



Facile synthesis of oxo-/thioxopyrimidines and tetrazoles C–C linked to sugars as novel non-toxic antioxidant acetylcholinesterase inhibitors

J.A. Figueiredo^a, M.I. Ismael^{a,*}, J.M. Pinheiro^a, A.M.S. Silva^b, J. Justino^c, F.V.M. Silva^c, M. Goulart^c, D. Mira^c, M.E.M. Araújo^d, R. Campoy^d, A.P. Rauter^d

^a Departamento de Química, Unidade I&D Materiais Têxteis e Papeiros da Universidade da Beira Interior, Av. Marquês d'Ávila e Bolama, 6201-001 Covilhã, Portugal

^b Departamento de Química & QOPNA, Universidade de Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal

^c Escola Superior Agrária, Instituto Politécnico de Santarém, Complexo Andaluz, 2001-904 Santarém, Portugal

^d Universidade de Lisboa, Faculdade de Ciências, Centro de Química e Bioquímica/Departamento de Química e Bioquímica, Carbohydrate Chemistry Group, Ed. C8, 5º Piso, Campo Grande, 1749-016 Lisboa, Portugal

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ABSTRACT

Microwave-assisted synthesis of oxo-/thioxopyrimidines and tetrazoles linked to furanoses with D-xyllo and D-ribo configuration, and to a D-galacto pyranose is reported and compared to conventional methods. Reaction of dialdofuranoses and dialdopyranoses with a β -keto ester and urea or thiourea under microwave irradiation at 300 W gave in 10 min the target molecules containing the 2-oxo- or 2-thioxo-pyrimidine ring in high yield. The tetrazole-derived compounds were obtained in two steps by reaction of the formyl group with hydroxylamine hydrochloride, copper sulfate, triethylamine and dicyclohexylcarbodiimide to give an intermediate nitrile, which was then treated with sodium azide. The use of microwave irradiation in the latter step also resulted in a considerably shorter reaction time (10 min) compared to hours under conventional heating to obtain a complete starting materials conversion. Acetylcholinesterase inhibition ranged from 20% to 80% for compounds concentration of 100 μ g/mL, demonstrating the potential of this family of compounds for the control of Alzheimer's disease symptoms. Most of the compounds showed antioxidant activity in the β -carotene/linoleic acid assay, some of them exhibiting IC₅₀ values in the same order of magnitude as those of gallic acid. The bioactive compounds did not show cytotoxic effects to human lymphocytes using the MTT method adapted for non-adherent cells, nor genotoxicity determined by the short-term in vitro chromosomal aberration assay.

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1. Introduction

Alzheimer's disease (AD) is a progressive dementia in which memory and other cognitive functions, such as language and general intellectual performance, also become impaired. It is one of the most common age-related neurodegenerative diseases, considered an urgent public health problem. Although the primary cause of AD is still debated,¹ research on its molecular pathogenesis has provided a robust framework to guide the search for effective new pharmacological treatments, which is one of the major challenges facing modern medicine. Acetylcholinesterase (AChE) inhibitors were the first group of compounds that showed some promise in the treatment of AD and have been effective for the control of mild to moderate AD.^{2,3}

Preliminary reports on compounds with a thiazolidinone ring C–C linked to the 4' position of a sugar residue showed that they also act as cholinesterase inhibitors.⁴ Compounds possessing

heterocycles C–C linked to a non-anomeric position are known for a variety of biological activities as pharmaceutical and agriculture applied products.^{5–8} As part of our ongoing search for bioactive compounds derived from sugars, we have reported the synthesis and bioactivity of pseudo-C-nucleosides with the heterocycles pyrazole,⁹ triazole⁹ and thiazole⁴ C–C linked to furanoses.^{4,9} We now report on the synthesis of related compounds-type with the oxo- and thioxopyrimidine rings synthesized by Biginelli reaction,¹⁰ which has been extended for the preparation of a large number of oxopyrimidines¹¹ starting from β -ketoesters or β -diketones, arylaldehyde and (thio)urea under photochemical¹² or microwave irradiation.^{13,14} These compounds have been investigated by a large number of research groups due to their biological properties, namely as antibacterial or antifungal agents,¹⁵ and as HIV inhibitors^{16,17} of gp-120-CD4.¹⁸ They are active against cancer,¹⁹ and present antihypertensive and anti-inflammatory activities.¹⁹ Hence, an easy access to this new class of compounds embodying these heterocycles C–C linked to sugar residues is of high importance.

The medicinal, biochemical and pharmacological properties of tetrazole compounds support a vast progress in their chemistry

* Corresponding author.

E-mail address: iismael@ubi.pt (M.I. Ismael).

over the last years.^{20–22} Tetrazoles have a wide variety of applications as pharmaceutical products and explosives, in photography, in information recording systems and as precursors of a variety of nitrogen heterocycles.^{23–25} 5-Substituted 1,2,3,4-tetrazoles are described to possess antibacterial,²⁶ antifungal,²⁷ antiviral,²⁸ analgesic,²⁹ anti-inflammatory,³⁰ antiulcer^{31,32} and antihypertensive³³ properties. It is known that the tetrazole ring can serve as an isosteric substitute for the carboxylic group in biologically active molecules, since both groups possess comparable acidity and size. The tetrazole unit, however, has proven to be superior in resisting metabolic degradation.^{34–37} Synthesis of tetrazoles C–C linked to sugars is also accomplished in this work and the target compounds are evaluated regarding their ability to inhibit acetylcholinesterase. Oxidative stress is associated with Alzheimer's and vascular dementias, as well as with diabetes type II, among other pathologies.³⁸ Hence, evaluation of the antioxidant activity³⁹ of the new compounds by the β -carotene/linoleic acid assay is also reported.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of 4-glycosyl-2-oxo-/2-thioxopyrimidines

Reaction of dialdofuranoses **1–3** with ethyl acetoacetate and urea/thiourea was accomplished under conventional heating or microwave irradiation giving the 2-oxopyrimidines **5–7** and the 2-thioxopyrimidines **8–10** (Scheme 1, Table 1), in high yield. Conventional heating required 5 h for reaction completion with xylofuranosyl-heterocycles, while the ribofuranosyl and galactosyl analogues required 5 h at 75 °C and additional stirring for 72 h at room temperature. However the microwave-assisted reactions were completed within 10 min in the presence of 10 times smaller volume of solvent than that used in the method described in the literature,⁴⁰ giving slightly higher yields.

The first reaction step consists of condensation of the aldehyde with urea to generate an iminium intermediate which acts as an electrophile for the nucleophilic addition of the keto enol ester, and the ketone carbonyl of the resulting adduct undergoes condensation with NH_2 to give the cyclized product. The formation of a

Table 1

Yields obtained by methods A, B and C^a (for compounds **5–10**) and D, E and F^b (for compounds **15–18**)

Compound number	Yield (%)		
	Conventional heating		Microwave irradiation Method C
	Method A	Method B	
5	77	—	80
6	79	—	80
7	—	82	84
8	—	78	80
9	—	80	81
10	—	79	81
	Method D	Method E	Microwave irradiation Method F
	15	96	98
	16	99	98
	17	97	99
	18	98	99

^a Method A: 75 °C, 5 h; Method B: 75 °C, 5 h and 72 h, room temp; Method C: MW (300 W), 10 min.

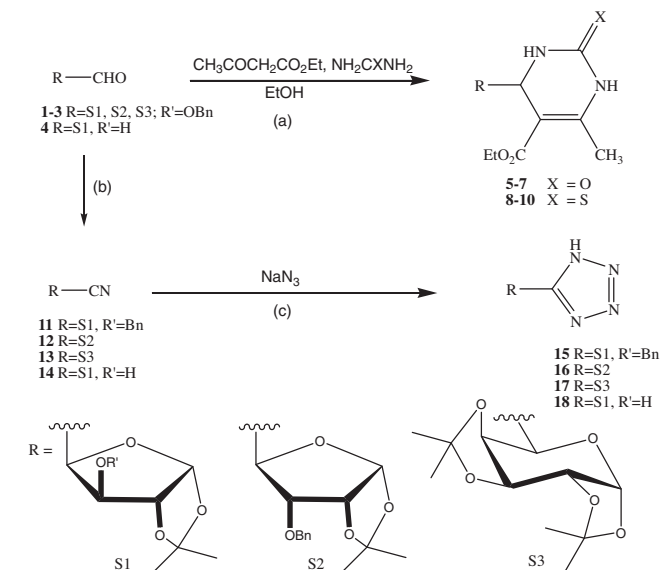
^b Method D: NaN_3 , NH_4Cl , DMF, 120 °C, 3 h; Method E: NaN_3 , ZnCl_2 , H_2O , 100 °C, 2 h 30 min; Method F: NaN_3 , ZnCl_2 , H_2O , MW (300 W), 10 min.

single reaction product may result from the exocyclic attack of the keto enol ester to the iminium species which seems to be stabilized by a hydrogen bond between the free amino group and the endocyclic ring oxygen. The formation of one single diastereoisomer with assigned (*R*)-configuration for the new stereogenic center, also confirms the proposed mechanism, in alternative to the described acid-promoted enolization and aldol addition, followed by addition of urea to the formed conjugated system, as described by Li.⁴¹ These results are in full agreement with data reported in the literature for similar reactions.⁴⁰

The oxypyrimidine ring in compound **5** was confirmed by the singlet at δ 8.92 (NH-1), the doublet at δ 5.66 (NH-3), the doublet at δ 4.57 (H-4), the signals of the ethyl carboxylate group at δ 4.11 (quartet, CH_2) and 1.18 (triplet, CH_3), in addition to the singlet of the methyl group CH_3 -6 at δ 2.26. In the ^{13}C NMR spectrum of **5** the ester carbonyl group appeared at δ 165.8, while the quaternary carbons C-2, C-6 and C-5 appeared at δ 155.0, 149.3, and 97.0, respectively. The chemical shift of C-4 is assigned at δ 51.42 and the signal of the methyl group linked to C-6 emerged at δ 18.41. NMR spectra of compounds **6** and **7** exhibited a similar pattern related to the presence of this heterocycle, while compounds **8–10** showed the signal of C-2, which belongs to a thiocarbonyl group, at higher field (δ 176.6–174.6).

2.1.2. Synthesis of 4-glycosyltetrazoles

The tetrazole ring was formed by the pathway presented in Scheme 1. Sugar nitriles **11–14** were obtained by the reaction of appropriate aldehydes with hydroxylamine hydrochloride, triethylamine, copper(II) sulfate pentahydrate, and the dehydration agent DCC,⁹ using dichloromethane as solvent, at room temperature for 2 h. After workup and purification by flash chromatography, compounds **11–14** were obtained in 95–97% yield. The IR band at 2257 – 2259 cm^{-1} , and the ^{13}C NMR signals at δ 114–115 confirmed the formation of compounds **11–14**. Reaction of the nitriles **11–14** with sodium azide, ammonium chloride in DMF at 120 °C for 3 h (method D) led to the formation of tetrazoles **15–18** in 96–99% yield. The reaction conditions using sodium azide, ZnCl_2 , water as solvent, at 100 °C for 2.5 h (method E) gave compounds **15–18** in similar yields (97–98%). However, when these reagents were submitted to microwave irradiation (method F), the tetrazoles were formed in 10 min and isolated in 98–99% yield. H-4' resonated at δ 5.25–5.74, shifted downfield to the corresponding proton of the nitrile precursor, and the presence of the C=N



Scheme 1. (a) Method A: 75 °C, 5 h; Method B: 75 °C, 5 h and 72 h, rt; Method C: MW (300 W), 10 min; (b) $\text{NH}_2\text{OH}\cdot\text{HCl}$, Pyr, H_2O ; $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, Et_3N , DCC, CH_2Cl_2 , 2 h, rt; (c) Method D: NaN_3 , NH_4Cl , DMF, 120 °C, 3 h; Method E: NaN_3 , ZnCl_2 , H_2O , 100 °C, 2 h 30 min; Method F: NaN_3 , ZnCl_2 , H_2O , MW (100 W), 10 min.

signal at δ 152–156 confirmed the expected structure for compounds **15**–**18**.

Table 1 summarizes compounds' yields synthesized either by conventional heating or by microwave irradiation and illustrates the advantages of the latter for the synthesis of this family of compounds.

2.2. Biological activity

2.2.1. Inhibition of acetylcholinesterase activity

The inhibitory activity of the new synthetic compounds against AChE (acetylcholinesterase), from bovine erythrocytes, was evaluated using the Ellman's spectrophotometric method⁴² to determine the rate of hydrolysis of acetylthiocholine in the presence of the inhibitor. The generated thiocholine reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to produce a yellow compound that is detected spectrophotometrically ($\lambda = 410$ nm). The enzyme activity (%) and the enzyme inhibition (%) were calculated from the change of absorbance with time ($V = \Delta\text{Abs}/\Delta t$) as follows:

$$\text{Enzyme activity(\%)} = 100 \times V/V_{\max}$$

$$\text{Enzyme inhibition(\%)} = 100 - \text{Enzyme activity(\%)}$$

Maximum rate ($V_{\max}$) is obtained when no inhibitor is used while V is the rate obtained in the presence of the inhibitor.

The most promising compounds **8** and **9** have the thioxopyrimidine ring in their structure linked to the furanose moiety and promoted 69% and 83% enzyme inhibition (Fig. 1). All other compounds gave inhibition lower than 54% with the exception of the tetrazole **18**, which also led to a considerable enzyme inhibition.

2.2.2. β -Carotene/linoleic acid bleaching test

The antioxidant activity of compounds **5**–**10** and **15**–**18** was evaluated by the β -carotene bleaching assay and the results obtained are presented in Figure 2. This test is appropriate for lipophilic compounds and measures their ability to inhibit lipid peroxidation. For comparative purposes the antioxidant activity of two standards, gallic acid ($\text{IC}_{50} = 0.833 \pm 0.044$ mg/mL) of natural origin and the synthetic antioxidant BHT (butylated hydroxytoluene) ($\text{IC}_{50} = 0.0045 \pm 0.0008$ mg/mL), was also evaluated. The latter has been used in the food industry but doubts about its safety for human health led to the investigation of new antioxidants. Compounds **9**, **16** and **17** presented $\text{IC}_{50} \geq 1.5$ mg/mL and were considered not active. However compounds **8** ($\text{IC}_{50} = 0.103 \pm 0.002$ mg/mL), **10** ($\text{IC}_{50} = 0.130 \pm 0.037$ mg/mL) and **15** ($\text{IC}_{50} = 0.144 \pm 0.0002$ mg/mL) show a considerable antiox-

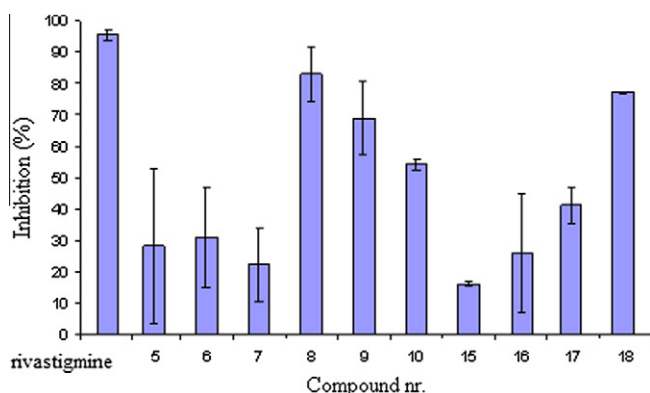


Figure 1. Inhibition (%) of acetylcholinesterase activity by compounds **5**–**10** and **15**–**18**.

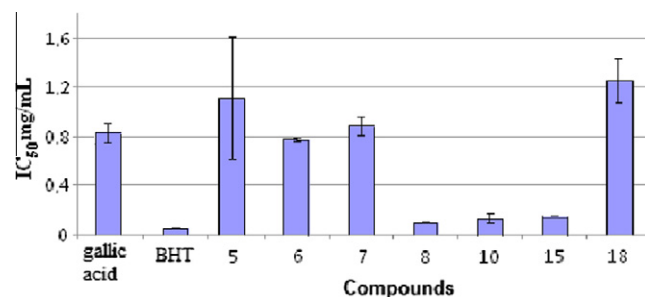


Figure 2. Antioxidant activity of compounds **5**–**8**, **10**, **15** and **18**, evaluated by the β -carotene/linoleic acid bleaching test.

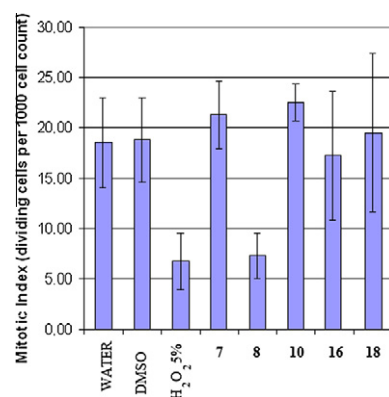


Figure 3. Cell proliferation after exposure to compounds **7**, **8**, **10**, **16**, and **18**.

idant activity, when compared to BHT and also to gallic acid, which is a polar antioxidant.

2.2.3. Toxicity studies

Acute toxicity assessment was performed by the MTT method⁴⁵ adapted for non-adherent cells.⁴⁶

As shown in Figure 3, only compound **8** has a mitotic index indicative of toxicity. All other compounds, including the most active ones **9** and **18**, do not present any toxicity. Viability of the cells was not affected (Fig. 4) and no evidence of genotoxic risk was observed for all the bioactive compounds.

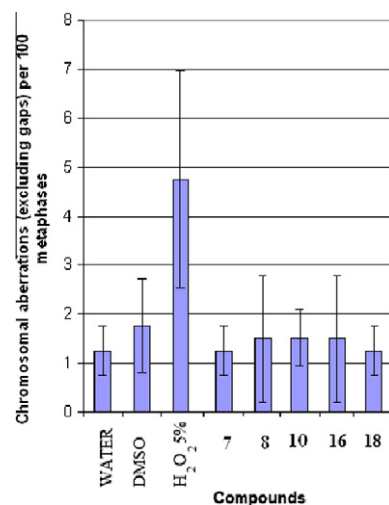


Figure 4. Genotoxicity of compounds **7**, **8**, **10**, **16**, and **18**.

3. Conclusions

Synthetic approaches for new 4-glycosyl(oxo- and thio-oxo)pyrimidines and 4-glycosyl-1H-tetrazoles starting from dialdo sugars were exploited and standard thermal heating/flash heating with microwave irradiation were used for the construction of the heterocycle. The latter heating source proved to be the most appropriate leading to the target molecules in very high yield and in a short time (10 min).

Most of the studied compounds inhibited acetylcholinesterase to some extent (20–83% inhibition at 100 µg/mL compound concentration). The thioxopyrimidine derivatives seem to be more effective than the oxopyrimidine analogues with the same glycosyl moiety, and the most active ones have this heterocycle linked to furanose moieties. They also showed antioxidant activity higher than that of gallic acid for the β -carotene/linoleic acid assay. The tetrazole linked to a 1,2-O-isopropylidene-xylosyl moiety unprotected at position 3 also showed considerable inhibition but low antioxidant activity. With the exception of the cytotoxic 2-thioxopyrimidine derivative **8**, the bioactive compounds do not present genotoxicity nor cytotoxicity. Hence, this family of compounds shows some promise regarding their application as acetylcholinesterase inhibitors for the control of neurodegenerative disease symptoms.

4. Experimental

4.1. Chemistry

Melting points were determined with a melting point apparatus (Leitz-Biomed) with platinum plate and are uncorrected. Optical rotations were measured with an Atago Polax-D polarimeter and IR spectra were recorded with a FT-IR Mattson Genesis II spectrophotometer. ^1H NMR spectra were run with a Bruker AC-250 P spectrometer (250 MHz). Chemical shifts are expressed in parts per million downfield from TMS. The ^{13}C NMR spectra were run at 62.90 MHz in the same spectrometer. The experiments were performed in chloroform-*d*. Elemental analyses were obtained with a Vario EL CHN analyser and were in good agreement ($\pm 0.4\%$) with the calculated values. Domestic microwave oven (Electrolux, 850 W, 20 L capacity) was used in the reactions carried out under microwave irradiation. The progress of all reactions was monitored by thin layer chromatography (TLC) using aluminum sheets precoated with silica gel 60F₂₅₄ to a thickness of 0.2 mm (Merck). Preparative TLC was performed with aluminum plates coated with silica gel 60F₂₅₄ to a thickness of 0.5 mm (Merck). Compounds were detected with UV light (254 nm) and/or by spraying the sheets with a 3% vanillin in ethanol/sulfuric acid (100 mL/1.5 mL) followed by heating. Column chromatography (CC) was conducted under low pressure by elution of the columns filled with silica gel (0.040–0.063 mm, Merck). All other chemicals and solvents used were obtained from commercial sources and used as received or dried using standard procedures.

4.2. General procedure to obtain oxo-/thioxopyrimidines

Method A: A solution of urea/thiourea (1.43 mmol) in ethyl acetoacetate (2.14 mmol, 0.279 g, 0.3 mL) was added at rt to a solution of the respective aldehyde (1 mmol) in anhydrous ethanol (3 mL). The reaction mixture was heated at 75 °C for 5 h. Cooling to rt and solvent evaporation afforded a residue that was washed with ethyl acetate and filtered off. Evaporation of the solvent afforded a residue, which was purified by CC to give the corresponding 2-oxo-/2-thioxopyrimidines.

Method B: A solution of urea/thiourea (1.43 mmol) in ethyl acetoacetate (2.14 mmol, 0.279 g, 0.3 mL) was added at rt, to a

solution of the respective aldehyde (1 mmol) in anhydrous ethanol (3 mL). The reaction mixture was heated at 75 °C for 5 h and then 72 h at rt. After solvent evaporation the residue was washed with ethyl acetate and filtered off. Evaporation of the solvent afforded a residue, which was purified by CC to give the corresponding 2-oxo-/2-thioxopyrimidines.

Method C: A solution of urea/thiourea (1.43 mmol) in ethyl acetoacetate (2.14 mmol, 0.279 g, 0.3 mL) was added at rt, to a solution of the corresponding aldehyde (1 mmol) in anhydrous ethanol (0.3 mL). The reaction mixture was submitted to microwave, at 300 W for 10 min. Evaporation of the solvent afforded a residue that was washed with ethyl acetate and filtered off. Evaporation of the solvent gave a residue, which was purified by CC to give the corresponding 2-oxo-/2-thioxopyrimidines.

4.2.1. Ethyl (4R)-[(1R,2R,3S,4S)-3-O-benzyl-1,2-O-isopropylidene-tetrafuranos-4-yl]-1,2,3,4-tetrahydro-6-methyl-2-oxopyrimidine-5-carboxylate (5)

CC eluted with ethyl acetate/toluene 1/6 (v/v). Yield: (Method A = 77%, 2.27 g; Method B = 80%, 2.35 g) R_f = 0.38 (ethyl acetate/toluene 5/1); syrup; IR (neat) cm^{-1} : 3400; 3240 (N–H), 1700; 1680 (C=O), 1640 (C=C); $[\alpha]_D^{25}$ = +190° (c 0.5, CHCl_3) U.V. (λ_{max} , nm) ethanol: 284 (ϵ = 8935), 208 (ϵ = 14413). Anal. Calcd for ($\text{C}_{21}\text{H}_{28}\text{O}_7\text{N}_2$): C, 59.99; H, 6.71; N, 6.66. Found: C, 59.60; H, 6.84; N, 6.31. ^1H NMR (δ ppm, J Hz, CDCl_3): 8.92 (s, 1H, NH-1), 7.33–7.30 (m, 5H, Ph of Benzyl), 6.01 (d, 1H, H-1', $J_{1',2'} = 2.9$), 5.66 (d, 1H, NH-3, $J_{3,4} = 1.3$), 4.76 (AB, 1H, CH_2 of Benzyl, $J = 12$), 4.62 (d, 1H, H-2', $J_{2',1'} = 2.9$), 4.57 (d, 1H, H-4, $J_{4,3} = 1.3$), 4.43 (AB, 1H, CH_2 of Benzyl, $J = 12$), 4.28 (d, 1H, H-4', $J_{4',3'} = 4.4$), 4.11 (q, 2H, CH_2CH_3 , $J = 7$), 4.03 (d, 1H, H-3', $J_{3',4'} = 4.4$), 2.26 (s, 3H, 6- CH_3), 1.43 and 1.30 (2s, $2 \times 3\text{H}$, $2 \times \text{CH}_3$ -isop), 1.18 (t, 3H, CH_2CH_3 , $J = 7$). ^{13}C NMR (δ ppm, CDCl_3): 165.8 (Cq, C=O of CO_2Et), 155.0 (Cq, C-2), 149.3 (Cq, C-6), 137.1 (Cq-Ph of Benzyl), 128.6, 128.1, 127.7 (CH-Ph of Benzyl), 111.6 (Cq, Cisop), 105.2 (CH, C-1'), 97.0 (Cq, C-5), 84.3 (CH, C-3'), 83.4 (2 CH, C-2', C-4'), 71.9 (CH_2 of Benzyl), 59.6 (CH_2CH_3), 51.4 (CH, C-4), 26.8 and 26.2 ($2 \times \text{CH}_3$ -Cisop), 18.4 (6- CH_3), 14.2 (CH_2CH_3).

4.2.2. Ethyl (4R)-[(1R,2R,3R,4S)-3-O-benzyl-1,2-O-isopropylidene-tetrafuranos-4-yl]-1,2,3,4-tetrahydro-6-methyl-2-oxopyrimidine-5-carboxylate (6)

CC eluted with ethyl acetate/toluene 1/5 (v/v). Yield: (Method A = 79%, 2.32 g; Method B = 80%, 2.35 g); Melting point: 155.5–157 °C; R_f = 0.64 (ethyl acetate/toluene: 8/1); $[\alpha]_D^{25}$ = +29° (c 3.25, CHCl_3); IR (KBr) cm^{-1} : 3320, 3220 (N–H), 1710; 1670 (C=O), 1630 (C=C); U.V. (λ_{max} , nm) ethanol: 282 (ϵ = 10304), 209 (ϵ = 13365). Anal. Calcd for ($\text{C}_{21}\text{H}_{28}\text{O}_7\text{N}_2$): C, 59.99; H, 6.71; N, 6.66. Found: C, 59.89; H, 6.70; N, 6.68. ^1H NMR (δ ppm, J Hz, CDCl_3): 8.85 (s, 1H, NH-1), 7.36–7.23 (m, 5H, Ph Benzyl), 6.61 (s, 1H, NH-3), 5.70 (d, 1H, H-1, $J_{1,2} = 3.2$), 4.70 (AB, 1H, CH_2 Benzyl, $J = 11.7$), 4.56–4.46 (m, 3H, H-2', H-4, 1H- CH_2 of Benzyl), 4.19–4.06 (m, 3H, H-4' and CH_2CH_3), 3.83 (dd, 1H, H-3, $J_{2,3} = 4.1$ and $J_{3,4} = 8.6$), 2.22 (s, 3H, 6- CH_3), 1.53 and 1.29 (2s, $2 \times 3\text{H}$, $2 \times \text{CH}_3$ -isop), 1.17 (t, 3H, CH_2CH_3 , $J = 7$). ^{13}C NMR (δ ppm, CDCl_3): 165.2 (Cq, C=O of CO_2Et), 154.4 (Cq, C-2), 148.4 (Cq, C-6), 137.1 (Cq of Benzyl), 127.4, 126.8, 126.6 (CH-Ph of Benzyl), 112.0 (Cq, Cisop), 103.2 (CH, C-1'), 96.2 (Cq, C-5), 79.5 (CH, C-4'), 77.4 (CH, C-3'), 76.6 (CH, C-2'), 70.9 (CH_2 of Benzyl), 59.1 (CH_2CH_3), 51.2 (CH, C-4), 25.8 and 25.7 ($2 \times \text{CH}_3$, Cisop), 18.0 (6- CH_3), 13.4 (CH_2CH_3).

4.2.3. Ethyl (4R)-[(1R,2R,3S,4S,5S)-1,2:3,4-Di-O-isopropylidene-pentopyranos-5-yl]-1,2,3,4-tetrahydro-6-methyl-2-oxopyrimidine-5-carboxylate (7)

CC eluted with ethyl acetate/toluene 1/5 (v/v). Yield: (Method B) = 82% (0.338 g); (Method C) = 84% (0.346 g); mp: 231–232° C; R_f = 0.34 (ethyl acetate/toluene: 5/1); IR (net) (cm^{-1}): 3420

(N–H), 3254 (N–H), 1720 (C=O), 1712 (C=O ester), 1645 (C=C). Anal. Calcd for (C₁₉H₂₃O₈N₂): C, 55.33; H, 6.84; N, 6.79; O, 31.03. Found: C, 55.34; H, 6.85; N, 6.78; O, 31.02. ¹H NMR (δ ppm, J Hz, CDCl₃): 7.47 (s, 1H, NH-1), 5.82 (s, 1H, NH-3), 5.55 (d, 1H, H-1', J_{1,2} = 5), 4.60 (d, 1H, H-4, J_{4,5'} = 5.2 Hz), 4.57 (dd, 1H, H-5', J_{5',4'} = 2.5 Hz), 4.30–4.27 (m, 2H, H-4' and H-2'), 4.20–4.09 (m, 2H, CH₂CH₃), 3.73 (s, 1H, H-3'), 2.34 (s, 3H, 6-CH₃), 1.54, 1.48, 1.37, 1.33 (4 s, 4 × 3H, CH₃ isop); 1.26 (t, 3H, CH₂CH₃, J = 7.0 Hz). ¹³C NMR (δ ppm, CDCl₃): 174.9 (Cq, C=O of CO₂Et), 165.2 (Cq, C-2), 149.4 (Cq, C-6), 110.1, 108.6 (Cq, 2 isop), 98.4 (Cq, C-5), 96.5 (CH, C-1'), 74.1 (CH, C-4'), 72.3 (CH, C-2'), 70.5 (CH, C-3'), 68.8 (CH, C-5'), 60.2 (CH₂CH₃), 54.0 (CH, C-4), 26.3, 25.3, 25.0, 24.2 (4 CH₃, 2 isop), 18.4 (6-CH₃), 14.3 (CH₂CH₃).

4.2.4. Ethyl (4R)-[(1R,2R,3S,4S)-3-O-benzyl-1,2-O-isopropylidene-tetrafuranos-4-yl]-1,2,3,4-tetrahydro-6-methyl-2-thioxo pyrimidine-5-carboxylate (8)

CC eluted with ethyl acetate/toluene 1/6 (v/v). Yield (Method B) = 78% (0.349 g); (Method C) = 80% (0.358 g) mp: 135–136 °C; R_f = 0.54 (ethyl acetate/toluene: 5/1); IR (net) (cm⁻¹): 3422, 3255 (N–H), 1721 (C=S), 1716 (C=O ester), 1642 (C=C). Anal. Calcd for (C₂₂H₂₈O₆N₂S): C, 58.91; H, 6.29; N, 6.25; S, 7.15; O, 21.40. Found: C, 58.92; H, 6.28; N, 6.24; S, 7.16; O, 21.41. ¹H NMR (δ ppm, J Hz, CDCl₃): 7.68 (s, 1H, NH-1); 7.42–7.22 (m, 5H, Ph of Benzyl), 7.18 (s, 1H, NH-3), 6.04 (d, 1H, H-1', J_{1,2} = 3.9 Hz), 4.84 (AB, 1H, CH₂ of Benzyl, J = 12.1), 4.66 (d, 1H, H-2', J = 3.9), 4.55–4.48 (m, 2H, H-4, 1H of CH₂ of Benzyl), 4.29 (d, 1H, H-4', J_{3',4'} = 5.1), 4.21–4.05 (m, 3H, CH₂CH₃ and H-3'), 2.27 (s, 3H, 6-CH₃), 1.46, 1.32, (2 s, 2 × 3H, CH₃ isop); 1.21 (t, 3H, CH₂CH₃, J = 7.6). ¹³C NMR (δ ppm, CDCl₃): 176.6 (Cq, C-2), 165.4 (Cq, C=O of CO₂Et), 145.7 (Cq, C-6), 136.4 (Cq, Ph of Bn), 128.5, 128.2, 127.5 (3 × CH, Ph of Bn), 111.9 (Cq, 2 isop), 105.1 (CH, C-1'), 98.5 (Cq, C-5), 83.8 (CH, C-3'), 82.6 (CH, C-2'), 82.3 (CH, C-4'), 71.8 (CH₂, Benzyl), 60.0 (CH₂CH₃), 52.1 (CH, C-4); 26.8; 26.2 (2 CH₃, isop); 18.2 (6-CH₃), 13.8 (CH₂CH₃).

4.2.5. Ethyl (4R)-[(1R,2R,3R,4S)-3-O-benzyl-1,2-O-isopropylidene-tetrafuranos-4-yl]-1,2,3,4-tetrahydro-6-methyl-2-thioxo pyrimidine-5-carboxylate (9)

CC eluted with ethyl acetate/toluene 1/5 (v/v). Yield (Method B) = 80% (0.358 g); (Method C) = 81% (0.363 g) m p: 109–110 °C; R_f = 0.49 (ethyl acetate/toluene: 5/1); IR (net) (cm⁻¹): 3419, 3251 (N–H), 1723 (C=S), 1714 (C=O of ester), 1639 (C=C). Anal. Calcd for (C₂₂H₂₈O₆N₂S): C, 58.91; H, 6.29; N, 6.25; S, 7.15; O, 21.40. Found: C, 58.90; H, 6.30; N, 6.26; S, 7.14; O, 21.41. ¹H NMR (δ ppm, J Hz, CDCl₃): 8.44 (s, 1H, NH-1), 7.46 (s, 1H, NH-3), 7.36–7.14 (m, 5H, Ph of Benzyl), 5.68 (d, 1H, H-1', J_{1,2} = 3.2), 4.73 (AB, 1H, CH₂ of Benzyl, J = 11.5), 4.57 (d, 1H, H-2'), 4.55 (d, 1H, H-4, J_{4,5'} = 6.0), 4.50 (AB, 1H, CH₂ of Benzyl, J = 11.5), 4.19–4.02 (m, 3H, CH₂CH₃ and H-4'), 3.80 (dd, 1H, H-3', J_{2',3'} = 4 Hz, J_{3',4'} = 8.9), 2.25 (s, 3H, 6-CH₃), 1.51, 1.29, (2 s, 2 × 3H, CH₃ isop), 1.08 (t, 3H, CH₂CH₃, J = 7.6). ¹³C NMR (δ ppm, CDCl₃): 176.3 (Cq, C-2), 165.4 (Cq, C=O of CO₂Et), 145.8 (Cq, C-6), 137.6 (Cq, Ph of Benzyl), 129.0, 128.2, 127.9 (3 × CH, Ph of Benzyl), 113.1 (Cq, 2 isop), 104.1 (CH, C-1'), 99.0 (Cq, C-5), 80.3 (CH, C-4'), 77.3 (CH, C-3'), 77.2 (CH, C-2'), 71.9 (CH₂, of Bn), 60.4 (CH₂CH₃), 52.0 (CH, C-4), 26.8, 26.5 (2 CH₃, isop), 18.5 (6-CH₃), 14.2 (CH₂CH₃).

4.2.6. Ethyl (4R)-[(1R,2R,3S,4S,5S)-1,2:3,4-Di-O-isopropylidene-pentopyranos-5-yl]-1,2,3,4-tetrahydro-6-methyl-2-thioxo pyrimidine-5-carboxylate (10)

CC eluted with ethyl acetate/toluene 1/4 (v/v). Yield (Method B) = 79% (0.338 g); yield (Method C) = 81% (0.347 g); mp: 107–108 °C; R_f = 0.31 (ethyl acetate/toluene: 5/1); IR (net) (cm⁻¹): 3420, 3254 (N–H), 1718 (C=S), 1714 (C=O ester), 1640 (C=C). Anal. Calcd for (C₁₉H₂₈O₇N₂S): C, 55.26; H, 6.59; N, 6.54; S, 7.48; O, 26.15. Found: C, 55.25; H, 6.58; N, 6.53; S, 7.49; O, 26.15; ¹H

NMR (δ ppm, J Hz, CDCl₃): 7.80 (s, 1H, NH-1), 7.48 (s, 1H, NH-3), 5.57 (d, 1H, H-1', J_{1,2} = 5); 4.63 (d, 1H, H-4, J_{4,5'} = 6.0), 4.57 (dd, 1H, H-5', J_{5',4'} = 3.0), 4.34–4.28 (m, 2H, H-4' and H-2'), 4.17–4.13 (m, 2H, CH₂CH₃), 3.70 (s, 1H, H-3'), 2.36 (s, 3H, 6-CH₃), 1.61, 1.36, 1.33, 1.30 (4 s, 4 × 3H, CH₃ isop), 1.26 (t, 3H, CH₂CH₃, J = 7.0). ¹³C NMR (δ ppm, CDCl₃): 174.6 (Cq, C-2), 165.7 (Cq, C=O of CO₂Et), 145.8 (Cq, C-6), 110.0 and 108.5 (Cq, 2 isop), 98.3 (Cq, C-5), 96.4 (CH, C-1'), 73.7 (CH, C-4'), 71.6 (CH, C-2'), 70.4 (CH, C-3'), 68.7 (CH, C-5'), 60.2 (CH₂CH₃), 54.0 (CH, C-4), 26.4, 25.8, 25.0, 24.3 (4 CH₃, 2 isop), 18.3 (CH₃), 14.3 (CH₂CH₃).

4.3. General procedure to obtain nitriles

A solution of the appropriate aldehyde (7.25 mmol) in pyridine (4 mL) was slowly added to a solution of hydroxylamine hydrochloride (7.93 mmol, 0.55 g) in water (2 mL). After stirring for 15 min at rt, pyridine (0.4 mL) was added to dissolve the precipitated oxime. The mixture remained under stirring at rt for 1 h. CuSO₄·5H₂O (14.4 mmol, 3.6 g) was added, followed by a solution of triethylamine (14.4 mmol, 2 mL) in dichloromethane (3.6 mL) and DCC (8.72 mmol, 1.8 g) in dichloromethane (15 mL). After 2 h at rt, formic acid (1.3 mL) was added to eliminate DCC. The mixture was filtered, and the filtrate was concentrated under vacuum. The residue was treated with water (50 mL), and then extracted with dichloromethane (3 × 50 mL). The organic phase was washed with a solution of HCl 5% (v/v) (50 mL), dried with sodium sulfate, and concentrated under vacuum to give the nitrile.

4.3.1. 3-O-Benzyl-1,2-O-isopropylidene-α-D-xylofuranosurononitrile (11)

After purification by CC eluted with ethyl acetate/toluene (1/1) the nitrile was obtained in 75% (0.206 g) yield, as syrup, R_f = 0.77 (ethyl acetate/toluene 1/2). IR (net) (cm⁻¹): 2257 (CN) cm⁻¹, [α]_D²⁵ = +13° (c 2.5, CHCl₃). Anal. Calcd for (C₁₅H₁₇O₄N): C, 65.44; H, 6.22; N, 5.09; O, 23.25. Found: C, 65.43; H, 6.23; N, 5.10; O, 23.26. ¹H NMR (δ ppm, J Hz, CDCl₃): 7.39–7.27 (m, 5H, Ph of Benzyl), 5.98 (d, 1H, H-1, J_{1,2} = 3.3), 4.84 (d, 1H, H-4 J_{3,4} = 3.2 Hz), 4.73 (s, 2H, CH₂ of Benzyl), 4.60 (d, 1H, H-3, J_{4,3} = 3.2), 4.16 (d, 1H, H-2, J_{2,1} = 3.3), 1.45, 1.30 (2 s, 2 × 3H, 2 × CH₃ isop). ¹³C NMR (δ ppm, CDCl₃): 136.6 (Cq, Ph of Benzyl), 128.7, 128.3, 128.1 (CH, Ph of Benzyl), 114.1 (Cq, CN), 113.9 (Cq, isop), 105.7 (CH, C-1), 82.0 (CH, C-3), 81.6 (CH, C-2), 72.7 (CH₂ of Benzyl), 69.5 (CH, C-4), 27.0, 26.2 (2 × CH₃ isop).

4.3.2. 3-O-Benzyl-1,2-O-isopropylidene-α-D-ribofuranosurononitrile (12)⁴

Yield = 78% (1.54 g), mp 123–124 °C; R_f = 0.74 (ethylacetate/toluene 1:2); [α]_D²⁵ = +59 (c 1.9, CHCl₃); IR (KBr) (cm⁻¹): 2117 (CN); UV (λ_{max} nm) ethanol: 209 (ε = 6887); Anal. Calcd for C₁₅H₁₇O₄N: C, 65.44; H, 6.22; N, 5.09. Found: C, 64.95; H, 6.38; N, 5.52. ¹H NMR (250 MHz): 7.32 (s, 5H, Ph), 5.74 (d, 1H, H-1, J_{1,2} = 3.4), 4.78, 4.71 (AB system, OCH₂, Bn), 4.62 (d, 1H, H-4, J_{3,4} = 9), 4.53 (t, 1H, H-2, J_{2,3} = 4.1), 4.10 (dd, 1H, H-3), 1.56 (s, 3H, CH₃ isop), 1.33 (s, CH₃ isop); ¹³C NMR: 136.08 (Cq, Ph), 128.45, 128.23, 127.89 (CH, Ph), 116.82 (Cq, C-5), 113.98 (Cq, isop), 104.56 (CH, C-1), 80.03 (CH, C-3), 76.74 (CH, C-2), 72.55 (OCH₂, Bn), 66.07 (CH, C-4), 26.59 (CH₃, isop), 26.05 (CH₃, isop).

4.3.3. 1,2:3,4-Di-O-isopropylidene-α-D-galactopyranosurononitrile (13)

CC eluted with ethyl acetate/toluene 1/4 (v/v). Yield 78% (0.199 g), syrup, R_f = 0.55 (ethyl acetate/toluene 1/2); IR (neat) (cm⁻¹): 2259 (CN). Anal. Calcd for (C₁₂H₁₇O₅N): C, 56.46; H, 6.71; N, 5.49; O, 31.34. Found: C, 56.45; H, 6.70; N, 5.50; O, 31.35. ¹H NMR (δ ppm, J Hz, CDCl₃): 5.53 (d, 1H, H-1, J_{1,2} = 4.9), 4.69–4.65 (m, 2H, H-3 and H-5), 4.39–4.32 (m, 2H, H-2 and H-4), 1.54, 1.53,

1.38, 1.34 (4s, 4 × 3H, 4 × CH₃ isop). ¹³C NMR (δ ppm, CDCl₃): 115.1 (Cq, C-6), 110.8 and 109.2 (2 × Cq, isop), 95.8 (CH, C-1), 70.4 (CH, C-3), 70.0 (CH, C-2), 69.5 (CH, C-4), 59.8 (CH, C-5), 25.6, 25.6, 24.3 and 24.2 (4 × CH₃ isop).

4.3.4. 1,2-O-Isopropylidene-α-D-xylofuranosurionitrile (14)

CC eluted with ethyl acetate/toluene 1/4 (v/v). Yield 75% (0.139 g), syrup, *R_f* = 0.44 (ethyl acetate/toluene 1/2); IR (neat) (cm⁻¹): 2259 (CN). Anal. Calcd for (C₈H₁₁ON): C, 51.89; H, 5.99; N, 7.56; O, 34.56. Found: C, 51.88; H, 5.98; N, 7.57; O, 34.57. ¹H NMR (δ ppm, J Hz, CDCl₃): 6.01 (d, 1H, H-1, *J*_{1,2} = 3.4), 4.99 (d, 1H, H-4, *J*_{4,3} = 2.7), 4.59 (d, 1H, H-2, *J*_{2,1} = 3.4), 4.47 (d, 1H, H-3, *J*_{3,4} = 2.7), 3.76 (br s, 1H, OH-3), 1.47, 1.31 (2s, 2 × 3H, 2 × CH₃ isop). ¹³C NMR (δ ppm, CDCl₃): 115.3 (Cq, CN), 112.8 (Cq, isop), 105.2 (CH, C-1), 83.7 (CH, C-3), 74.9 (CH, C-2), 70.7 (CH, C-4), 26.5 and 25.7 (2 × CH₃ isop).

4.4. General procedure for preparation of tetrazoles

Method 1: A mixture of the nitrile (1.0 mmol) in anhydrous DMF (2 mL), sodium azide (1.1 mmol, 72 mg) and ammonium chloride (1.3 mmol, 70 mg) was refluxed (120 °C) under stirring for 3 h (until TLC showed complete conversion). After solvent evaporation, the residue was submitted to flash chromatography eluted with ethyl acetate/toluene 1/2 (v/v).

Method 2: A mixture of the nitrile (1 mmol) in water (3.0 mL), sodium azide (1.05 mmol, 68 mg) and zinc chloride (1.1 mmol, 15 mg) was refluxed (100 °C), under stirring for 2.5 h (until TLC showed complete conversion). After solvent evaporation, the residue was submitted to flash chromatography eluted with ethyl acetate/toluene 1/2 (v/v).

Method 3: A mixture of the nitrile (1 mmol) in water (3 mL), sodium azide (1.05 mmol, 68 mg), and zinc chloride (1.1 mmol, 15 mg) was heated under microwave irradiation (100 W) for 10 min (until TLC showed complete conversion). After solvent evaporation, the residue was submitted to flash chromatography eluted with ethyl acetate/toluene 1/2 (v/v).

4.4.1. 5-[(1R,2R,3S,4S)-3-O-benzyl-1,2-O-isopropylidene-tetrafurans-4-yl]tetrazole (15)

CC eluted with ethyl acetate/toluene 1/4 (v/v). Yield (Method 1) = 96% (305 mg), (Method 2) = 98% (312 mg), (Method 3) = 98% (312 mg), solid, mp 139–140 °C; *R_f* = 0.81 (ethyl acetate/methanol 1/1), IR (KBr) cm⁻¹ 3482 (NH), [*α*]_D¹⁷ = +1.58° (c 1.8, CH₃COCH₃). ESI(+)-HRMS: *m/z* 341.1228 (M+Na)⁺ (63), (341.1226 calcd for C₁₅H₁₈N₄O₄Na) 319.1406 (M+H)⁺ (100) (319.1407 calcd for C₁₅H₁₉N₄O₄). ¹H NMR (δ ppm, J Hz, CDCl₃): 7.29–7.26 (m, 3H, Ph of Benzyl), 6.99–6.96 (m, 2H, Ph of Benzyl), 6.08 (d, 1H, H-1', *J*_{1',2'} = 3.6), 5.74 (d, 1H, H-4', *J*_{3',4'} = 3.16), 4.74 (d, 1H, H-2', *J*_{2',1'} = 3.6), 4.47 (AB, CH₂ of Benzyl, *J* = 11.3 Hz), 4.30 (d, 1H, H-3' *J*_{2',3'} = 3.07 Hz), 4.26 (AB, CH₂ of Benzyl, *J* = 11.3 Hz), 1.55 and 1.37 (2s, 2 × 3H, 2 × CH₃ isop). ¹³C NMR (δ ppm, CDCl₃): 152.6 (Cq, C-5), 136.1 (Cq, Ph of Benzyl), 128.8, 128.5 and 127.9 (CH, Ph of Benzyl), 113.2 (Cq, isop), 105.5 (CH, C-1'), 82.6 (CH, C-3' and C-2'), 74.6 (CH, C-4'), 72.9 (CH₂ of Benzyl), 27.0, 26.3 (2 × CH₃ isop).

4.4.2. 5-[(1R,2R,3R,4S)-3-O-Benzyl-1,2-O-isopropylidenetetrafurans-4-yl]tetrazole (16)⁴

CC eluted with ethyl acetate/toluene 1/4 (v/v). Yield (Method 1) = 97% (308 mg), (Method 2) = 99% (315 mg), (Method 3) = 98% (312 mg).

4.4.3. 5[(1R,2R,3S,4S,5S)-1,2:3,4-Di-O-isopropylidene-pentopyranos-5-yl]tetrazole (17)

CC eluted with ethyl acetate/toluene 1/4 (v/v). Yield (Method 1) = 98% (294 mg), (Method 2) = 99% (297 mg), (Method 3) = 99%

(297 mg), solid; mp 235–236 °C *R_f* = 0.80 (ethyl acetate/methanol 1/2), IR (KBr) 3479 (C=N) cm⁻¹, [*α*]_D¹⁷ = -5.92 (c 19.5, CH₃COCH₃). FAB(+)-MS *m/z*: 299 (M+1)⁺ (12), 283 (M-CH₃)⁺ (100). ¹H NMR [(δ ppm, J Hz, (CD₃)₂CO): 5.54 (d, 1H, H-1', *J*_{1,2} = 4.9), 5.25 (d, 1H, H-4', *J*_{3',4'} = 1.96), 4.70 (dd, 1H, H-5', *J* = 5.2 and 7.7), 4.49 (dd, 1H, H-3', *J* = 2.4 and 7.8), 4.40 (dd, 1H, H-2', *J* = 2.4 and 4.9), 1.54, 1.53 1.38 and 1.34 (4s, 4 × 3H, 4 × CH₃ isop). ¹³C NMR [(δ ppm, (CD₃)₂CO): 154.5 (Cq, C-5), 110.3, 109.8 (2 × Cq, isop), 97.2 (CH, C-1'), 72.9 (CH, C-3'), 71.3 (CH, C-2'), 71.2 (CH, C-4'), 64.6 (CH, C-5'), 26.3, 26.0, 24.9 and 24.2 (4 × CH₃ isop).

4.4.4. 5-[(1R,2R,3S,4S)-1,2-O-isopropylidenetetrafurans-4-yl]tetrazole (18)

CC eluted with ethyl acetate/toluene 1/4 (v/v). Yield (Method 1) = 98% (225 mg), (Method 2) = 99% (228 mg), (Method 3) = 99% (228 mg); solid, mp 154–155 °C *R_f* = 0.60 (ethyl acetate/methanol 1/2), IR (KBr) cm⁻¹ 3583 (OH), 3477 (NH), [*α*]_D¹⁷ = -3.15° (c 10, CH₃COCH₃). ESI(+)-HRMS *m/z*: 251.0755 (M+Na)⁺ (61) (251.0757 calcd for C₁₅H₁₈N₄O₄Na); 229.0936 (M+H)⁺ (100) (229.0938 calcd for C₁₅H₁₉N₄O₄). ¹H NMR [(δ ppm, J Hz, (CD₃)₂CO): 6.08 (d, 1H, H-1', *J*_{1,2} = 3.2 Hz), 5.62 (d, 1H, H-4', *J*_{3,4} = 2.6), 4.72 (d, 1H, H-3', *J*_{4,3} = 3.3), 4.49 (d, 1H, H-2', *J*_{2,1} = 3.2), 1.50 and 1.32 (2s, 2 × 3H, 2 × CH₃ isop). ¹³C NMR [(δ ppm, (CD₃)₂CO): 153.6 (Cq, C-5), 112.8 (Cq, isop), 106.0 (CH, C-1'), 85.9 (CH, C-3'), 76.2 (CH, C-2'), 75.8 (CH, C-4'), 27.0 and 26.3 (2 × CH₃ isop).

4.5. Biological activity

4.5.1. Enzymatic studies

A double beam spectrophotometer Shimadzu® equipped with thermostatic cell holders was used on visible range and operated on the kinetic mode for the enzymatic studies. The absorbance data were acquired in a computer by means of UV Probe software. Appropriate disposable plastic cuvettes Plastibrand® were used in the kinetic experiments. The following materials were purchased from Aldrich: enzyme acetylcholinesterase (AChE) from bovine erythrocytes, substrate acetylthiocholine iodide (ATChI), and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). Other Aldrich reagents used for the preparation of buffers and solutions were KH₂PO₄, KOH and NaHCO₃. Deionized water was used to prepare the buffer pH 8.0, the substrate and DTNB solutions.

4.5.1.1. Solutions preparation.

Preparation of 0.1 M phosphate buffer pH 8.0: KH₂PO₄ (136.1 mg) was dissolved in water (10 mL) and adjusted with KOH to a pH of 8.0 ± 0.1. Buffer was freshly prepared and stored below 5 °C.

AChE solution 1.32 U/mL: the enzyme (1.02041 U, 4.4 mg) was dissolved in freshly prepared buffer pH 8.0 (1.0 mL).

DTNB solution 0.01 M: DTNB (3.96 mg) was dissolved in water (1 mL) containing sodium hydrogen carbonate (1.5 mg).

ATChI solution 0.022 M: ATChI (6.4 mg) was dissolved in water (1 mL).

All solutions were stored in Eppendorf caps (100 μL aliquots) in the refrigerator at below 5 °C. The pure compounds were dissolved in DMSO and diluted with distilled water until 4.4 mg/mL concentration to obtain the final compound concentration of 100 μg/mL in the enzymatic test. No inhibition was detected by residual DMSO (<0.5%) at the reaction cuvette.

4.5.1.2. AChE inhibitory activity assay.

A mixture of the buffer (200 μL), enzyme (5 μL), DTNB (5 μL) and new synthetic compound (5 μL) solutions was prepared and kept for 15 min at 30 °C in a heated water bath, and then the substrate ATChI solution (5 μL) was added to start the enzymatic reaction. The absorbance was read along reaction time for 4 min under a controlled temperature of 30 °C. At least four replicates were made. Several blank

assays without the new synthetic compounds were carried out in order to determine the average $V_{\max}$. Also assays without the enzyme and the inhibitor compound were carried out to check for any non-enzymatic hydrolysis of the substrate. The final concentrations in the test were as follows: [AChE] = 0.03 U/mL, [compound] = 100 µg/mL, [DTNB] = 0.0002273 M, [ATCh] = 0.0005 M.

4.5.2. β -Carotene/linoleic acid bleaching test

The antioxidant activity of pure compounds was evaluated using a β -carotene-linoleic acid model system⁴³ with a slight modification of the described procedure.⁴⁴ Briefly, a solution of β -carotene dissolved in chloroform (0.5 mL, 2 mg/mL) was pipetted into a round-bottomed flask (50 mL) and Tween 40 (200 mg) was added. After removing the chloroform with a rotary evaporator, aerated distilled water (50 mL) and linoleic acid (25 µL) were added to the flask under vigorous stirring and the mixture sonicated for 30 min for homogenization. Aliquots (2.5 mL) of the prepared emulsion were transferred to a test tube containing a solution of the compound in ethanol (300 µL) and were further shaken. Each type of sample was prepared in duplicate. The test systems were placed in the cell compartment of a Shimadzu 1603 UV–Vis spectrophotometer thermostated to 50 ± 1 °C and absorbance of each sample was recorded at 470 nm immediately after sample preparation ($t = 0$ min) and at 30 s intervals until the end ($t = 120$ min). A blank containing aerated water (300 µL) instead of sample was prepared.

The antioxidant activity (AA) of the compounds under investigation was expressed as:

$$AA\% = 1 - \frac{A(t=0) - A(t=120)}{A_0(t=0) - A_0(t=120)} \times 100$$

where AA is the antioxidant activity, $A(t=0)$ is the absorbance (470 nm) of the solution in investigation at 0 min, $A(t=120)$ is the absorbance (470 nm) of the same solution at $t = 120$ min, $A_0(t=0)$ is the absorbance (470 nm) of the aqueous control sample at 0 min and $A_0(t=120)$ is the absorbance (470 nm) of the aqueous control sample at $t = 120$ min. Solution concentration providing 50% inhibition (IC_{50}) for each compound was obtained plotting inhibition percentage versus extract solution concentration.

4.5.3. Toxicity studies

Acute toxicity assessment was performed by the MTT method⁴⁵ adapted for non-adherent cells.⁴⁶ The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to quantify metabolically viable cells in all samples. Suspension cells (K562 human erythroid cell line) were seeded onto 96-well plates, allowed to divide for 24 h and exposed to the test compound for the following 24 h. Positive controls (hydrogen peroxide) and negative controls (pure solvent) were also included. At 48 h of culture, MTT was added to the cells at a final concentration of 0.5 mg/mL, followed by an incubation period of 3 h to allow the formazan crystals to form. After the incubation time, DMSO (100 µL) was added to each well. Solubilization of formazan crystals was performed by agitation in a 96-well plate shaker for 20 min at room temperature. Absorbance of each well was quantified at 550 nm using 620 nm as reference wavelength on a scanning multiwell spectrophotometer (automated plate reader).

Genotoxicity was assessed by the short-term in vitro chromosomal aberration assay.⁴⁷ Briefly, cell cultures of peripheral blood lymphocytes from healthy donors were set up, and lympho-proliferation was induced with phytohemagglutinin (2% v/v, 24 h incubation). Cells were exposed to the test compounds for 24 h, and then colcemid, at final concentration of 0.5 µg/mL, was added during the final 3 h of culture, to stop the dividing cells in the metaphase stage of mitosis. Metaphase spreads were obtained in standard glass microscopy slides, pre-washed and covered with a

thin water film. Scoring was performed in a Zeiss optical microscope at 1,250 $\times$ magnification, by observing 100 complete metaphases (presenting 46 centromeres) per case. Classification of chromosomal aberrations was done according to criteria described in Rueff et al.⁴⁷ The mitotic index was also quantified by counting the number of metaphases per 1000 nuclei.

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