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Original article

Microwave-assisted synthesis of 3,5-disubstituted isoxazoles and evaluation of their anti-ageing activity

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ABSTRACT

One-pot uncatalysed microwave-assisted 1,3-dipolar cycloaddition reactions between *in situ* generated nitrile oxides and alkynes bearing protected antioxidant substituents, were regioselectively afforded 3,5-disubstituted isoxazoles. The yields were moderate, based on the starting aldehydes, while the reaction times were in general shorter than those reported in the literature.

The cytoprotective and anti-ageing effect of the final deprotected compounds was evaluated *in vitro*, on cellular survival following oxidative challenge and *in vivo*, on organismal longevity using the nematode *Caenorhabditis elegans*. The activity of the isoxazole analogues depends on the nature and the number of the antioxidant substituents. Analogue **17** bearing a phenolic group and a 6-OH-chroman group is a promising anti-ageing agent, since it increased survival of human primary fibroblasts following treatment with H_2O_2 and extended *C. elegans* lifespan.

1. Introduction

Isoxazoles, have attracted an increasing research interest, as non classical amide or ester bioisosteres and potential pharmacophores endowed with anticancer [1], neuroprotective [2], anti-obesity [3], antidepressant [4], insecticidal [5], antidiabetic [6] and anti-inflammatory [7] activities. The major synthetic strategies to construct this heterocycle are: i) condensation of a 1,3-dicarbonyl compound with hydroxylamine and ii) 1,3-dipolar cycloaddition of an alkyne with a nitrile oxide, which is regioselective in the presence of copper(I), giving 3,5-disubstituted isoxazoles [8].

Nitrile oxides can react with simple terminal alkynes without the need of a catalyst, because of their increased reactivity, compared to azides. Very recently, several groups [1a,c,d,3,6,9–12] accessed 3,5-disubstituted isoxazoles through a metal-free cycloaddition of alkynes with nitrile oxides, usually in modest yields or long reaction times.

In general the 3,5-regioisomer was favoured under uncatalyzed conditions. Use of organocatalysts [7] or hypervalent iodine [13] reagents improved the yield/regioselectivity of the reaction. Moreover, the regiospecific synthesis of novel isoxazolines and isoxazoles of N-substituted saccharin derivatives, using a microwave oven, was also described [14].

Our group has been involved in the synthesis of neuroprotective antioxidants and we reported that the presence of isoxazole scaffold results in higher *in vitro* neuroprotective activity, compared to other nitrogen heteroaromatics. Isoxazole analogues were prepared by conventional Cu catalyzed cycloadditions [2] or by using dual-frequency ultrasound irradiation [15].

Although the isoxazole pharmacophore has been incorporated into a wide range of bioactive agents, the effect of isoxazole analogues on the cellular or organismal lifespan has not yet been reported.

Ageing is an inevitable natural biological process that is linked to the gradual deterioration of organismal homeostasis and the increasing accumulation of damaged macromolecules [16]. The progression of ageing has been highly correlated with increased levels of reactive oxygen species (ROS) and the extent of ROS formation and oxidative damage has been inversely correlated with longevity in different species [17]. Increased oxidative stress promotes the deterioration of all biomolecules including DNA, lipids and proteins thus leading to a global failure of cellular and organismal homeostasis [18]. There are various models used to study ageing *in vitro* and *in vivo*. Human primary fibroblasts that age *in vitro*, the so called replicative senescence model constitute the best accepted model to study human ageing *in vitro* [19]. Moreover, human primary fibroblasts can easily be used in different assays to

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reveal antioxidant properties on top of anti-ageing properties of different compounds. The nematode *Caenorhabditis elegans* is also a prominent model to study organismal ageing due to its short lifespan, the fast generation time and the multiple experimental applications [20,21].

The discovery of agents which could slow down the deleterious effects of ageing *in vitro* and/or *in vivo* has attracted an increasing research interest. Since ageing is associated with increased incidence of diseases related to elevated levels of reactive oxygen species (ROS), dietary phenolic antioxidants have emerged as promising candidates [22–27] while there is one recent report on the anti-ageing properties of synthetic compounds applying the aforementioned *in vivo* model [28]. Therefore bioactive isoxazoles bearing antioxidant groups would represent an interesting approach towards the development of anti-ageing compounds.

Collectively, the aim of the present study was i) the green regiospecific microwave-assisted one-pot synthesis of isoxazoles from *in situ* generated nitrile oxides and alkynes, in the presence or absence of Cu(I) as catalyst and ii) the investigation of the effects of the derived compounds on the cellular survival following oxidative challenge and on organismal longevity.

2. Results

We first investigated the cycloaddition reaction between the *in situ* generated 4-methoxy-phenyl nitrile oxide and phenyl acetylene under conventional heating and microwave irradiation in the presence or absence of Cu catalyst. The reaction was performed in a mixture of *tert*-butanol/water (Scheme 1). Specifically, 4-methoxybenzaldehyde was first converted to the corresponding aldoxime via reaction with hydroxylamine. Without isolation, the aldoxime was converted to the corresponding nitrile oxide using chloramine-T trihydrate which acts as both a halogenating agent and a base. The results are shown in Table 1.

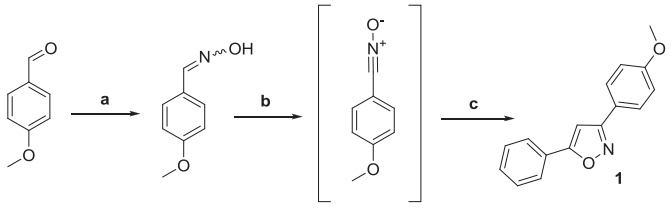
The Cu catalyzed reaction at ambient temperature gave 45% of the desired isoxazole after 24 h (entry 1). The yield was slightly improved when the temperature was increased to 90 °C (entry 2) whereas the use of microwave irradiation significantly improved the yield and shortened the reaction time (entry 7).

Concerning the amount of the catalyst, the use of 0.3 equivalents of $CuSO_4$ and 0.6 equivalents of sodium ascorbate (entry 6) gave higher yields than lower catalyst loading (entry 5).

On the contrary, the use of larger excess of chloramine-T (1.5 equivalents, entry 8) did not affect the yield of the reaction.

The best results of the Cu catalyzed reaction were obtained at 90 °C and 80 W. Lower (60 °C, entry 6) or higher (100 and 110 °C) temperatures (entries 13 and 14) gave lower yields. The yield was further decreased when 120 °C and 100 W were applied (entry 15). Solid additives [29] such as silica gel, Al_2O_3 or NaCl had a detrimental effect on the reaction yield.

Although washing with NH_4OH, ensures quantitative removal of the copper salt during the reaction work-up, Cu-free cycloaddition strategy, not requiring metals and additives, is a promising approach. Thus, we set out to examine the feasibility and the regioselectivity of the microwave-assisted 1,3-dipolar cycloaddition reactions, *tert*-butanol/water, under metal free conditions.



Scheme 1. One-pot isoxazole synthesis. Reagents and conditions: **a**: $NH_2OH{\cdot}HCl$, *t*-BuOH:H_2O (1:1), NaOH 1 N, rt, **b**: $TsN(Cl)Na{\cdot}3H_2O$, *t*-BuOH:H_2O (1:1), rt, **c**: $CuSO_4{\cdot}5H_2O$/Sodium ascorbate, phenyl acetylene, MW irradiation.

Table 1
Formation of isoxazole **1** under conventional heating or microwave irradiation in the presence or absence of Cu catalyst.

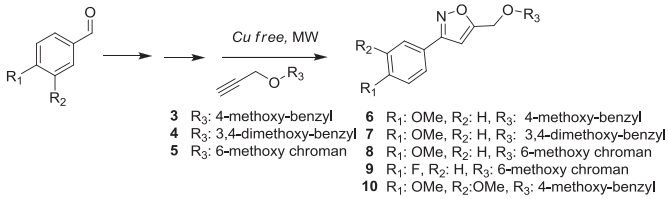
Entry	Chloramine-T	Catalyst	Method	Time	Yield %[b]
1	1.05 eq	*a*[#]	Room temperature	24 h	45
2	1.05 eq	*a*[#]	90 °C	24 h	55
3	1.05 eq	*a*[#]	90 °C	30 min	35
4	1.05 eq	*Cu free*	90 °C	30 min	47
5	1.05 eq	*a*	MW (60 °C, 80 W)	30 min	30
6	1.05 eq	*a*[#]	MW (60 °C, 80 W)	30 min	68
7	**1.05 eq**	***a*[#]**	**MW (90 °C, 80 W)**	**30 min**	**72**
8	1.5 eq	*a*[#]	MW (90 °C, 80 W)	30 min	72
9	1.05 eq	*a*[#]	+Silica gel MW (90 °C, 80 W)	30 min	Traces
10	1.05 eq	*a*[#]	+Al_2O_3 MW (90 °C, 80 W)	30 min	45
11	1.05 eq + Al_2O_3	*a*[#]	MW (90 °C, 80 W)	30 min	45
12	1.05 eq	*a*[#] + NaCl	MW (90 °C, 80 W)	30 min	50
13	1.05 eq	*a*[#]	MW (100 °C, 80 W)	30 min	58
14	1.05 eq	*a*[#]	MW (110 °C, 80 W)	30 min	62
15	1.05 eq	*a*[#]	MW (120 °C, 100 W)	30 min	53
16	1.05 eq	*Cu free*	MW (90 °C, 80 W)	30 min	57
17	**1.05 eq**	***Cu free***	**MW (90 °C, 80 W)**	**45 min**	**68**
18	1.05 eq	*Cu free*	MW (90 °C, 80 W)	60 min	62
19	1.05 eq	*Cu free* + NaCl	MW (90 °C, 80 W)	30 min	26
20	**1.05 eq**	***Cu free***	**MW (90 °C, 100 W)**	**22 min**	**65**
21	1.05 eq	*Cu free*	MW (90 °C, 100 W)	30 min	45

Solvent: *t*-BuOH:H_2O (1:1), (*a*) $CuSO_4{\cdot}5H_2O$/Sodium ascorbate (0.05 eq/0.1 eq), [#](0.3 eq/0.6 eq). [b]isolated yields based on 4-methoxy-benzaldehyde, after column chromatography. Optimal conditions for catalyzed and Cu free reaction are shown in bold.

Microwave irradiation increased the yield of the uncatalysed reaction (entries 4 and 16). The optimum reaction time at 90 °C and 80 W was 45 min (entry 17) giving 68% of isoxazole. Similar yields were obtained using 90 °C, 100 W for 22 min (entry 20).

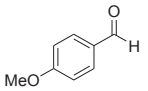
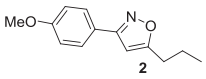
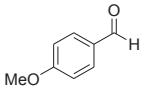
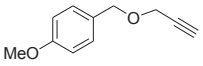
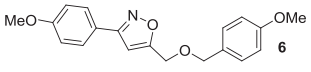
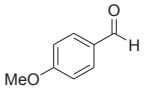
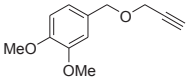
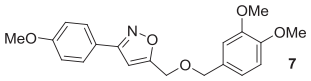
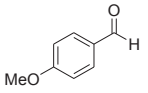
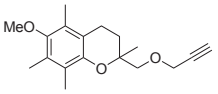
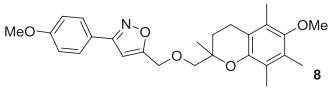
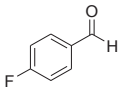
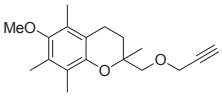
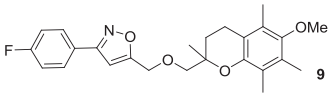
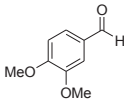
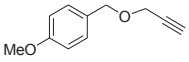
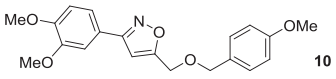
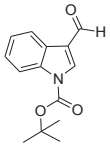
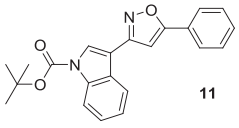
Using the optimal conditions for the metal free reaction, we synthesized the compounds depicted in Scheme 2 and Table 2. Although reaction time of 22 min and 100 W, did not significantly affect the yield of the reaction in the case of phenyl acetylene (Table 1 entry 20), when aliphatic alkyne was used (Table 2, entries 1, 4) the yield was decreased. The low yield of the entry 6 of Table 2 (compound **10**), is due to the removal of the 4-methoxybenzyl group, under these reaction conditions. After purification by column chromatography, compound **10** and 3-(3,4-dimethoxyphenyl)-5-isoxazolyl-methanol were isolated.

It should be noted that all the yields are based on the aldehydes and not on oximes or imidoyl chlorides. Thus, the low to moderate yields of the Cu-free reactions are overall yields of a three step reaction. As we have previously reported [15], in our experiments the *in situ* generation of hydroximoyl chlorides and their conversion to nitrile oxides was fast, followed by addition of terminal alkynes which are trapping agents to avoid the dimerization of nitrile oxides to furoxans.



Scheme 2. Uncatalysed synthesis of isoxazoles.

Table 2
Copper free examples of the optimized conditions (80 W/90 °C/45 min).[a]

Entry	Aldehyde	Acetylene	Isoxazole	Yield (%)
1			 **2**	52 (34)
2			 **6**	47
3			 **7**	29
4			 **8**	49 (15)
5			 **9**	30
6			 **10**	18
7			 **11**	26

[a] In parentheses are the yields of isoxazoles using 100 W/90 °C/22 min.

In general, the reaction times of the metal free reactions were shorter of those reported in the literature (max 45 min instead of hours). In the case of analogue **8** the yield of the microwave (80 W/90 °C/45 min) uncatalysed reaction was comparable to that we have reported [2] for this compound using $CuSO_4 \cdot 5H_2O$/copper turnings, overnight and based on the oxime.

Since the synthesized methoxy analogues are not expected to possess antioxidant activity and keeping in mind that antioxidant properties are usually linked to anti-ageing properties [23–28], compounds **1, 2** and **6**–**9** were deprotected using $BF_3.SMe_2$ as previously described [2]. In the case of derivatives **6** and **7** the known alcohol (3-phenylisoxazol-5-yl) methanol **14** was obtained due to the removal of the 4-methoxybenzyl or the 3,4-dimethoxybenzyl group under these reaction conditions. The structures of the deprotected analogues **12**–**17** are depicted in Fig. 1.

Ageing is associated with increased levels of reactive oxygen species (ROS) thus, we sought to test the antioxidant and/or anti-ageing properties of the isoxazole derivatives (**12**–**17**) in two model systems; human primary fibroblasts (*in vitro* model) and the nematode *C. elegans* (*in vivo* model). The first model was used to test cell survival following oxidative stress, while the second one was used to reveal possible longevity-promoting effects of our compounds in a eukaryotic multicellular organism. More specifically, we subjected young human primary fibroblasts to oxidative stress (H_2O_2) in the presence or absence of our compounds and we then tested their survival ability.

As shown in Fig. 2A, cells treated with compound **17** exhibited significantly enhanced viability after oxidative insult as compared to the control cultures. In contrast, the rest of the compounds (**12**–**16**) demonstrated low or no cytoprotective properties. Following the identification of analogue **17** as the most potent, we then used a reference standard compound namely quercetin, the most abundant dietary flavonol that has been shown previously to be a potent antioxidant by others [30] as well by us [26], to compare its effects with the effects of compound **17**. As shown in Fig. 2B, compound **17** was more potent as compared to quercetin at the same concentrations (2 and 5 μM). Therefore, the *in vitro* model revealed that compound **17** enhances the ability of the cells to cope

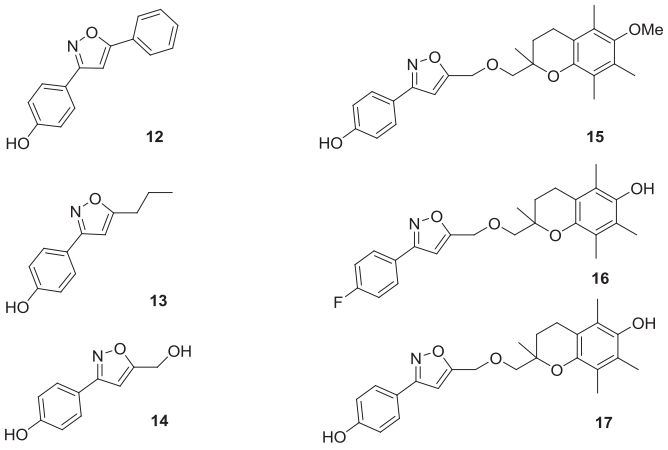


Fig. 1. Structures of the tested compounds.

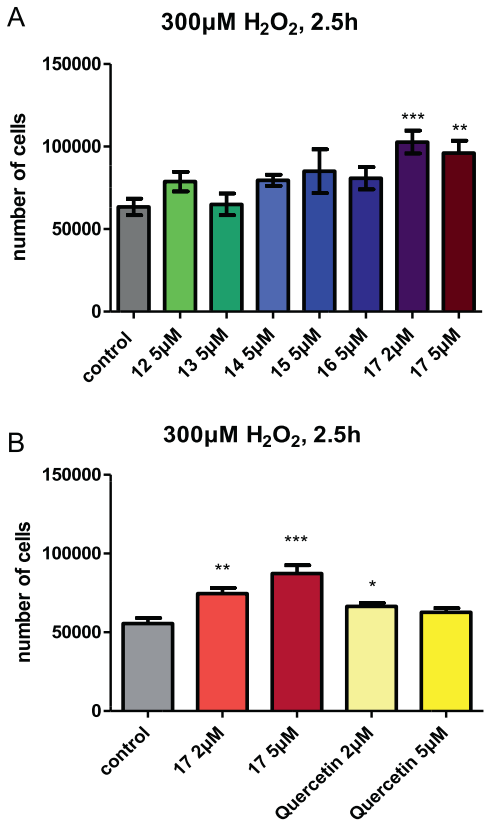


Fig. 2. Treatment with compound **17** increases cellular resistance to oxidative stress. Number of cells treated with (A) 2 μM compound **17** or 5 μM of compounds **12**–**17** or DMSO (solvent control) and (B) 2 and 5 μM compound **17** or quercetin or DMSO (solvent control) for 24 h following treatment with 300 μM H_2O_2 for 2.5 h and a five-day recovery period. Results with *p*-values, $p < 0.05$, $p < 0.01$ or $p < 0.001$ are denoted in graphs by a single (*), double (**) or triple (***) asterisk, respectively.

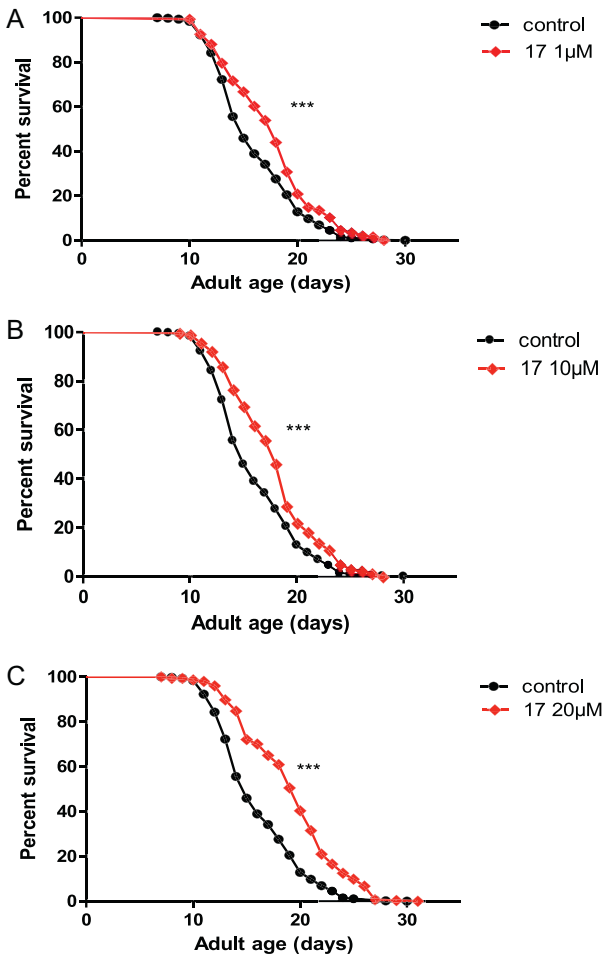


Fig. 3. Treatment with compound **17** extends the lifespan of wt *C. elegans*. (*A*–*C*) Survival curves of wt N2 worms treated with (A) 1 μM, (B) 10 μM and (C) 20 μM compound **17** as compared to the relative control cultures (DMSO). The percentage of animals remaining alive is plotted against animal age. (A) Control: mean = 15 ± 0.85, n = 471/489 (number of animals that died/total; see *Materials and Methods*), compound **17** 1 μM: mean = 18 ± 0.33, n = 304/313, $P < 0.0001$, **17** 10 μM: mean = 18 ± 0.5, n = 319/325, $P < 0.0001$, **17** 20 μM, 20 ± 0.33, n = 297/308, $P < 0.0001$.

better with oxidative stressors while, more interestingly, it does so better as compared to a standard antioxidant compound such as quercetin, at lower concentrations. We then examined whether our antioxidant compounds also exhibit anti-ageing properties in a multicellular organism. To this end, we fed wild type nematode worms with different concentrations of each compound and the relative diluent (DMSO). Compounds **12**–**16** did not promote any differences in the lifespan of the nematodes (data not shown), thus coinciding with the results from the cell survival assays where no cytoprotective effects were scored. In bright contrast, analogue **17** presented anti-ageing activity. More specifically, as shown in Fig. 3, treatment with various concentrations of **17** (ranging from 1 μM to 20 μM) resulted in significant extension of animal lifespan, with 20 μM being the most effective concentration.

Given that we had previously shown the anti-ageing properties of quercetin using human fibroblasts [26] while others have revealed its anti-ageing effects in *C. elegans* [31], we have also compared the anti-ageing effects of compound **17** with the relative anti-ageing effects of quercetin in *C. elegans*. We therefore fed wild type nematode worms with the most potent concentrations of compound **17** that we have identified in our initial experiments, namely 10 and 20 μM, and with equal concentrations of quercetin.

As shown in Fig. 4A, treatment of nematodes with 10 μM compound **17** resulted in significant extension of animal lifespan while treatment with 10 μM quercetin did not promote lifespan extension. Likewise, treatment of nematodes with 20 μM compound **17** resulted in significant lifespan extension that was more enhanced as compared to the relative extension induced by 20 μM quercetin (Fig. 4B). In total, compound **17** was revealed to act protectively against oxidative stress in human primary fibroblasts and to promote lifespan extension in *C. elegans*. More interestingly, **17** was identified to be more potent in lower concentrations as compared to a well established natural antioxidant such as quercetin.

3. Discussion

In this study, we have achieved the green regiospecific microwave-assisted one-pot synthesis of bioactive isoxazoles bearing antioxidant groups and we have shown for the first time their impact on cellular resistance to stress and on organismal ageing. More specifically, we have revealed that the newly derived compounds possess anti-ageing properties depending on the number and the nature of the antioxidant isoxazole substituents. The most potent of these isoxazoles, namely analogue **17**, confers resistance to oxidative stress in human primary fibroblasts while in its presence an extended lifespan of the wild type *C. elegans* is observed. The increased resistance to oxidative stress along with the anti-ageing effects is strongly linked to the aforementioned antioxidant properties.

Compounds **12**–**15** and **17** bear the 3-(4-hydroxyphenyl)isoxazole moiety. The presence of an alkyl group at 5-position of the

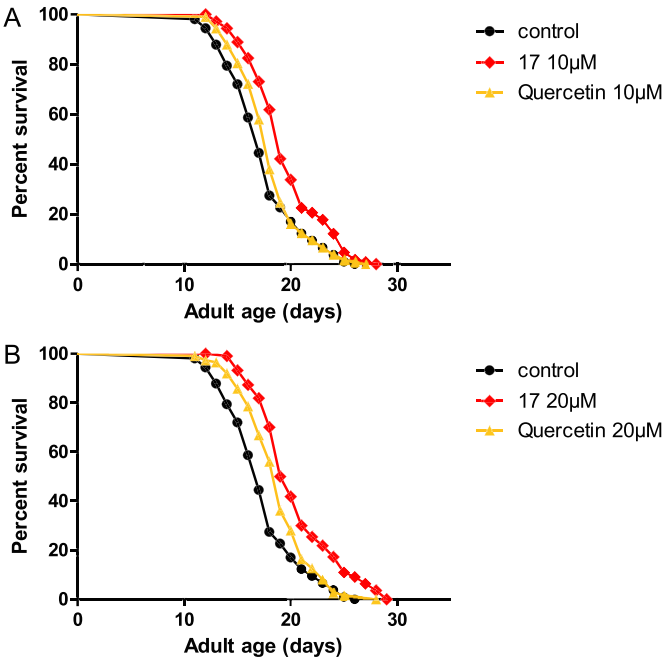


Fig. 4. Comparative treatment of *C. elegans* with compound **17** and quercetin. (*A*,*B*) Survival curves (1 experiment) of wt N2 worms treated with (A) 10 μM and (B) 20 μM of compound **17**, quercetin or the relevant amount of DMSO (control). The percentage of animals remaining alive is plotted against animal age. (A–B) Control: mean = 17, n = 106/109 (number of animals that died/total; see *Materials and Methods*), (A) compound **17** 10 μM: mean = 19, n = 107/111, $P < 0.0001$ compared with control, 10 μM quercetin: mean = 18, n = 106/108, $P = 0.1927$ compared with control, $P < 0.0001$ 10 μM compound **17** compared with 10 μM quercetin: (B) 20 μM compound **17**: mean = 20, n = 111/121, $P < 0.0001$ compared with control, 20 μM quercetin: mean = 19, n = 111/115, $P = 0.0051$ compared with control, $P = 0.0004$ 20 μM compound **17** compared with 20 μM quercetin.

isoxazole ring (compound **13**) or a phenyl group (compound **12**) had no impact on the antioxidant activity of the compounds. Isoxazoles **15** and **17** can be considered as derivatives of **14**. The 4-hydroxyphenyl-substituted isoxazoles **14** and **15** exhibited similar activity in cells indicating that the protected chroman does not affect the possible cytoprotective activity of these compounds. In contrast, deprotection of the chroman hydroxyl group gave the most active analogue **17**. The significance of the antioxidant groups is demonstrated by the fact that, the cytoprotective activity in the 6-OH-chroman derivative **16** that lacks a second antioxidant group is lost as compared to the relative activity of compound **17** that carries both antioxidant groups. Thus the presence of two antioxidant moieties, a phenolic and a 6-OH-chroman group in compound **17** results in significantly elevated cell survival under oxidative stress. However, the number of hydroxyl groups does not seem to be the only requisite for the cytoprotective properties against oxidative stress. More specifically, quercetin which bears five free hydroxyl groups that contribute to its strong antioxidant activity, was nevertheless less active than compound **17** at the cellular level (*in vitro* model).

Resistance to oxidative stress has been linked to longevity in *C. elegans*. More specifically, many long-lived mutants exhibit increased resistance to a variety of stressors i.e. oxidants [32] or heat [33]. Therefore, given that we detected resistance to oxidative stress in our cellular model we sought to investigate the possible effects of our compounds on the lifespan of the *C. elegans*. In accordance to the results reported in the cell assays, compound **17** was the only one that exhibited lifespan-extending properties, thus further advocating for a conserved positive effect of this isoxazole among species. Few natural antioxidants have been revealed previously to promote extension of lifespan in *C. elegans*, i.e. epigallocatechin gallate [23], the flavonoids myricetin, quercetin, kaempferol and naringenin [24] and tyrosol [25] among others. However, it is noteworthy that our synthetic compound was able to promote longevity at significantly lower doses as compared to the dosages of natural antioxidants that have been shown to be necessary to exert beneficial effects on the ageing process in *C. elegans* [23–25,31]. Therefore, compound **17** is more potent and efficient than other known natural antioxidants both *in vitro* and *in vivo*, a highly advantageous characteristic in case of product application.

Our results suggest that the antioxidant activities of our compounds are responsible for the cellular stress resistance and the extension of organismal lifespan. Nonetheless, it is possible that these isoxazole analogues may differentially affect various cellular signal cascades. Thus, further studies are needed to determine the effects of the synthesized compounds on signalling cascades *in vivo*.

4. Conclusion

A series of 3,5-disubstituted isoxazoles were synthesized by microwave-assisted, Cu free, 1,3-dipolar cycloaddition reaction between *in situ* generated nitrile oxides and alkynes bearing protected antioxidant substituents. Uncatalysed reactions, in *tert*-butanol/water, were regioselective giving low to moderate yields of the three step reaction, based on starting aldehydes. The reaction times were significantly shorter compared to those reported in the literature.

The biological evaluation of the deprotected compounds showed a correlation of their antioxidant properties with stress resistance in human primary fibroblasts (*in vitro* model) and with the extended longevity of the nematode *C. elegans* (*in vivo* model). Clearly, the activity of our isoxazole analogues at the cellular and organismal level depends on the nature and the number of the antioxidant substituents. Compound **17** bearing a phenolic group and a 6-OH-chroman group was revealed to be a potent antioxidant against oxidative stress at a very low dose in human primary fibroblasts and a promising anti-ageing agent as shown in the *in vivo* model used. Additional studies using various strains of *C. elegans* bearing mutations in molecular pathways that are key to the progression of ageing are required to reveal the exact pathway through which analogue **17** functions as an anti-ageing agent.

5. Experimental

5.1. Chemistry

5.1.1. Materials and methods

All starting materials and common laboratory chemicals were purchased from commercial sources and used without further purification. ^{1}H NMR spectra were recorded on Varian spectrometers operating at 300 MHz or 600 MHz and ^{13}C spectra were recorded at 75 MHz using $CDCl_3$ or $(CD_3)_2CO$ as solvent. Silica gel plates Macherey–Nagel Sil G-25 UV_{254} were used for thin layer chromatography. Chromatographic purification was performed with silica gel (200–400 mesh). Mass spectra were obtained on HPLC–MS^n Fleet-Thermo, in the ESI mode. HRMS spectra were recorded, in the ESI mode, on UPLC–MS^n Orbitrap Velos-Thermo. The microwave-assisted experiments were carried out with a CEM Discover 300 W monomode microwave instrument. The closed vessels used were special glass tubes with self-sealing septa that controlled pressure with appropriate sensors on the top (outside the vial). The temperature was monitored through a non-contact infrared sensor centrally located beneath the cavity floor.

Magnetic stirring was provided to assure complete mixing of the reactants.

5.1.2. General procedure for preparation of 3,5-disubstituted isoxazoles

To a solution of aldehyde (1 eq) and hydroxylamine hydrochloride (1.05 eq) in a mixture of *t*-BuOH and H_2O (1:1) was added 1 M aqueous NaOH (1.05 eq). The reaction mixture was stirred at ambient temperature until thin-layer chromatography indicated consumption of the aldehyde. After completion of oxime formation, 1.05 eq of chloramine-T [TsN(Cl)Na·$3H_2O$] was added, followed (after 3 min) by the appropriate alkyne (1.05 eq), the pH of the reaction medium was adjusted to 6 (by addition of few drops of 1 M aqueous NaOH) and the mixture was microwave irradiated as indicated in Tables 1 and 2. The reaction mixture was extracted with AcOEt, the organic layer was washed with saturated NaCl, dried over Na_2SO_4 and concentrated *in vacuo*. The crude residue was purified by column chromatography (pet. ether/ethyl acetate, 90:10 to 80:20 affording products as colourless oils).

Compounds **1**, **2**, **8** were prepared according to the procedure described above and their analytical data are in accordance with those previously reported [15].

*5.1.2.1. 5-(4-Methoxybenzyloxy)methyl-3-(4-methoxyphenyl)-isoxazole (**6**).* TLC (pet. ether/ethyl acetate, 85:15) R_f = 0.2, ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.73 (d, J = 8.7 Hz, 2H, Ar*H*), 7.29 (d, J = 8.5 Hz, 2H, Ar*H*), 6.96 (d, J = 8.7 Hz, 2H, Ar*H*), 6.89 (d, J = 8.5 Hz, 2H, Ar*H*), 6.50 (s, 1H, *H*-isoxazole), 4.61 (s, 2H, CH_2), 4.56 (s, 2H, CH_2), 3.84(s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), ^{13}C NMR (75 MHz, $CDCl_3$) δ: 169.7, 162.0, 161.0, 159.5, 129.7, 129.2, 128.2, 121.5, 114.3, 113.9, 100.8, 72.6, 62.5, 55.3, 55.2, MS *m*/*z*: 326.36 $(M+H)^+$, 348.35 $(M+Na)^+$, 672.96 $(2M+Na)^+$, HRMS: calcd for $C_{19}H_{20}NO_4$ $(M+H)^+$ 326.1387, $C_{19}H_{19}NO_4Na$ $(M+Na)^+$ 348.1206; found: 326.1381, 348.1200.

*5.1.2.2. 5-(3,4-Dimethoxybenzyloxy)methyl-3-(4-methoxyphenyl)-isoxazole (**7**).* TLC (pet. ether/ethyl acetate, 80:20) R_f = 0.15, ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.72 (d, J = 8.8 Hz, 2H, Ar*H*), 6.96 (d, J = 8.8 Hz, 2H, Ar*H*), 6.92–6.89 (m, 2H, Ar*H*), 6.83 (d, J = 8.0 Hz, 1H, Ar*H*), 6.50 (s, 1H, *H*-isoxazole), 4.62 (s, 2H, CH_2), 4.56 (s, 2H, CH_2), 3.88 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), ^{13}C NMR (75 MHz, $CDCl_3$) δ: 169.6, 162.0, 161.0, 149.1, 148.9, 129.6, 128.2, 121.4, 120.7, 114.3, 111.2, 111.0, 100.9, 72.9, 62.4, 55.9, 55.8, 55.3, MS *m*/*z*: 356.25 $(M+H)^+$, 378.28 $(M+Na)^+$, 732.91 $(2M+Na)^+$, HRMS: calcd for $C_{20}H_{22}NO_5$ $(M+H)^+$ 356.1492, $C_{20}H_{21}NO_5Na$ $(M+Na)^+$ 378.1312; found: 356.1492, 378.1307.

*5.1.2.3. 5-{[(3,4-Dihydro-6-methoxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl)methoxy] methyl}-3-(4-fluorophenyl)-isoxazole (**9**).* TLC (pet. ether/ethyl acetate, 90:10) R_f = 0.3, ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.77–7.75 (m, 2H, Ar*H*), 7.13 (t, J = 8.6 Hz, 2H, Ar*H*), 6.46 (s, 1H, *H*-isoxazole), 4.74 (ABq, 2H, $\Delta\nu_{AB}$ = 12.9 Hz, J_{AB} = 13.9 Hz −O−CH_2−), 3.61 (s, 3H, −OCH_3), 3.58 (ABq, 2H, $\Delta\nu_{AB}$ = 27.7 Hz, J_{AB} = 10 Hz −CH_2−O−), 2.58 (t, J = 6.8 Hz, 2H, −CH_2), 2.17 (s, 3H, Ar−CH_3), 2.12 (s, 3H, Ar−CH_3), 2.08 (s, 3H Ar−CH_3), 2.02–1.97 (m, 1H, −C*H*H), 1.79–1.76 (m, 1H, −C*H*H), 1.30 (s, 3H, −CH_3), ^{13}C NMR (75 MHz, $CDCl_3$) δ: 170.2, 162.1, 161.4, 149.8, 147.3, 128.8, 128.7, 128.0, 125.9, 125.2, 122.8, 117.4, 116.2, 115.9, 100.7, 74.9, 64.6, 60.4, 28.4, 22.0, 20.2, 12.6, 11.9, 11.7, ^{19}F NMR δ: −110.6, MS *m*/*z*: 426.17 $(M+H)^+$, 872.75 $(2M+Na)^+$, HRMS: calcd for $C_{25}H_{29}FNO_4$ $(M+H)^+$ 426.2075, $C_{25}H_{28}FNO_4Na$ $(M+Na)^+$ 448.1895; found: 426.2079, 448.1895.

*5.1.2.4. 5-(4-Methoxybenzyloxy)methyl-3-(3,4-dimethoxyphenyl)-isoxazole (**10**).* TLC (pet. ether/ethyl acetate, 85:15) R_f = 0.1, ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.40 (s, 1H, Ar*H*), 7.31–7.28 (m, 1H, Ar*H*), 6.91 (d, J = 8.3 Hz, 1H, Ar*H*), 6.52 (s, 1H, *H*-isoxazole), 4.61 (s, 2H, −CH_2−O−), 4.57 (s, 2H, −OCH_2−), 3.93 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), ^{13}C NMR (75 MHz, $CDCl_3$) δ: 169.8, 162.1, 159.5, 150.6, 150.2, 149.3, 129.7, 129.1, 126.4, 121.6, 119.9, 113.9, 111.0, 109.3, 100.9, 72.6, 62.5, 56.1, 56.0, 55.9, MS *m*/*z*: 356.31 $(M+H)^+$, 378.28 $(M+Na)^+$, 732.84 $(2M+Na)^+$, HRMS: calcd for $C_{20}H_{22}NO_5$ $(M+H)^+$ 356.1492, $C_{20}H_{21}NO_5Na$ $(M+Na)^+$ 378.1312; found: 356.1489, 378.1307.

5.1.3. General procedure for deprotection of methoxy groups

A solution of the appropriate protected compound (**1**, **2** and **6**–**9**, 1 equiv) in anhydrous CH_2Cl_2 (3 mL), was cooled at 0 °C and BF_3·SMe_2 (10 equiv for each methoxy group) was added. After stirring for 24 h, the solvent and excess reagent were evaporated under argon stream. The residue was taken up in EtOAc and washed with H_2O and saturated NaCl. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*.

*5.1.3.1. 3-(4-Hydroxyphenyl)-5-phenyl-isoxazole (**12**) [34].* Compound **1** was treated as described in the general procedure. Purification by column chromatography (pet. ether/ethyl acetate, 90:10 to 85:15), Yield: 84%, white solid, mp 178–180 °C, TLC (pet. ether/ethyl acetate, 85:15) R_f = 0.2, ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.87 (d, J = 7.1 Hz, 2H, Ar*H*), 7.77 (d, J = 8.7 Hz, 2H, Ar*H*), 7.51–7.45 (m, 3H, Ar*H*), 6.95 (d, J = 8.7 Hz, 2H, Ar*H*), 6.78 (s, 1H, *H*-isoxazole), 5.20 (bs, 1H, −O*H*), ^{13}C NMR (75 MHz, $CDCl_3$) δ: 170.1, 162.8, 158.5, 130.1, 128.9, 128.3, 127.4, 125.8, 120.6, 115.8, 97.3, MS *m*/*z*: 236.08 $(M-H)^-$, HRMS: calcd for $C_{15}H_{12}NO_2$ $(M+H)^+$ 238.0863, $C_{15}H_{11}NO_2Na$ $(M+Na)^+$ 260.0682; found: 238.0860, 260.0680.

*5.1.3.2. 3-(4-Hydroxyphenyl)-5-propyl-isoxazole (**13**).* Compound **13** was obtained by deprotection of **2**. Purification by column chromatography (pet. ether/ethyl acetate, 80:20), Yield: 75%, white solid, mp 107–109 °C, TLC (pet. ether/ethyl acetate, 90:10) R_f = 0.25, ^{1}H NMR (600 MHz, $(CD_3)_2CO$) δ: 8.85 (bs, 1H, −O*H*), 7.70 (d, J = 8.0 Hz, 2H, Ar*H*), 6.93 (d, J = 8.0 Hz, 2H, Ar*H*), 6.50 (s, 1H, *H*-isoxazole), 2.74 (t, J = 7.5 Hz, 2H, $CH_3CH_2CH_2$−), 1.75–1.72 (m, 2H, $CH_3CH_2CH_2$−), 0.98 (t, J = 7.4 Hz, 3H, $CH_3CH_2CH_2$−), ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 173.6, 161.8, 158.9, 128.1, 120.9, 115.8, 98.3, 28.2, 20.8, 13.1, MS *m*/*z*: 204.12 $(M+H)^+$, 428.80 $(2M+Na)^+$, HRMS: calcd for $C_{12}H_{14}NO_2$ $(M+H)^+$ 204.1019, $C_{12}H_{13}NO_2Na$ $(M+Na)^+$ 226.0838; found: 204.1018, 226.0839.

*5.1.3.3. 3-(4-Hydroxyphenyl isoxazol-5-yl)methanol (**14**) [35].* Compound **6** was treated as described in the general procedure. Purification by column chromatography (pet. ether/ethyl acetate, 85:15), Yield: 18%, white solid, mp 148–150 °C, TLC (pet. ether/ethyl acetate, 90:10) R_f = 0.2, ^{1}H NMR (600 MHz, $(CD_3)_2CO$) δ: 8.87 (bs, 1H, −O*H*), 7.71 (d, J = 8.7 Hz, 2H, Ar*H*), 6.93 (d, J = 8.7 Hz, 2H, Ar*H*), 6.66 (s, 1H, *H*-isoxazole), 4.70 (s, 2H, −CH_2OH), ^{13}C NMR (75 MHz, $(CD_3)_2CO$) δ: 173.1, 161.7, 128.1, 120.7, 115.7, 99.1, 55.5, MS *m*/*z*: 190.09 $(M-H)^-$, 380.79 $(2M)^-$, HRMS: calcd for $C_{10}H_{10}NO_3$ $(M+H)^+$ 192.0655; found: 192.0657.

*5.1.3.4. 5-{[(3,4-Dihydro-6-methoxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl)methoxy]-methyl}-3-(4-hydroxyphenyl)-isoxazole (**15**) [2].* Compound **8** was treated according to the general procedure described above giving a mixture of analogues **15** and **17** which was purified by column chromatography (pet. ether/ethyl acetate, 70:30). Yield: 27% yellowish foam, ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.67 (d, J = 8.3 Hz, 2H, Ar*H*), 6.90 (d, J = 8.3 Hz, 2H, Ar*H*), 6.46 (s, 1H, *H*-isoxazole), 5.53 (bs, 1H, −O*H*), 4.72 (ABq, 2H,

$\Delta\nu_{AB}$ = 14.9 Hz, J_{AB} = 13.9 Hz, −O−CH_2−), 3.63 (s, 3H, OCH_3), 3.57 (ABq, 2H, $\Delta\nu_{AB}$ = 29.9 Hz, J_{AB} = 9.8 Hz −CH_2−O−), 2.60 (t, J = 6.7 Hz, 2H, −CH_2−), 2.18 (s, 3H, Ar−CH_3), 2.13 (s, 3H, Ar−CH_3), 2.09 (s, 3H, Ar−CH_3), 2.03–1.98 (m, 1H, −CHH), 1.80–1.75 (m, 1H, −CHH), 1.31 (s, 3H, −CH_3), ^{13}C NMR (75 MHz, $CDCl_3$) δ: 169.8, 161.9, 157.3, 149.7, 147.3, 128.4, 128.0, 125.9, 122.8, 121.5, 117.5, 115.8, 100.7, 76.2, 75.0, 64.6, 60.4, 28.3, 22.1, 20.1, 12.6, 11.9, 11.7, MS m/z: 422.14 $(M-H)^-$, 844.31 $(2M)^-$ HRMS: calcd for $C_{25}H_{30}NO_5$ $(M+H)^+$ 424.2118; found: 424.2103.

*5.1.3.5. 2-{[(3-(4-Fluorophenyl)isoxazol-5-yl)methoxy]-methyl}-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol (**16**).* Compound **16** was obtained by deprotection of **9**. Purification by column chromatography (pet. ether/ethyl acetate, 90:10 to 80:20), Yield: 50%, yellowish oil, TLC (pet. ether/ethyl acetate, 90:10) R_f = 0.1, ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.77–7.75 (m, 2H, Ar*H*), 7.13 (t, J = 8.7 Hz, 2H, Ar*H*), 6.45 (s, 1H, *H*-isoxazole), 4.73 (ABq, 2H, $\Delta\nu_{AB}$ = 12.5 Hz, J_{AB} = 14 Hz, −O−CH_2−), 4.20 (bs, 1H, −O*H*), 3.58 (ABq, 2H, $\Delta\nu_{AB}$ = 22.1 Hz, J_{AB} = 10 Hz, −CH_2−O−), 2.59 (t, J = 6.8 Hz, 2H, −CH_2−), 2.17 (s, 3H, Ar−CH_3), 2.12 (s, 3H, Ar−CH_3), 2.08 (s, 3H, Ar−CH_3), 2.03–1.97 (m, 1H, −CHH), 1.79–1.75 (m, 1H, −CHH), 1.30 (s, 3H, −CH_3), ^{13}C NMR (75 MHz, $CDCl_3$) δ: 170.3, 161.4, 145.0, 144.9, 128.8, 128.7, 122.5, 121.2, 118.6, 117.2, 100.6, 76.2, 74.7, 64.6, 28.6, 22.0, 20.3, 12.2, 11.9, 11.3, ^{19}F NMR δ: −110.6, MS m/z: 412.17 $(M+H)^+$, 434.33 $(M+Na)^+$, 457.08 $(M+2Na)^+$, 844.92 $(2M+Na)^+$, HRMS: calcd for $C_{24}H_{27}FNO_4$ $(M+H)^+$ 412.1919, $C_{24}H_{26}FNO_4Na$ $(M+Na)^+$ 434.1738; found: 412.1916, 434.1731.

*5.1.3.6. 2-{[(3-(4-Hydroxyphenyl)isoxazol-5-yl)methoxy]-methyl}-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol (**17**).* Yield: 57% white foam, ^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.64 (d, J = 8.7 Hz, 2H Ar*H*), 6.90 (d, J = 8.7 Hz, 2H, Ar*H*), 6.44 (s, 1H, *H*-isoxazole), 5.03 (bs, 1H, −O*H*), 4.72 (ABq, 2H, $\Delta\nu_{AB}$ = 13.4 Hz, J_{AB} = 13.9 Hz, −O−CH_2−), 4.20 (bs, 1H, −O*H*), 3.57 (ABq, 2H, $\Delta\nu_{AB}$ = 23.5 Hz, J_{AB} = 10 Hz, −CH_2−O−), 2.62 (t, J = 6.8 Hz, 2H, −CH_2−), 2.15 (s, 3H, Ar−CH_3), 2.11 (s, 3H, Ar−CH_3), 2.10 (s, 3H, Ar−CH_3), 2.02–1.95 (m, 1H, −CHH), 1.80–1.75 (m, 1H, −CHH), 1.30 (s, 3H, −CH_3), ^{13}C NMR (75 MHz, $CDCl_3$) δ: 169.8, 162.0, 157.4, 145.1, 144.9, 128.4, 122.5, 121.3, 118.6, 117.3, 115.9, 100.6, 76.2, 74.7, 64.6, 28.6, 22.0, 20.3, 12.2, 11.9, 11.3, MS m/z: 408.16 $(M-H)^-$, 816.44 $(2M)^-$, HRMS: calcd for $C_{24}H_{28}NO_5$ $(M+H)^+$ 410.1962; found: 410.1959.

5.2. Biology

5.2.1. Cell culture

HFL-1 human embryonic fibroblasts were obtained by the European Collection of Cell Cultures and were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% foetal bovine serum (v/v; Invitrogen), 2 mM glutamine, and 1% non-essential amino-acids. HFL-1 cells were subcultured at 37 °C, 5% CO_2 and 95% humidity and were fed approximately 16 h prior to each assay.

5.2.2. Cell survival

Cell survival was assessed by counting the number of cells after treatment with 300 μM H_2O_2 for 2.5 h. Briefly, 10^4 HFL-1 cells were plated in 6-well plates and 24 h later were treated with 2 μM compound **17** and quercetin (Sigma Aldrich) and 5 μM compounds **12**–**17** and quercetin or DMSO (solvent control) for 24 h. Cells were then incubated with 300 μM hydrogen peroxide (H_2O_2) for 2.5 h in the presence of each compound and were then washed thoroughly with PBS. Treated cultures were left to recover in complete medium for 5 days and their numbers were determined in triplicates using a Coulter Z_2 counter. Each experiment was performed at least two times with the exception of quercetin that was repeated once in 5-plicates.

5.2.3. C. elegans strains, culture conditions and compounds treatments

The N2 (wt Bristol isolate) strain was used. It was provided by the Caenorhabditis Genetics Center, supported by the NIH National Center for Research Resources (NCRR). We followed standard procedures for *C. elegans* strain maintenance at 20 °C. The nematodes were grown on solid nematode growth medium (NGM) seeded with *E. coli* (OP50) for food. For compounds treatments, UV-irradiated NGM/OP50 plates were supplemented with different compounds concentrations (solubilized in DMSO) as indicated. DMSO was included as a solvent control in control cultures. UV-killing was used to avoid any adverse effects of live *E. coli* on the compounds. The compounds or DMSO were diluted in M9 reaching a volume of 200 μl and added over the agar and dead bacteria right after the UV irradiation. Plates were allowed to dry for 24 h before use.

5.2.4. Lifespan analysis

Gravid N2 worms were allowed to lay eggs for 3 h to produce synchronized populations. At L4 larval stage, 80–120 animals/condition were transferred to fresh plates containing either the compound or DMSO. Day 1 of adulthood was set as t = 0. Animals were maintained at 20 °C, were transferred to fresh plates containing the compound or DMSO every 2 days to avoid confounding of generation and starvation and were examined every day for touch-provoked movement and pharyngeal pumping until death. Animal populations were maintained in the respective compounds throughout their lifespans. Each survival assay was repeated at least twice unless otherwise indicated and representative assays are shown. Survival curves were created using the product-limit method by Kaplan and Meier. The log-rank (Mantel–Cox) test was used to evaluate differences between survivals and to determine P values for all available independent data. The n in lifespan figures is the number of animals that died/total where total equals the animals number that died plus the number of censored animals (due to internally hatched eggs, extruded gonad or desiccation due to crawling off the plates). Median lifespan values are expressed as mean ± SEM.

5.2.5. Statistical analysis

Statistical analyses were performed using Prism (GraphPad Software, San Diego, California USA) and Microsoft Office 2003 Excel (Microsoft Corporation) software packages.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.06.046.

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