

Aspergillus niger, *A. flavus*, *Penicillium digitatum*, *Trichoderma harzianum* and *Fusarium solani* were used for the screening of antifungal activity of the products tested by using the disc diffusion method [40]. A conidial suspension of the tested fungi was prepared (10^4 - 10^5 CFU/mL) and added to PDA medium cooled at 45 °C and poured uniformly into Petri plates (diameter 90 mm). Sterilized paper discs (6 mm, Whatman No. 1 filter paper) were impregnated with 20 μ L of the product dissolved in DMSO at different concentrations (250, 500 and 1000 μ g/mL) and placed on the culture plates whereas the negative control plates had no product added to the filter paper. In the positive control plates, discs were imbibed with the same volume of a Carbendazim suspension (0.5 mg/mL). The diameter of the inhibition zone (mm) around the disc was measured after incubation at 25 °C for 4 days. The test was performed in triplicate.

2.2.4. Statistical analyses

Data of the antifungal and the antibacterial tests were analyzed separately. Data were subjected to one-way analysis of variance (ANOVA) according to a factorial design where fungal (or bacterial agents), compounds and the concentrations used were the three fixed factors. Means were separated using Student-Newman-Keul's (SNK) test at $p \leq 0.05$.

2.2.5. Acetylcholinesterase inhibition

The acetylcholinesterase (AChE) inhibition of the two series of the separated dihydroisoxazoles **2a-h** and **5a-h** was determined using an adaptation of the method described in the literature [41]. 90 μ L of 50 mM Tris-HCl buffer, pH = 8, 30 μ L of the sample dissolved in MeOH and 7.5 μ L of acetylcholinesterase solution containing 0.26 U/mL were mixed in a microwell plate and left to incubate for 15 min. Subsequently, 22.5 μ L of a solution of acetylcholine iodide (AChI) (0.023 mg/mL) and 142 μ L of 3 mM DTNB were added. The absorbance was read at 405 nm in the presence (A_{sample}) and in the absence (A_{control}) of the tested products and when the reaction reached equilibrium. Eserine was used as a positive control and water served as a negative control and it was considered 100% activity. The inhibition percentage (IP) is given as follow:

$$IP = 100 - (A_{\text{sample}} / A_{\text{control}}) \times 100 \quad (1)$$

where A_{control} is the absorbance of the control reaction containing all reagents except the tested sample, and A_{sample} is the absorbance of the tested compounds. Tests were carried out in triplicate.

3. Results and discussion

3.1. Chemistry

We report here, a novel one-step synthesis of isoxazolines by 1,3-dipolar cycloaddition reaction by condensing (*R*)-limonene **1** with different aryl nitrile oxides in the presence of triethylamine in refluxing anhydrous toluene for four hours to give a number of new structural analogues of 5-methyl-5-(4-methylcyclohex-3-enyl)-3-phenyl-4,5-dihydroisoxazoles **2a-h** (Scheme 1 and Table 1). Under these experimental conditions,

the reaction revealed in all cases the formation of a mixture of two diastereoisomers (*R,R*) and (*R,S*) not easily separable which were assigned as isoxazolines **2a-h** based on their spectral data. The structure of compounds **2a-h** has been assigned from their analytical data.

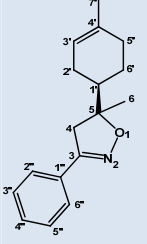
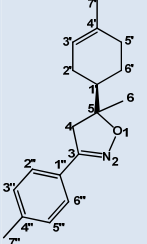
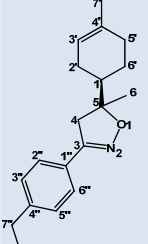
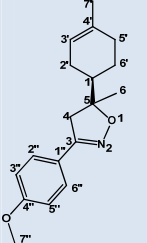
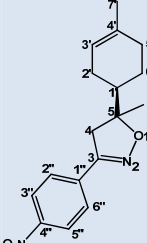
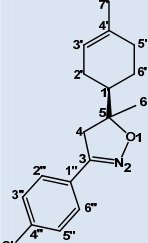
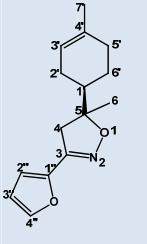
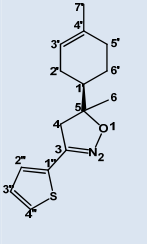
In fact, ES-HRMS of compound **2d**, given as an example, gave a pseudo-molecular ion peak $[M+Na]^+$ at m/z 308.1630 which is consistent with the molecular formula $C_{18}H_{23}NO_2$. Furthermore, the 1H NMR spectrum of this compound was compatible with the proposed structure. In addition to the signals corresponding to the protons introduced by the cyclic part of (*R*)-limonene **1**, we observed the presence of new signals consequent to the methoxy group (δ_H 3.83 ppm, s), to the methylene protons H-4a,b (δ_H 2.94 ppm, d, $J = 16.5$ Hz, H-4a and δ_H 3.25 ppm, d, $J = 16.5$ Hz, H-4b). A characteristic AA'BB' pattern for aromatic protons was observed in the same spectrum. Examination at 300 MHz offered excellent resolution with two doublets at δ_H 6.89 ppm (2H, d, $J = 8.7$ Hz; H-3'', H-5'') and at δ_H 7.61 ppm (2H, d, $J = 8.7$ Hz; H-2'', H-6'') all relative to the isoxazoline moiety. The ^{13}C NMR spectrum confirmed the above spectral data by the observation of signals at δ_C 55.40 ppm, δ_C 160.82 ppm and at δ_C 114.08-127.95 ppm attributable to the methoxy, to the iminic carbon (C-3) in the dihydroisoxazole ring and to the aromatic carbons, respectively. The duplication of the most indicated signals confirms the formation of the two diastereoisomers (*R, R*) and (*R, S*). The reaction was diastereoselective.

The β -lactam **3** was prepared in good yield (78%) by treating (*R*)-limonene **1** with chlorosulfonyl isocyanate (CSI) in anhydrous ether at room temperature for nine hours (Scheme 2). The structure of compound **3** was established on the basis of its spectroscopic data. Its positive ES-HRMS showed a pseudo-molecular ion peak at $[M+H]^+$ at m/z 180.1380 which is consistent with the molecular formula $C_{11}H_{17}NO$. The 1H NMR spectrum of this compound showed the appearance of two new signals at δ_H 2.42 ppm (1H, d, $J=14.4$ Hz) and δ_H 2.66 ppm (1H, d, $J=14.4$ Hz) attributable to the non-equivalent methylenic protons H-3a and H-3b of the lactam system, respectively, in addition to the characteristic signals of the protons introduced by the cyclic fragment of limonene **1**. Its ^{13}C NMR spectrum reinforced the proposed structure by the appearance of the signals of C-2 (CO) and C-3 (CH_2) at δ_C 167.69 ppm and δ_C 39.92 ppm, respectively, in addition to the carbon signals introduced by the limonene skeleton. The diastereo selectivity of the reaction was ascertained by the duplication of the most signals indicated above.

The required dipolarophile **4** was prepared by *N*-allylation (Scheme 2). Indeed, in our investigation, anhydrous dimethylformamide was found to be an excellent solvent for the reaction of allylbromide with lactam **3** in the presence of NaH used as a base. The *N*-allyllactam **4** was obtained in 88% yield and the reaction is completed after one hour at room temperature.

The structure of compound **4** was established on the basis of its spectroscopic data. Its ES-HRMS spectrum gave a pseudo-molecular ion peak $[M+H]^+$ at m/z 220.1693 compatible with the molecular formula $C_{14}H_{21}NO$. The 1H NMR spectrum of compound **4** indicates the presence of characteristic signals of the precursor **3** skeleton which can be, according to their chemical shifts and multiplicities, readily assigned to H-3' (δ_H 5.37 ppm, 1H), H-7' (δ_H 1.71 ppm, 3H), H-5 (δ_H 1.35 ppm, 3H). In addition of the signals corresponding to the protons introduced by the lactam **3**, we revealed the appearance of signals at δ_H 2.42 ppm (d, 1H, $J = 14.4$ Hz) and at δ_H 2.66 ppm (d, 1H, $J = 14.4$ Hz) relative to the non-equivalent protons H-3a and H-3b, of the lactam system, respectively. The two doublet of doublets at δ_H 5.12 ppm (dd, 1H, $J_{\text{cis}}=10.2$ Hz, $J_2=1.2$ Hz) and at δ_H 5.22 ppm (dd, 1H, $J_{\text{trans}}= 17.1$ Hz, $J_2=1.8$ Hz) was attributed to the terminal methylenic protons H-8a and H-8b.

Table 1. Synthesis of compounds 2a-h.

Entry	Compound	Entry	Compound	Entry	Compound	
1	 2a 67%	2	 2b 73%	3	 2c 76%	
4	 2d 80%	5	 2e 82%	6	 2f 87%	
7	 2g 68%	8	 2h 70%			

The multiplet at δ_{H} 5.77 ppm (m, 1H) was assigned to the ethylenic proton H-7. On the other hand, C-2 (167.19 ppm), C-3 (38.58 ppm), C-6 (46.10 ppm), C-7 (133.42 ppm) and C-8 (116.23 ppm) were readily assigned from the ^{13}C NMR spectrum.

The dipolarophile **4** was then treated with various aryl nitrile oxides generated *in situ* from aromatic oxime precursors under conventional conditions furnished the desired isoxazolines **5a-h** in good yields (68-87%) (Scheme 3 and Table 2). The reaction was regioselective and diastereoselective.

The structures of these compounds were confirmed according to their spectral data. The ^1H NMR spectra of compounds **5a-h** shows duplication of most signals indicating the formation of a mixture of diastereoisomers due to the apparition of two additional stereogenic centers, (C-4 and C-5). Attempts to separate these diastereoisomers by chromatography were not successful. The ES-HRMS of compound **5h** as an example gave a pseudo-molecular ion peak $[\text{M}+\text{H}]^+$ at m/z 345.1649 which is consistent with the molecular formula $\text{C}_{19}\text{H}_{24}\text{N}_2\text{O}_2\text{S}$. In the ^1H NMR spectrum of compound **5h** we observed a multiplet centered at 5.83 ppm attributable to the stereogenic center protons (H-5) system and another multiplet at δ_{H} 2.75-2.86 (m, 2H) assigned to from the non-equivalent methylenic protons H-4 both from the isoxazoline moiety. The N-CH₂ protons (H-6) appeared as a multiplet at 3.38-3.73 ppm. Signals at δ_{H} 2.51 ppm (d, 1H, $J=13.5$ Hz) and at δ_{H} 2.80 ppm (d, 1H, $J=13.5$ Hz) were attributed to the non-equivalent protons H-3'a and H-3'b of the lactam moiety. We revealed the appearance of signals at δ_{H} 7.39 ppm (d, 1H, $J=3.9$ Hz, H-4'''), at δ_{H} 7.19 ppm (d, 1H, $J=3.9$ Hz, H-2''') and at δ_{H} 7.05 ppm (t, 1H, $J=3.9$ Hz, H-3''') relative to the protons H-4''', H-2''' and H-

3''' of the thiophene system, respectively. ^{13}C NMR spectrum of compound **5h** exhibited a signal at 61.36 ppm corresponding to the N-CH₂ carbon (C-6), two signals at 39.25 ppm and 168.12 attributable to the lactam carbons C-3' and C-2', respectively, and two signals at 39.9 ppm and 79.43 ppm relative to the isoxazoline carbons C-4 and C-5, respectively. The C=N carbon C-3 resonated at 167.02 ppm. The signals observed between 127.37-128.66 ppm are attributable to the thiophene carbons. The duplication of some signals confirmed the diastereoselectivity of the reaction.

3.2. Biological activities

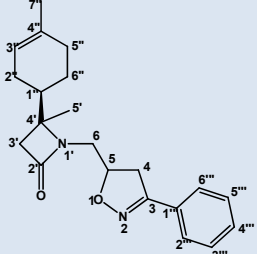
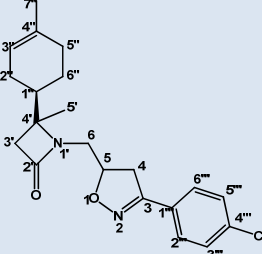
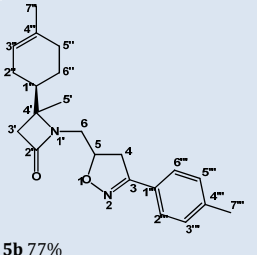
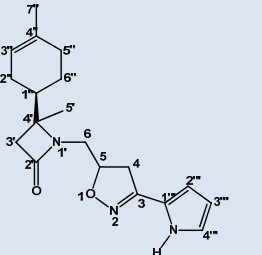
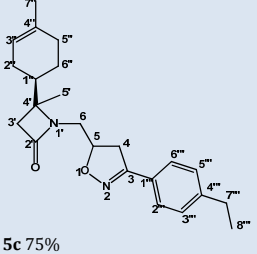
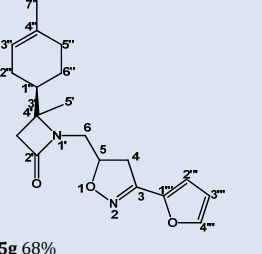
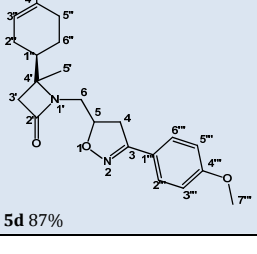
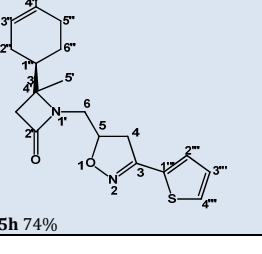
3.2.1. Antibacterial activity

Data presented in Table 3 show that the inhibition zones induced by (*R*)-Limonene **1**, lactam **3**, *N*-alkyllactam **4** and the different isoxazolines (**2a-h** and **5a-h**) tested against three bacteria *Burkholderia Glathei* 153, *Bacillus pumilus* 420 and *Pseudomonas aureofaciens* 499 vary significantly (at $p \leq 0.01$) depending on compounds, concentrations and the bacterial agents used. A significant interaction was noted between the three fixed factors.

Table 3 indicates also that the antibacterial activity of the tested compounds varied upon the concentrations used. In fact, visible inhibition zones were more evident since the concentration of 250 $\mu\text{g}/\text{mL}$ and the highest inhibitory effect was recorded at the concentration of 1000 $\mu\text{g}/\text{mL}$ with all compounds tested.

Burkholderia Glathei 153 and *Bacillus pumilus* 420 were very sensitive to (*R*)-limonene **1**, which gave inhibition zones varying from 32.5 to 33.75 mm.

Table 2. Synthesis of compounds 5a-h.

Entry	Compound	Entry	Compound
1	 5a 70%	5	 5e 85%
2	 5b 77%	6	 5f 75%
3	 5c 75%	7	 5g 68%
4	 5d 87%	8	 5h 74%

On the other hand, *Pseudomonas aureofaciens* 499 shows a resistance to (*R*)-limonene **1** with an inhibition zone of 15.25 mm.

4,5-Dihydroisoxazoles **2a**, **2d**, **2e**, **2g**, **5a**, **5d**, **5e** and **5g** showed significant inhibition zones (12.5-14 mm, concentration of 1000 µg/mL) against *Burkholderia Glathei* 153. This activity may be related to the nature of the aromatic ring (phenyl, 4-methoxyphenyl, 4-chlorophenyl and furanyl, respectively) fixed at C-3 of the dihydroisoxazole moiety in these compounds.

The growth of bacterial *Bacillus pumilus* 420 is inhibited mainly by compounds **2b**, **2d**, **2g**, **5b**, **5d** and **5g** showing inhibition zones varying between 12.5 and 15 mm. The nature of the aromatic ring (methylphenyl, 4-methoxyphenyl and furanyl, respectively) attached to C-3 of the isoxazoline system could be the origin of the noted activity in these compounds compared to the others cycloadducts.

Pseudomonas aureofaciens 499 was found to be remarkably sensitive towards compounds **2a**, **2c**, **2d**, **2f**, **2g**, **2h**, **5a**, **5c**, **5d**, **5f**, **5g** and **5h** with inhibition zones ranging between 12 and 17.25 mm at the concentration of 1000 µg/mL. Compounds **2h** (IZ = 17.25 mm) and **5h** (IZ = 17.25

mm) both bearing a thiophene ring at C-3 of the dihydroisoxazole moiety, showed higher antibacterial activity against *Pseudomonas aureofaciens* 499 (IZ = 15.25 mm) compared to the other cycloadducts and to (*R*)-limonene **1**. The presence of the thiophenyl ring in both **2h** and **5h** could be the origin of this activity.

Our results were in good concordance with that cited in the literature showing the important antibacterial activity of some dihydroisoxazoles [21,22]. Table 3 also revealed that compounds **2d**, **5d**, **2g** and **5g** exhibited antibacterial effect towards all the used bacterial agents.

3.2.2. Antifungal activity

Data shown in Table 4 revealed that, (*R*)-limonene **1** and all synthesized compounds were tested against five fungal: *Aspergillus niger*, *Aspergillus flavus*, *Penicillium digitatum*, *Trichoderma harzianum* and *Fusarium solani*. As above mentioned for the antibacterial activity, the inhibitory effect of the tested compounds became visible since the concentration of 250 µg/mL and the highest inhibition of the tested fungi was recorded at 1000 µg/mL.

Table 3. Antibacterial activity of compounds **1**, **3**, **4**, **2a-h** and **5a-h** depending on the concentrations used^a.

Compounds	Concentration (µg/mL)	<i>Burkholderia glathei</i>	<i>Bacillus pumilus</i>	<i>Pseudomonas aureofaciens</i>
1	250	27.75	24.25	10.25
	500	29.75	25.75	11.75
	1000	33.75	32.5	15.25
3	250	7	-	7.5
	500	9.75	6.5	9.75
	1000	10.75	7.75	15.75
4	250	-	-	9.5
	500	7.75	6.5	9.75
	1000	13	10.25	16.25
2a	250	7.5	6.25	7.25
	500	10.5	7	8.5
	1000	12.5	9	13.5
2b	250	-	8	-
	500	-	10	-
	1000	7	12.5	8.25
2c	250	6.75	6.75	10
	500	8	6.75	11.5
	1000	10	8.75	14
2d	250	7.75	7.25	6.5
	500	10	10	9.75
	1000	12.5	15	12.5
2e	250	8.5	6.75	7.25
	500	10.75	7.5	9
	1000	13.25	10.75	10.5
2f	250	-	6.5	6.25
	500	7.75	9.25	8.5
	1000	8.75	11.75	13.5
2g	250	9	9.25	9.75
	500	12.75	12.75	10.25
	1000	14	14.25	12
2h	250	-	6.5	8.75
	500	-	7.5	10.5
	1000	8	9	17.25
5a	250	7.5	6.25	7.25
	500	10.5	7	8.5
	1000	12.5	9	13.5
5b	250	-	8	-
	500	-	10	-
	1000	7	-	8.25
5c	250	6.75	6.75	10
	500	8	6.75	11.5
	1000	10	8.75	14
5d	250	7.75	7.25	6.5
	500	10	10	9.75
	1000	12.5	15	12.5
5e	250	8.5	6.75	7.25
	500	10.75	7.5	9
	1000	13.25	10.75	10.5
5f	250	-	6.5	6.25
	500	7.75	9.25	8.5
	1000	8.75	11.75	13.5
5g	250	9	9.25	9.75
	500	12.75	12.75	10.25
	1000	14	14.25	12
5h	250	-	6.5	8.75
	500	-	7.5	10.5
	1000	8	9	17.25
Ampicillin ^b	5000	32.5	30.5	31.5
DMSO		-	-	-

^a Diameter of inhibition zone (IZ) expressed in mm, "-": Not active.^b Positive control.

Limonene **1** was unable to reduce the growth of *Aspergillus flavus* and *Trichoderma harzianum* but it exhibited a significant effect towards *Aspergillus niger* (IZ = 24.25 mm) and *Fusarium solani* (IZ = 25 mm). All synthesized compounds demonstrated variable inhibition zones, which dominated by the activity **2a** and **2b** towards *Trichoderma harzianum* (IZ = 13.25 and 12.75 mm, respectively), **2b**, **2d** and **2g** against *Penicillium digitatum* (IZ = 13, 12.25 and 13 mm, respectively) and **5b** towards *Aspergillus flavus* (IZ = 13.75 mm). The recorded results did not show any clear structure-antifungal activity relationship and could be explained by the relative susceptibility of the microorganism to each compound tested.

3.2.3. Anti-acetylcholinesterase activity

Inhibition of acetylcholinesterase (AChE), the key enzyme in the breakdown of acetylcholine, is considered one of the treatment strategies against several neurological disorders such as Alzheimer's disease, senile dementia, ataxia and myasthenia gravis [42,43]. The acetylcholinesterase (AChE) inhibition was determined using an adaptation of the method described in the literature [41]. Only dihydroisoxazoles **2a-h** and **5a-h** were assayed for inhibition of acetylcholinesterase and the obtained results are shown in Table 5. The IC₅₀ of compounds **2a-h** ranged between 82 and 280 µg/mL, and those of compounds **5a-h** varied between 99 and 500 µg/mL.

Table 4. Antifungal activity of compounds **1**, **3**, **4**, **2a-h** and **5a-h** depending on the concentrations^a.

Compounds	Concentration (µg/mL)	<i>Aspergillus niger</i>	<i>Penicillium digitatum</i>	<i>Trichoderma harzianum</i>	<i>Fusarium solani</i>	<i>Aspergillus flavus</i>
1	250	15	7.5	-	13.75	-
	500	21	9	-	23.25	-
	1000	24.25	10.25	-	25	-
3	250	8.5	-	6.5	6	7.5
	500	9.5	-	7.75	8.25	8.75
	1000	11.5	-	8.5	9	11.25
4	250	8.25	-	6	-	6.5
	500	8.75	-	7	-	7.75
	1000	10.25	8.25	8.75	7.75	8.75
2a	250	8.5	-	9.5	-	7
	500	11	6.25	10.5	9	8.25
	1000	12	8	13.25	11.5	10.25
2b	250	8.5	-	8.75	-	7.5
	500	9.5	7.75	11.25	-	8.25
	1000	11.75	13	12.75	8.75	9.5
2c	250	7	-	-	7.75	-
	500	9	-	7.5	9.5	-
	1000	10.25	-	8.5	10.5	8.5
2d	250	9.5	-	7	7.75	8.75
	500	11	9	9.5	10.25	9.5
	1000	12.25	12.25	11.5	11.75	11
2e	250	8	-	-	-	-
	500	9.25	7	-	-	7
	1000	10.25	8	8	8.5	9
2f	250	8	-	-	-	-
	500	9.25	-	-	8.25	-
	1000	11	8.75	-	10.75	8.25
2g	250	8.75	7.25	8.25	7.25	7.5
	500	11.5	10	11	9.5	10
	1000	11.5	13	13	11	10
2h	250	8.75	-	7.25	7.75	-
	500	10.5	-	8.5	8.25	8
	1000	10.25	8	12	10.75	10.5
5a	250	6.5	-	-	8.25	6.5
	500	7.25	-	-	9.75	7.5
	1000	8.25	-	-	12.5	9.5
5b	250	7	-	-	-	8
	500	7.75	7.5	7	-	10
	1000	9.25	10	8.75	8.75	13.75
5c	250	8.5	-	-	-	-
	500	9	6.25	9	-	7.25
	1000	10.5	7.75	11.5	8	9
5d	250	7.5	-	-	-	6.5
	500	8.25	-	-	-	7
	1000	10	7.5	-	9.25	9.25
5e	250	7.25	-	-	-	-
	500	9.5	-	-	-	8.25
	1000	10.5	7.5	-	9.25	9.25
5f	250	7.25	6	7.25	-	-
	500	8	6	9.25	7.75	-
	1000	9.5	7.75	11.5	10.25	8
5g	250	7.5	6	-	-	-
	500	8.5	6	9	8.25	7
	1000	9.75	7.75	10.25	10.75	8.75
5h	250	7.75	6	-	-	6.75
	500	9.5	6	7.25	7.5	8.75
	1000	11.5	6	9.25	9.25	10.25
Carbendazim ^b	500	35	-	34	-	35 ^a
DMSO ^c		-	-	-	-	-

^a Diameter of inhibition zone (IZ) expressed in mm, “-”: Not active.^b Positive control.^c Solvent (negative control).**Table 5.** Acetylcholinesterase inhibition capacity, represented by IC₅₀ (mg/mL) of **2a-h** and **5a-h**^a.

Compounds	IC ₅₀ (mg/mL)	Compounds	IC ₅₀ (mg/mL)
2a	175±2	5a	99±1
2b	172±3	5b	190±1
2c	170±1	5c	110±2
2d	230±2	5d	175±4
2e	173±1	5e	140±1
2f	82±3	5f	450±1
2g	250±2	5g	500±3
2h	280±1	5h	310±1
Eserine ^b	0.018±0.002		

^a Average±SD were obtained from three different experiments.^b Positive control.

The most significant inhibition of acetylcholinesterase was induced by compounds **2f** (IC₅₀ = 82±3 µg/mL) in which the aryl group fixed at C-3 of the dihydroisoxazole is the 4-

chlorophenyl, and **5a** (IC₅₀ = 99±1 µg/mL) with a *p*-tolyl group at the same position. The compounds **2g**, **2h**, **5d**, **5e** and **5g** have a close inhibition.

IC₅₀ values represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurements, each performed in triplicate.

4. Conclusion

In this paper, we described the synthesis of a new series of dihydroisoxazoles from the (*R*)-limonene via the 1,3-dipolar cycloaddition reactions exploring its disubstituted terminal double bond. On the other hand, the limonene-lactam, previously prepared in this work, was *N*-allylated to be used in a 1,3-dipolar cycloaddition reaction affording an original new series of regiospecific dihydroisoxazoles. All synthesized compounds were evaluated for their antimicrobial and anti-acetylcholinesterase activities. The fusion of two biologically relevant systems as they are the limonene skeleton along with the dihydroisoxazole ring may explain some significant activities noted.

Acknowledgement

The authors acknowledge the Ministry of Higher Education, Scientific Research and Technology in Tunisia for the financial support.

References

- Guenther, A.; Geron, C.; Pierce, T.; Brian, L.; Harley, P.; Fall, R. *Atmos. Environ.* **2000**, *234*, 2205-2230.
- Crowell, P. L. *J. Nutr.* **1999**, *129*, 775S-778S.
- Singh, P.; Shukla, R.; Prakash, B.; Kumar, A.; Singh, S.; Mishra, P. K.; Dubey, N. K. *Food Chem. Toxicol.* **2010**, *48*, 1734-1740.
- Ferrarini, S. R.; Duarte, M. O.; DaRosa, R. G.; Rolim, V.; EiflerLima, V. L.; VonPose, G.; Ribeiro, V. L. S. *Vet. Parasitol.* **2008**, *157*, 149-153.
- Elson, C. E.; Maltzman, T. H.; Boston, J. L.; Tanner, M. A.; Gould, M. N. *Carcinogenesis* **1988**, *9*, 331-332.
- Elegbede, J. A.; Maltzman, T. H.; Verma, A. K.; Tanner, M. A.; Elson, C. E.; Gould, M. N. *Carcinogenesis* **1999**, *7*, 2047-2049.
- Dietrich, D. R.; Swenberg, J. A. *Cancer. Res.* **1991**, *51*, 3512-3521.
- Wattenberg, L. W.; Coccia, J. B. *Carcinogenesis* **1991**, *12*, 115-117.
- Nakaizumi, A.; Baba, M.; Uehara, H.; Iishi, H.; Tatsuta, M. *Cancer Lett.* **1997**, *117*, 99-103.
- Chen, J.; Lu, M.; Jing, Y.; Dong, J. *Bioorg. Med. Chem.* **2006**, *14*, 6539-6547.
- Maltzman, T. H.; Hurt, L. M.; Elson, C. E.; Tanner, M. A.; Gould, M. N. *Carcinogenesis* **1989**, *10*, 781-783.
- Yang, Y. Q.; Zhao, G. *Chem. Eur. J.* **2008**, *14*, 10888-10891.
- Cimarelli, C.; Fratoni, D.; Palmieri, G. *Tetrahedron-Asymmetr.* **2009**, *20*, 2234-2239.
- Hensen, K.; Mahaim, C.; Holderich, W. F. *Appl. Catal. A-Gen.* **1997**, *149*, 311-329.
- Chang, S. S.; Peterson, R. J. *J. Food Sci.* **1977**, *42*, 298-305.
- Midori, A. A.; Toshio, S.; Takayoshi, A.; Hiroaki, S. *Yuki Gosei Kagaku Kyokaiishi* **2004**, *62*, 59-69.
- Quadrelli, P.; Scrocchi, R.; Caramella, P.; Rescifina, A.; Piperno, P. *Tetrahedron* **2004**, *60*, 3643-3651.
- Lam, P. Y. S.; Adams, J. J.; Clark, C. G.; Calhoun, W. J.; Luetttgen, J. M.; Knabb, R. M.; Wexler, R. R. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1795-99.
- Kai, H.; Mastsumoto, H.; Hattori, N.; Takase, A.; Fujiwara, T.; Sugimoto, H. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1997-2000.
- Tangallapally, R. P.; Sun, D.; Rakesh; Budha, N.; Lee, R. E.; Lenaerts, A. J.; Meibohm, B.; Lee, R. E. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 6638-6642.
- Jadhav, S. B.; Shastri, R. A.; Gaikwad, K. V.; Gaikwad, S. V. *E-J. Chem.* **2009**, *6(S1)*, S183-S188.
- Jayashankara, B.; LokanathaRai, K. M. *Arkivoc* **2008**, *11*, 75-85.
- Prasad, Y. R.; Ravikumar, P.; Ramesh, B. *Int. J. Chem. Sci.* **2007**, *5*, 542-548.
- Amici, M. D.; Conti, P.; Vistoli, G.; Carrea, G.; Ottolina, G.; DeMicheli, C. *Med. Chem. Res.* **2001**, *10*, 615-633.
- Park, K. K.; Ko, D. H.; You, Z.; Khan, M. O.; Lee, H. J. *Steroids* **2006**, *71*, 183-188.
- Hwang, I. T.; Kim, H. R.; Jeon, J. D.; Hong, K. S.; Song, J. H.; Cho, K. Y. *J. Agric. Food Chem.* **2005**, *53*, 8639-8643.
- Li, X. F.; Feng, Y. Q.; Xu, M. *Acta. Crystallogr. E* **2003**, *59*, O675-O676.
- Pedro, A. C.; Volker, J.; Albrecht, L.; Rodolfo, D. B. *Tetrahedron* **2003**, *44*, 1071-1074.
- Nair, V.; Suja, T. D. *Tetrahedron* **2007**, *63*, 12247-12275.
- Pellissier, H. *Tetrahedron* **2007**, *63*, 3235-3285.
- Feddaoui, A.; Aitlto, M. Y.; AitAli, M.; Hasnoui, A.; Riahi, A. *Synth. Commun.* **2006**, *36*, 3617-3624.
- Clemett, D.; Markham, A. *Drugs* **2000**, *59*, 815-827.
- GarciaMoreno, M. I.; DiazPérez, P.; Ortiz, M. C.; Garcia, F. J. M. *J. Org. Chem.* **2003**, *68*, 8890-8901.
- Dighade, S. R.; Patil, S. D.; Chincholkar, M. M.; Dighade, N. R. *Asian J. Chem.* **2003**, *15*, 450-454.
- Barbachyn, M. R.; Cleek, G. J.; Dolak, L. A.; Garmon, S. A.; Morris, J.; Seest, E. P.; Thomas, R. C.; Toops, D. S.; Watt, W.; Wishka, D. G.; Ford, C. W.; Zurenko, G. E.; Hamel, J. C.; Schaadt, R. D.; Stapert, D.; Yagi, B. H.; Adams, W. J.; Friis, J. M.; Slatter, J. P.; Oien, N. L.; Zaya, M. J.; Wienkers, L. C.; Wynalda, M. A. *J. Med. Chem.* **2003**, *46*, 284-302.
- Basappa, Sadashiva, M. P.; Mantelingu, K.; Nanjunda, S. S.; Rangappa, K. S. *Bioorg. Med. Chem.* **2003**, *11*, 4539-4544.
- Gyonfalvi, S.; Szakonyi, Z.; Fulop, F. *Tetrahedron-Asymmetr.* **2003**, *14*, 3965-3972.
- Woznica, M.; Butkiewicz, A.; Grzywacz, A.; Kowalska, P.; Masnyk, M.; Michalak, K.; Luboradzki, R.; Furche, F.; Kruse, H.; Grimme, S.; frelek, J. *J. Org. Chem.* **2011**, *76*, 3306-3319.
- Alcontres, G. S.; Caristi, C.; Ferlazzo, A.; Gattuso, M. *J. Chem. Soc. Perkin Trans.* **1976**, *1*, 1694-1696.
- Harhash, A. H.; Elnagdi, M. H.; Kassab, N. A. L.; Negm, A. M. *J. Chem. Eng. Data* **1975**, *20*, 120-122.
- Fale, P. L.; Borges, C.; AmorimMadeira, P. J.; Ascensao, L.; Araujo, M. E. M.; Florencio, M. H.; Serralheiro, M. L. M. *Food Chem.* **2009**, *114*, 798-805.
- Orhan, G.; Orhan, I.; Sener, B. *Lett. Drug. Des. Discov.* **2003**, *3*, 268-274.
- Hoves, M. J.; Houghton, P. J. *Pharmacol. Biochem. Behav.* **2003**, *75*, 513-527.