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Novel 1,2,4-Triazolo[1,5-*a*]pyridines and Their Fused Ring Systems Attenuate Oxidative Stress and Prolong Lifespan of *Caenorhabiditis elegans*

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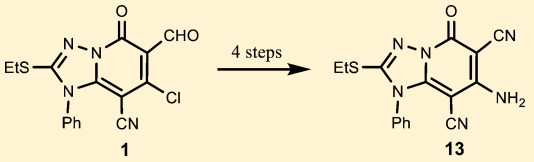
ABSTRACT: In this paper we report the synthesis of some novel 1,2,4-triazolo[1,5-*a*]pyridine and azolotriazolopyridine ring systems. The products were screened for various types of activity like antibacterial, antifungal, and antioxidative activity. Compound **13** was found to pose an antioxidative activity. In addition, this compound was found to extend the life span of *Caenorhabiditis elegans* under standard laboratory conditions and reduces both heat and chemical induced oxidative stress in *C. elegans* in a dose-dependent manner. Furthermore, treatment of worms with compound **13** was found to significantly attenuate the formation of advanced glycation end products and malondialdehyde in a dose-dependent manner.



1. INTRODUCTION

Aging is associated with increased incidence of a range of diseases, some of which are pathogenetically linked to elevated levels of reactive oxygen species (ROS).[1] The mitochondrial free radical theory of aging[2] proposes an association between ROS generated during mitochondrial respiration (mtROS, the principal source of ROS in cells), accumulation of oxidative damage in mitochondrial DNA (mtDNA), and occurrence of mutations in the mitochondrial genome. These molecular changes lead to damaged or misfolded proteins, mitochondrial dysfunction, decline of cellular and tissue functions, enhanced formation of advanced glycation end products (AGE), and shortened life span. ROS generated due to mitochondrial dysfunction are thought to further induce mtDNA mutations, thus contributing to a vicious cycle of aging.[3−5] The extent of ROS formation and oxidative damage to mtDNA is inversely correlated with longevity across species.[6] Moreover, several studies have demonstrated an association between oxidative stress, mtDNA mutations, AGE formation, and age-related organ dysfunction.[4−7]

Antioxidants may play an important role in preventing free radical damage associated with aging by interfering directly in the generation of radicals or by scavenging them. Previously, Brown et al.,[8] Bartholome et al.,[9] and Zhang et al.[10] have been indicated that antioxidants like Epigallocatechin gallate and α-lipoic acid have the ability to attenuate oxidative stress and prolong life span of wild-type *Caenorhabditis elegans* (*C. elegans*) under both standard and induced stress conditions. Recently, Wilson et al.[11] have demonstrated that proanthocyanidin, a potent antioxidant, reduced oxidative stress and extend the life span of wild-type *C. elegans*. In 2012, Grünz and his co-workers[12] have proved that myricetin, quercetin, kaempferol, and naringenin improved the antioxidant status of wild-type *C. elegans*, which results in life span extension.

We designed a study to determine whether the new synthesized 1,2,4-triazol[1,5-*a*]pyridine derivatives can prolong lifespan in a whole organism. For these studies, we required an organism with relatively short lifespan that could be assayed reproducibly and robustly and for which the genetic and environmental factors affecting lifespan were well-defined. The experimental organism that could best accommodate these requirements was the nematode, *C. elegans*, which has become a popular model for studying aging and longevity due to its short 2−3-week lifespan, rapid generation time, and experimental flexibility.[13]

1,2,4-Triazolo[1,5-*a*]pyridines constituted an important class of heterocyclic compounds, which are of considerable interest due to their uses as active ingredients in antihypertensive, bronchodilatory, antiinflammatory, analgesic, and positive inotropic agents.[14−16]

Isoxazoles, pyrroles, and pyrazoles are well-known examples of heteroaromatic organic compounds associated with diverse biological and pharmacological properties. Isoxazoles constitute an important family of five-membered hetrocycles in view of their use in many natural products syntheses[17,18] and occurrence in pharmaceutical agents such as COX-2 inhibitor Bextra.[19] The pyrrole skeleton is of great importance to chemists as well as biologists, as it is found naturally in plants and in animal cell constituents.[20] Pyrazole derivatives are synthetic targets of utmost importance in the pharmaceutical industry

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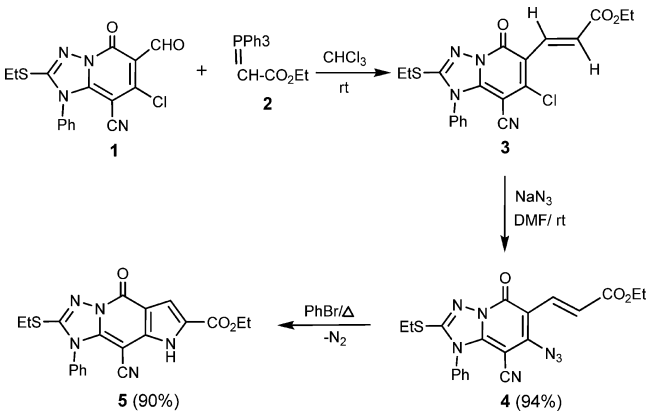
because the pyrazole ring has been known as an important framework in a large number of drugs.[21−25] Moreover, recent reports display the pyrazole chemotype as the structural motif of a number of highly potent inhibitors of coagulation factor Xa[26] and antitumor CDK.[27]

Considering the above very interesting pharmacological properties and as a part of our studies on the design of new routes for the synthesis of novel heterocyclic ring systems containing the 1,2,4-triazole skeleton, with potential pharmaceutical activities,[28] we have now designed and synthesized some novel functionalized 1,2,4-triazolo[1,5-*a*]pyridines and their fused tricyclic ring systems, with important heterocycles such as pyrrole, isoxazole, and pyrazole. The products were screened for various types of activity like antibacterial, antifungal, and antioxidative activity. Compound **13** was found to pose an antioxidative activity. In addition, this compound was found to extend the life span of *C. elegans* under standard laboratory conditions and reduces both heat and chemical induced oxidative stress in *C. elegans* in a dose-dependent manner. Furthermore, treatment of worms with compound **13** was found to significantly attenuate the formation of advanced glycation end products and malondialdehyde in a dose-dependent manner.

2. RESULTS AND DISCUSSION

2.1. Chemistry. 7-Chloro-6-formyl-1,2,4-triazolo[1,5-*a*]-pyridine derivative **1**[29] served as a starting point for studying all the reactions leading to the construction of novel linearly tricyclic systems incorporating pyrrole, isoxazole, and pyrazole nucleus in addition to the triazolopyridine moiety as illustrated in Schemes 1−3. Scheme 1, shows the first synthesis of the

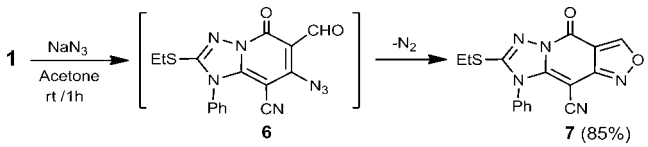
Scheme 1



hitherto unknown ethyl 9-cyano-2-ethylthio-5-oxo-1-phenyl-5,8-dihydro-1*H*-pyrrolo[2,3-*d*]-1,2,4-triazolo[1,5-*a*]pyridine-7-carboxylate (**5**) in which 7-chloro-6-ethoxycarbonylvinyl-1,2,4-triazolo[1,5-*a*]pyridines **3** was prepared, as described previously by Mekheimer et al.,[29] by treatment of compound **1** with an equimolar amount of the stabilized yield **2** in $CHCl_3$ at room temperature. Azidation of **3** with sodium azide in DMF at room temperature gave the corresponding 7-azido-triazolopyridine derivative **4**. Ring closure to the hitherto unknown ethyl 9-cyano-2-ethylthio-5-oxo-1-phenyl-5,8-dihydro-1*H*-pyrrolo-[2,3-*d*]-1,2,4-triazolo[1,5-*a*]pyridine-7-carboxylate (**5**) could be achieved by thermolysis of the azide **4** in refluxing bromobenzene for one hour, resulting in loss of nitrogen and subsequent intramolecular cyclization (the reaction may be monitored by the evolution of nitrogen or by disappearance of the intense azide absorption at 2130 cm^{-1} in the IR spectrum). The structure of **5** is supported by analytical and spectral data. Thus, the IR spectrum of **5** revealed the absence of the azido group and the presence of absorption band at 3210 cm^{-1}, attributed to the NH group. While in the ^1H NMR spectrum, the pyrrole NH proton resonates as a singlet at δ = 12.65 ppm. The signal attributable to the H-6 aromatic proton of the pyrrole ring is found at δ = 7.33 ppm as singlet besides the set of signals due to phenyl and ethyl protons. Additionally, its structure was fully supported by ^{13}C NMR, which was compatible with the assigned structure. Furthermore, in its mass spectrum, this product has the molecular ion *m*/*z* 407 (M^+, 88) and other peaks (see Experimental Section), confirming its presumed structure. Analytical data are in accordance with the proposed structure for compound **5**. A mechanism for the transformation of **4** into **5** could involve a well-known cyclization of a singlet nitrene.[30,31]

Next, we have examined the azidation of the chloroaldehyde **1** with sodium azide with a view to synthesize the interesting isoxazolotriazolopyridine **7** as a novel tricyclic system. Thus, when compound **1** was reacted with sodium azide in acetone for one hour at room temperature, the new 7-ethylthio-4-oxo-8-phenyl-4,8-dihydro-isoxazolo[3,4-*d*]-1,2,4-triazolo[1,5-*a*]-pyridine-9-carbonitrile (**7**) was directly obtained without isolation of the azide intermediate **6** (see Scheme 2). To our

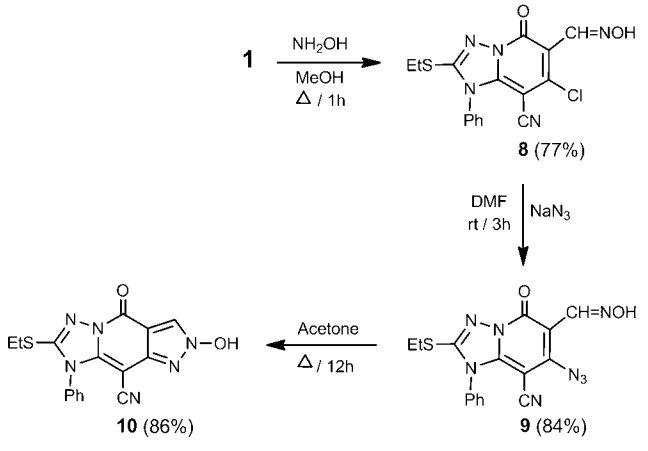
Scheme 2



knowledge, compound **7** is the first example of the linear isoxazolotriazolopyridine ring system.

Attention was next turned to synthesize the azido compound **9** possessing the azomethine moiety, as another cyclization partner, at the 6-position, which was expected to rearrange with loss of nitrogen to the novel pyrazolotriazolopyridines **10**. Thus, the chloroaldehyde **1** was reacted with hydroxylamine in MeOH at reflux temperature for one hour to generate the oxime **8**. The 7-azido-triazolopyridine **9** was readily formed from the 7-chloro derivative **8** by displacement with azide ion (Scheme 3). The azide

Scheme 3



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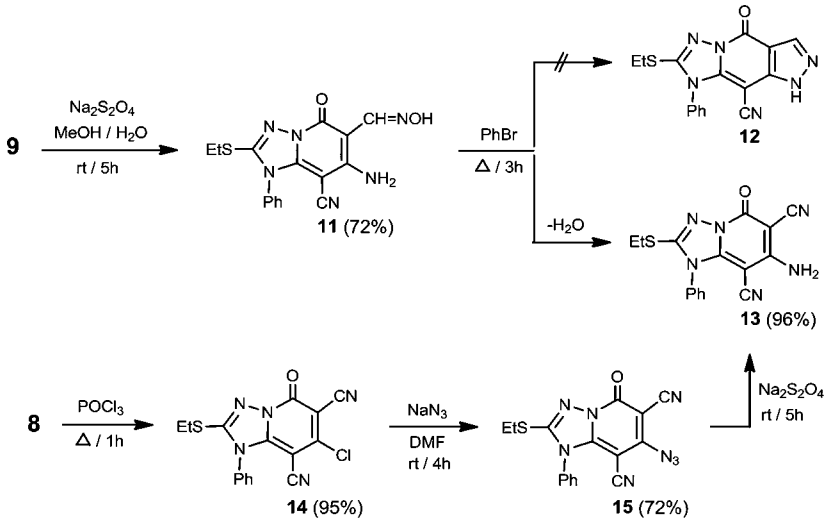
Scheme 4

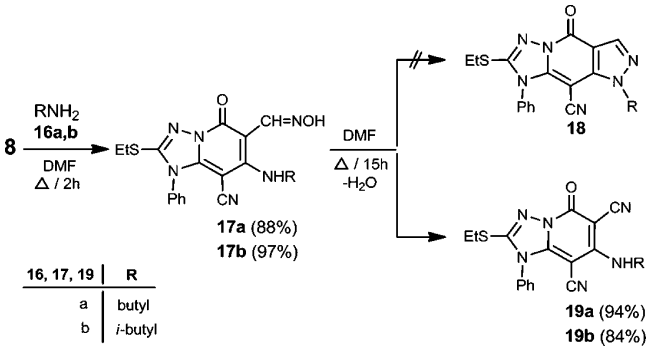


9 underwent smooth thermal decomposition in refluxing acetone to afford the novel 7-ethylhio-2-hydroxy-4-oxo-8-phenyl-4,8-dihydro-2*H*-pyrazolo[3,4-*d*]-1,2,4-triazolo[1,5-*a*]pyridine-9-carbonitrile (**10**) in good yield. A mechanism for the formation of the isoxazolotriazolopyridines **7** and pyrazolotriazolopyridines **10** could be explained in the terms of neighboring group attack on the azido moiety with concurrent loss of nitrogen without transient formation of the nitrene, as previously reported.[31−34]

To construct new derivatives of the interesting tricyclic systems of type **10**, we attempted thermal intramolecular cyclization of the amino compound **11**, utilizing the azido compound **9** as a good starting material for this purpose. Reduction of the azide moiety **9** with $Na_2S_2O_4$ gave the corresponding 7-amino-1,2,4-triazolo[1,5-*a*]pyridines **11**. We envisaged that the thermal cyclization of **11** in bromobenzene might afford the hitherto unknown tricyclic pyrazolotriazolopyridines **12**. Interestingly, however, an unexpected new compound, 7-amino-1,2,4-triazolo-[1,5-*a*]pyridine-6,8-dicarbonitriles **13**, was obtained (Scheme 4). Variation of solvents, temperature, and reaction time gave no the desire pyrazolotriazolopyridine derivative **12**. To obtain unequivocal evidence for the structure, compound **13** could also be obtained by an alternative route: dehydration of oxime **8** with $POCl_3$ afforded the corresponding 7-chloro-1,2,4-triazolo[1,5-*a*]pyridine-6,8-dicarbonitriles **14**. Reacting chloro compound **14** with excess of sodium azide in DMF at room temperature gave the azido compound **15**. Reduction of **15** with $Na_2S_2O_4$ in MeOH/H_2O mixture at room temperature afforded the 7-amino-1,2,4-triazolo[1,5-*a*]pyridine derivative **13** (Scheme 4).

In an attempt to synthesize new derivatives of the interesting pyrazolotriazolopyridine of type **18**, we further examined the thermal cyclization of the oximes **17a,b**, which prepared, as depicted in Scheme 5, by refluxing chloro compound **8** with an excess of alkylamines **16a,b** in DMF for 2 h. We first tried the cyclization of **17a,b** under various conditions for extended periods. However, when only starting material was recovered, we attempted the cyclization in a more basic and higher boiling point solvent such as DMF. However, the thermal cyclization of **17a,b** in boiling DMF for 15 h also failed to yield the tricyclic compound **18**, instead forming 7-alkyl-amino-1,2,4-triazolo[1,5-*a*]pyridines **19a,b** as a new derivative of 1,2,4-triazolo[1,5-*a*]-pyridine (Scheme 5).

Scheme 5



2.2. Biological Activity. To study the effect of the compounds **5**, **8**, **13**, and **17b** on the lifespan of wild type *C. elegans*, N2 worms were cultivated on agar plates under standard laboratory conditions at 20 °C either in the absence (control) or presence of different concentrations of these compounds (500 nM, 50 nM and 1 μM). A significantly prolonged lifespan of wild-type *C. elegans* under standard laboratory conditions in a dose-dependent manner was obtained with compound **13** (P = 0.012 for 1 μM, 0.034 for 500 nM, and 0.065 for 50 nM) as indicated in Figure 1. Compounds **5**, **8**, and **17b** do not affect the lifespan of the wild-type animals under standard laboratory conditions (data not shown). The life span extending effect of all concentrations of compound **13** was still lower than the effect of proanthocyanidin.

In *C. elegans*, there is a good correlation between stress resistance and lifespan because most genetic mutants with a longevity phenotype have increased resistance to a variety of acute stressors like oxidative stress[35] and heat.[36] For this reason, acute stressors have been successfully used as surrogates to identify new gerontogenes in genetic screens[37] and it was discussed whether such a surrogate assay might also be helpful in identifying compounds that affect lifespan.[38]

One possible explanation for the beneficial effects of 1,2,4-triazolo[1,5-*a*]pyridine compounds on aging in *C. elegans* is that these compounds may able to increase cellular stress resistance. To test this possibility, we examined stress resistance of **5**, **8**, **13** and **17b** treated animals. Resistance to oxidative stress was examined by exposing animals to paraquat, an intracellular

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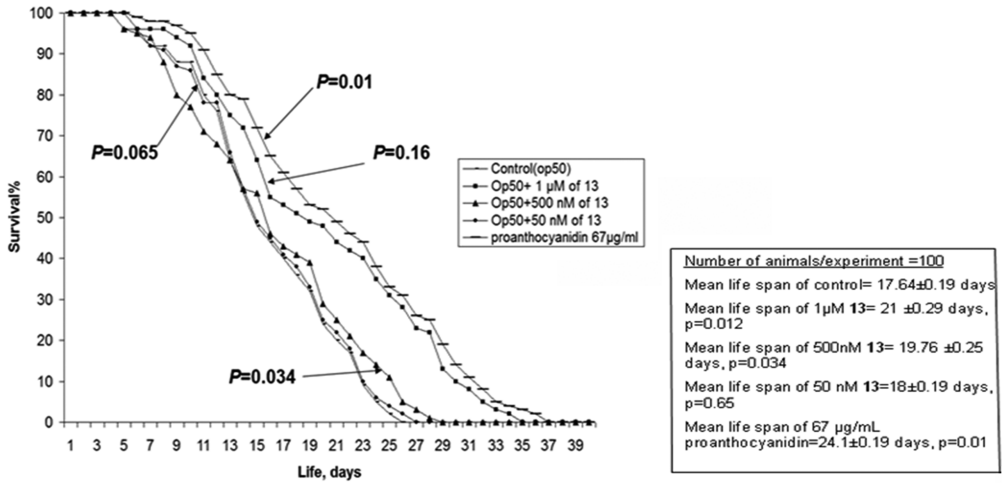

Figure 1. Survival of *C. elegans* on agar cultures of N2 worms in absence of treatment (control) and in presence of the indicated concentrations of **13** or 67 μg/mL proanthocycanidin. The curves represent the means of the survival curves from six individual experiments. Values represent the mean from three independent experiments, each including 100 worms.

free-radical-generating compound. Compound **13** treatment significantly increased survival time in the presence of paraquat (P = 0.011 for 1 μM, 0.04 for 500 nM, and 0.057 for 50 nM) as indicated in Figure 2. Compounds **5**, **8**, and **17b** have no effect on the paraquat induced oxidative stress.

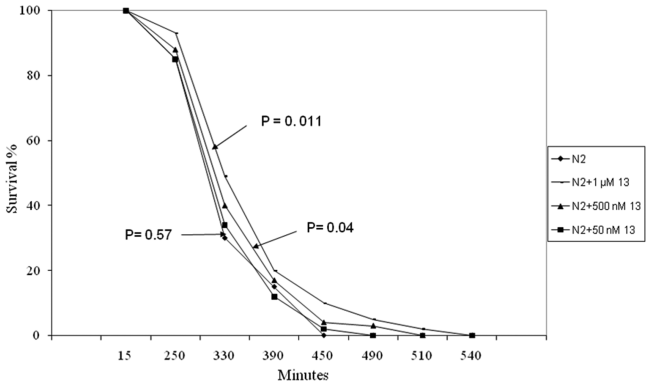

Figure 2. Kaplan–Meier curve. Treatment with **13** significantly improved survival rates in the presence of 40 μM paraquat of seven-day-old N2 worms. Worms were either left untreated (control) or treated with different concentrations of **13**. Three independent experiments with an average of 30 individual animals (7 days old) were used per experiment.

In addition, thermotolerance was increased significantly (P < 0.01) as a result of compound **13** treatment in a dose-dependent manner. In wild-type animals, treatment with **13** was correlated with a 1.4, 1.8, 2, and 2.5-fold increase in 16 h survival at 35 °C, respectively, as shown in Figure 3.

In this study, we found such a correlation between stress resistance and lifespan because compound **13** conferred an increased resistance in a surrogate assay using heat and paraquat as well as an extension of lifespan. In our hands, heat and paraquat were the suited surrogate because the worms died within a few hours under conditions of an acute lethal thermal and oxidative stress and the bacteria acting as a source of food were not affected by both. The thermal and paraquat stress caused an increase of ROS accumulation in the worms and therefore it is likely that the death of the worms was at least partially due to oxidative stress.

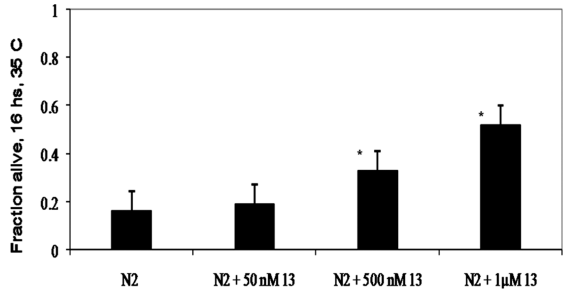

Figure 3. Average survival rates of *C. elegans*. Treatment with **13** improved thermotolerance. Fractional survival at 35 °C for seven-day-old N2 worms or worms treated with indicated treatments (500 nM, 50 nM, and 1 μM) of **13**. This is the average of four independent experiment with an average of 30 individual animals (7 days old) used per experiment. Error bars are the standard error of the mean. *Denotes a significant difference from N2 (Control) strain. p < 0.05.

Oxidative stress results in the formation of reactive oxygen species (ROS). ROS have the ability to react with proteins and macromolecules leading to formation of advanced glycation end products (AGEs), e.g., carboxymethyllysine (CML). CML plays a major role in decreasing lifespan of *C. elegans*.[4,39] To test the degree of CML formation in worms cultivated under standard laboratory conditions at 20 °C in the absence (control) and presence of different concentrations of compound **13**, CML concentration was determined using ELISA. The data in Figure 4 showed that treatment of worms with compound **13** significantly decreased CML formation in wild-type animals in a dose-dependent manner (P < 0.05).

Lipid peroxidation has been and remains one of the most widely used indicators of oxidant/free radical formation in vitro and in vivo. Reactive oxygen species (ROS) play a central role in the formation of active aldehydes, e.g., glyoxal, methyl glyoxal, and 3-deoxyglucosone. The role of **13** attenuating lipid peroxidation was tested by measuring the concentration of malondialdehyde (MDA) in the control and **13**-treated animals. Compound **13** was found to significantly attenuate MDA formation in treated animals. The effect was also dose dependent, as shown in Figure 5.

In the present study, we found that different concentrations of compound **13** significantly extended lifespan of *C. elegans*

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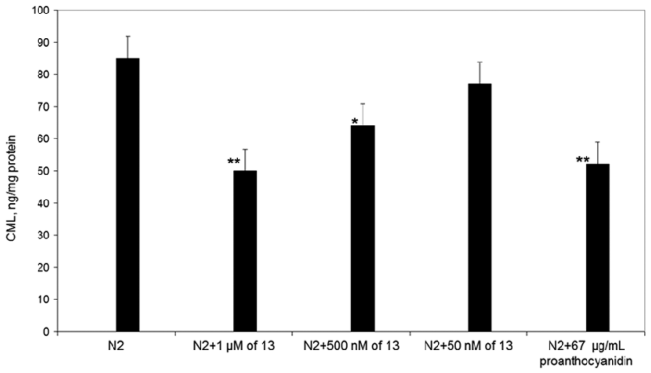

Figure 4. CML ELISA for seven-day-old worms in absence of treatment (control) or in presence of different concentration of **13** and 67 μg/mL proanthocyanidin as indicatred. **13** significantly attenuated CML formation in a dose-dependent manner. Vertical bars represent SEM from three independent experiments. *P < 0.05, **P = 0.01.

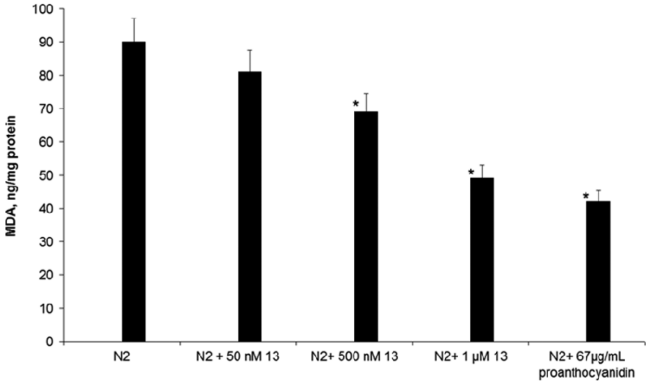

Figure 5. MDA contents in the seven-day-old worms in absence of treatment (control) or in presence of 500 nM, 50 nM, and 1 μM of **13** and 67 μg/mL proanthocyanidin. Vertical bars represent SEM from five replicate experiments. *Denotes a significant difference from N2/control strain (P < 0.05).

and increased their stress resistance against both heat and paraquate induced stress under standard culture conditions. These effects were found to be dose-dependent as shown in Figures 1, 2, and 3.

Compound **13** was also found to significantly modulate the formation of AGEs, in particular CML in the wild-type animals under standard laboratory conditions at 20 °C in a dose-dependent manner as shown in Figure 5. These observations indicated that glycosylation of proteins in the treated animals was significantly attenuated as a result of compound **13** supplementation.

In all trials, the effect of all concentrations of compound **13** was found to be lower than the effect of positive control proanthocyanidin.

Attenuation of AGE formation resulted in lifespan extension and improved stress resistance of the wild-type *C. elegans* under standard laboratory conditions. The obtained data were in line with previous work.[4,5,7−12,40,41] This suggests an antioxidant power of **13** or its ability to activate the antioxidant enzymes of the animals. This was also supported with the obtained data from lipid peroxidation. Compound **13** has the ability to attenuate lipid peroxidation resulting in the obtained concentration of MDA. Attenuation of lipid peroxidation results in attenuation of AGE formation and reduces oxidative stress.

Further studies must show which is the exact mechanism leading to the effects described above. Several pathways must be taken into account, including enhanced expression of antioxidant genes like superoxide dismutase, catalase, and others. It is also thinkable that compound **13** influences mitochondrial free radical generation by stabilization of the complex or reduced AGE modification, to which our data point.

Yet what other pathways might be influenced we do not know because these data are preliminary. The importance of these effects on mammals could not be judged yet, but many pathways in *C. elegans* are very similar to mammal and human systems.

Proanthocyanidin was selected as a positive control in this study because of its well-known antioxidant effect.

3. CONCLUSION

In conclusion, we have developed for the first time an efficient synthesis of previously unreported 1,2,4-triazolo[1,5-*a*]pyridine derivatives and some of their fused linear tricyclic ring systems and studied their biological activity. Compound **13** was found to extend lifespan of wild-type *C. elegans* under standard laboratory conditions. Lifespan extension of compound **13** in the nematode *C. elegans* might be attributed to its direct ROS scavenging activity and indirect free radical-scavenging activity through up-regulating stress-resistance-associated genes such as SOD, *hsp*, *daf 2*, *clk*, *mev*, and *skn-1*. These interesting findings highlight the therapeutic efficacy of compound **13** as an antioxidant and the possibility of its use as an antioxidant drug. Further studies using different mutants of *C. elegans* are required to examine the exact mechanism of compound **13** action and the optimal dose required.

4. EXPERIMENTAL SECTION

4.1. Chemistry. Melting points were determined on a Gallenkamp melting point apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on Bruker-DPX-300 (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR) and Bruker ARX500 (500.1 MHz for ^{1}H NMR and 125.8 MHz for ^{13}C NMR) spectrometers with DMSO-d_6 as solvent, and the chemical shifts were expressed in δ (ppm) values with trimethylsilane as an internal reference (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad peak). IR spectra were recorded in KBr disks using a Schimadzu 470 spectrophotometer. Mass spectra were measured on a Shimadzu GCMS-QP2010 mass spectrometer. Microanalyses were performed at the Microanalytical Data Unit, Cairo University, and results were within ±0.4% of the calculated values. Both the analytical and spectroscopic data confirmed ≥95% purity of the all prepared compounds.

*7-Azido-6-ethoxycarbonylvinyl-2-ethylthio-1,5-dihydro-5-oxo-1-phenyl-1,2,4-triazol[1,5-a]pyridine-8-carbonitrile (**4**).* Sodium azide (0.060 g, 0.932 mmol) was added to a solution of **3** (0.20 g, 0.466 mmol) in DMF (5 mL), and the mixture was stirred for 3 h at room temperature (25 °C). Then the reaction mixture was poured into H_2O and the precipitated solid product was collected by filtration, washed well with H_2O, dried, and recrystallized from $CHCl_3$ to give compound **4** (0.190 g, 94%) as yellowish crystals: mp 164−166 °C (dec.). ^{1}H NMR (DMSO-d_6) δ 1.25 (t, 3H, J = 7.2 Hz, CH_3), 1.41 (t, 3H, J = 7.2 Hz, CH_3), 3.30 (q, 2H, J = 7.2 Hz, CH_2), 4.17 (q, 2H, J = 7 Hz, CH_2), 7.15 (d, 1H, J = 16 Hz, CH), 7.69 (m, 5H$_{arom}$), 7.82 (d, 1H, J = 16 Hz, CH). ^{13}C NMR (DMSO-d_6) δ 14.1 (2CH_3), 26.3 (CH_2), 59.8 (CH_2), 67.0 (C-8), 103.4 (C_β), 110.2 (CN), 117.9 (C-6), 128.7, 129.8, 130.4, 132.0 (Ar−C), 134.8 (C_α), 145.8 (C-7), 147.9 (C-2), 152.6 (C-8a), 155.7 (CO, amide), 167.2 (CO, ester). IR (KBr) ν 3100 (arom CH), 2950 (aliph CH), 2220 (CN), 2130 (N_3), 1710 (CO, ester), 1680 (CO, amide) cm^{-1}. MS: m/z (%) = 407 ($M^+ - N_2$, 92). Anal. Calcd for $C_{20}H_{17}N_7O_3S$ (435.46): C, 55.16; H, 3.93; N, 22.52; S, 7.36. Found: C, 55.33; H, 4.12; N, 22.67; S, 7.19.

*Ethyl 9-Cyano-2-ethylthio-5-oxo-1-phenyl-5,8-dihydro-1H-pyrrolo[2,3-d]-1,2,4-triazolo[1,5-a]pyridine-7-carboxylate (**5**).* A solution of azide compound **4** (0.10 g, 0.229 mmol) in bromobenzene (5 mL)

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was refluxed for one hour. After concentration and cooling to room temperature, a small amount of EtOH was added. The resulting solid product was collected by filtration, dried, and recrystallized from EtOH to give compound **5** (0.085 g, 90%) as buff crystals: mp 316−318 °C. ^{1}H NMR (DMSO-d_6) δ 1.32 (t, 3H, J = 7 Hz, CH_3), 1.40 (t, 3H, J = 7 Hz, CH_3), 3.26 (q, 2H, J = 7 Hz, CH_2), 4.28 (q, 2H, J = 7 Hz, CH_2), 7.33 (s, 1H, pyrrole CH), 7.62−7.72 (m, 5H$_{arom}$), 12.65 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ 14.2 (CH_3), 14.2 (CH_3) 26.0 (CH_2), 56.6 (C-9), 60.2 (CH_2), 108.5 (C-5a), 111.8 (C-6), 112.0 (CN), 125.8 (C-7), 129.0, 129.7, 130.8, 131.5 (Ar−C), 139.3 (C-8a), 146.5 (C-2), 151.0 (CO), 153.8 (C-9a), 159.7 (CO). IR (KBr) ν 3210 (NH), 2965 (aliph CH), 2210 (CN), 1700 (CO, ester), 1670 (CO, amide) cm^{-1}. MS: m/z (%) = 408 (M^+ + 1, 20). Anal. Calcd for $C_{20}H_{17}N_5O_3S$ (407.45): C, 58.96; H, 4.21; N, 17.19; S, 7.87. Found: C, 59.07; H, 4.09; N, 17.28; S, 7.96.

*7-Ethylthio-4-oxo-8-phenyl-4,8-dihydro-isoxazolo[3,4-d]-1,2,4-triazolo[1,5-a]pyridine-9-carbonitrile (**7**).* Sodium azide (0.037 g, 0.569 mmol) was added to a solution of compound **1** (0.20 g, 0.557 mmol) in acetone (10 mL), and the reaction mixture was stirred for one hour at room temperature (25 °C). Then it was poured into H_2O, and the precipitated solid product was collected by filtration, washed well with H_2O, dried, and recrystallized from EtOH to give compound **7** (0.160 g, 85%) as yellow crystals: mp 245−246 °C (dec.). ^{1}H NMR (DMSO-d_6) δ 1.40 (t, 3H, J = 7.2 Hz, CH_3), 3.26 (q, 2H, J = 7.2 Hz, CH_2), 7.61−7.70 (m, 5H$_{arom}$), 10.26 (s, 1H, isoxazole CH). ^{13}C NMR (DMSO-d_6) δ 14.1 (CH_3), 26.0 (CH_2), 51.5 (C-9), 109.4 (C-3a), 111.8 (CN), 128.8, 129.9, 130.3, 131.8 (Ar−C), 149.9 (C-7a), 150.3 (C-9a), 155.3 (CO), 155.5 (C-8a), 164.8 (C-3). IR(KBr) ν 3100 (arom CH), 2928 (aliph CH), 2224 (CN), 1710 (CO) cm^{-1}. MS: m/z (%) = 338 (M^+ + 1, 18). Anal. Calcd for $C_{16}H_{11}N_5O_2S$ (337.36): C, 56.96; H, 3.29; N, 20.76; S, 9.50. Found: C, 56.83; H, 3.40; N, 20.89; S, 9.39.

*7-Chloro-2-ethylthio-6-[(hydroxyimino)methyl]-5-oxo-1-phenyl-1,5-dihydro-1,2,4-triazolo[1,5-a]pyridine-8-carbonitrile (**8**).* A mixture of compound **1** (0.10 g, 0.279 mmol) and hydroxylamine hydrochloride (0.038 g, 0.547 mmol) in MeOH (5 mL) was refluxed for one hour, during which a yellow solid product separated out. After cooling to room temperature, the resulting solid product was collected by filtration, washed with MeOH, dried, and recrystallized from DMF to give compound **8** (0.080 g, 77%) as yellow crystals: mp 271−272 °C. ^{1}H NMR (DMSO-d_6) δ 1.4 (t, 3H, J = 7 Hz, CH_3), 3.30 (q, 2H, J = 7 Hz, CH_2), 7.64−7.72 (m, 5H$_{arom}$), 8.17 (s, 1H, CH), 11.52 (s, 1H, OH). ^{13}C NMR (DMSO-d$_6$) δ 14.1 (CH_3), 26.2 (CH_2), 72.1 (C-8), 109.0 (C-6), 111.7 (CN), 128.7, 129.8, 130.0, 131.9 (Ar−C), 142.4 (C-2), 142.7 (oxime-carbon), 145.7 (C-7), 151.7 (C-8a), 155.7 (CO). IR (KBr) ν 3296 (OH), 3050 (arom CH), 2944 (aliph CH), 2220 (CN), 1692 (CO) cm^{-1}. MS: m/z (%) = 375 (M^+, 16). Anal. Calcd for $C_{16}H_{12}ClN_5O_2S$ (373.82): C, 51.41; H, 3.24; Cl, 9.48; N, 18.73; S, 8.58. Found: C, 51.53; H, 3.13; Cl, 9.64; N, 18.61; S, 8.72.

*7-Azido-2-ethylthio-6-[(hydroxyimino)methyl]-5-oxo-1-phenyl-1,5-dihydro-1,2,4-triazolo[1,5-a]pyridine-8-carbonitrile (**9**).* To a solution of compound **8** (0.20 g, 0.535 mmol) in DMF (5 mL), NaN_3 (0.07 g, 1.07 mmol) was added. The reaction mixture was stirred for 3 h at room temperature (25 °C), and then it was poured into H_2O. The precipitated solid product was collected by filtration, washed well with H_2O, dried, and recrystallized from $CHCl_3$ to give compound **9** (0.170 g, 84%) as colorless crystals: mp 162−164 °C (dec.). ^{1}H NMR (DMSO-d_6) δ 1.38 (t, 3H, J = 7 Hz, CH_3), 3.27 (q, 2H, J = 7 Hz, CH_2), 7.67 (m, 5H$_{arom}$), 8.18 (s, 1H, CH), 11.51 (s, 1H, OH). ^{13}C NMR (DMSO-d_6) δ 14.3 (CH_3), 26.3 (CH_2), 66.7 (C-8), 103.1 (C-6), 111.3 (CN), 128.8, 129.9, 130.1, 132.0 (Ar−C), 142.2 (C-2), 145.9 (oxime-carbon), 147.0 (C-7), 153.3 (C-8a), 155.7 (CO). IR (KBr) ν 3250 (OH), 2210 (CN), 2120 (N_3), 1670 (CO) cm^{-1}. MS: m/z (%) = 381 (M^+ + 1, 13). Anal. Calcd for $C_{16}H_{12}N_8O_2S$ (380.38): C, 50.52; H, 3.18; N, 29.46; S, 8.43. Found: C, 50.45; H, 3.27; N, 29.33; S, 8.57.

*7-Ethylthio-2-hydroxy-4-oxo-8-phenyl-4,8-dihydro-2H-pyrazolo-[3,4-d]-1,2,4-triazolo[1,5-a]pyridine-9-carbonitrile (**10**).* A solution of azide compound **9** (0.20 g, 0.526 mmol) in dry acetone (7 mL) was refluxed for 12 h. After concentration and cooling to room temperature, the reaction mixture was poured into H_2O. The obtained precipitate was collected by filtration, washed with H_2O, dried, and recrystallized from EtOH to give compound **10** (0.160 g, 86%) as yellow crystals: mp 242−243 °C. ^{1}H NMR (DMSO-d_6) δ 1.37 (t, 3H, J = 7 Hz, CH_3), 3.23 (q, 2H, J = 7 Hz, CH_2), 7.58−7.93 (m, 5H$_{arom}$), 8.68 (s, 1H, pyrazole CH), 13.81 (s, 1H, OH). ^{13}C NMR (DMSO-d_6) δ 15.0 (CH_3), 26.7 (CH_2), 56.9 (C-9), 105.0 (C-3a), 113.6 (CN), 122.5 (C-9a), 129.8, 130.5, 131.6, 132.2 (Ar−C), 144.4 (C-3), 147.9 (C-7), 154.5 (CO), 163.0 (C-8a). IR (KBr) ν 3550 (OH), 3100, 3056 (arom CH), 2928 (aliph CH), 2210 (CN), 1689 (CO) cm^{-1}. MS: m/z (%) = 353 (M^+ + 1, 21); Anal. Calcd for $C_{16}H_{12}N_6O_2S$ (352.37): C, 54.54; H, 3.43; N, 23.85; S, 9.10. Found: C, 54.67; H, 3.59; N, 23.72; S, 9.23.

*7-Amino-2-ethylthio-6-[(hydroxyimino)methyl]-5-oxo-1-phenyl-1,5-dihydro-1,2,4-triazolo[1,5-a]pyridine-8-carbonitrile (**11**).* Sodium dithionite (0.6 g, 3.45 mmol) was added portionwise to a stirred suspension of **9** (0.30 g, 0.788 mmol) in a 2:1 MeOH−H_2O (15 mL) mixture. Stirring was maintained at room temperature for 5 h; during this period of time, a solid product was formed. Then, the reaction mixture was poured into H_2O. The resulting solid product was collected by filtration, washed well with H_2O, dried, and recrystallized from $CHCl_3$ to give compound **11** (0.20 g, 72%) as yellowish crystals: mp 282−284 °C (dec.). ^{1}H NMR (DMSO-d_6) δ 1.37 (t, 3H, J = 7.2 Hz, CH_3), 3.28 (q, 2H, J = 7.2 Hz, CH_2), 7.65 (m, 7H, 5H$_{arom}$ + NH_2), 8.45 (s, 1H, CH), 10.71 (s, 1H, OH). ^{13}C NMR (DMSO-d_6) δ 14.2 (CH_3), 26.3 (CH_2), 73.2 (C-8), 102.1 (C-6), 111.4 (CN), 128.7, 129.7, 130.1, 131.9 (Ar−C), 142.2 (C-2), 142.6 (oxime-carbon), 148.7 (C-7), 151.4 (C-8a), 155.6 (CO). IR (KBr) ν 3392 (OH), 3289, 3184 (NH_2), 3054 (arom CH), 2912 (aliph CH), 2210 (CN), 1650 (CO) cm^{-1}. MS: m/z (%) = 355 (M^+ + 1, 29). Anal. Calcd for $C_{16}H_{14}N_6O_2S$ (354.39): C, 54.23; H, 3.98; N, 23.71; S, 9.05. Found: C, 54.39; H, 4.11; N, 23.62; S, 8.87.

*7-Amino-2-ethylthio-1,5-dihydro-5-oxo-1-phenyl-1,2,4-triazolo-[1,5-a]pyridine-6,8-dicarbonitrile (**13**). Route A.* A solution of compound **11** (0.110 g, 0.310 mmol) in bromobenzene (5 mL) was refluxed for 3 h. After cooling to room temperature, the mixture was evaporated to dryness in vacuum. The remaining oily residue was triturated with H_2O. The resulting solid product was collected by filtration, washed with H_2O, and dried.

Route B. To a stirred suspension of compound **15** (0.30 g, 0.789 mmol) in a methanol (10 mL)−water (5 mL) mixture, sodium dithionite (0.6 g, 3.45 mmol) was added portionwise. Stirring was maintained at room temperature for 1.5 h; during this period of time, a solid product was formed. Then, the reaction mixture was poured into H_2O. The resulting solid product was collected by filtration, washed well with H_2O, dried, and recrystallized from DMF to give compound **13** [0.10 g, 96% (route A); 0.240 g, 86% (route B)] as yellow crystals: mp 324−326 °C. ^{1}H NMR (DMSO-d_6) δ 1.35 (t, 3H, J = 7 Hz, CH_3), 3.20 (q, 2H, J = 7 Hz, CH_2), 7.25 (s, 2H, NH_2), 7.59−7.67 (m, 5H$_{arom}$). ^{13}C NMR (DMSO-d_6) δ 14.3 (CH_3), 26.2 (CH_2), 60.0 (C-6), 71.9 (C-8), 111.5 (CN), 116.0 (CN), 128.9, 129.8, 130.3, 131.9 (Ar−C), 148.0 (C-2), 153.9 (CO), 154.2 (C-8a), 159.0 (C-7). IR (KBr) ν 3360, 3230 (NH_2), 3050 (arom CH), 2900 (aliph CH), 2210 (CN), 1680 (CO) cm^{-1}. MS: m/z (%) = 337 (M^+ + 1, 30). Anal. Calcd for $C_{16}H_{12}N_6OS$ (336.37): C, 57.13; H, 3.60; N, 24.98; S, 9.53. Found: C, 57.29; H, 3.74; N, 24.79; S, 9.44.

*7-Chloro-2-ethylthio-1,5-dihydro-5-oxo-1-phenyl-1,2,4-triazolo-[1,5-a]pyridine-6,8-dicarbonitrile (**14**).* Compound **8** (0.20 g, 0.535 mmol) in $POCl_3$ (3 mL) was refluxed for one hour. The excess of $POCl_3$ was evaporated under reduced pressure. The remaining residue was triturated with cold H_2O. The resulting solid product was neutralized with a dilute KOH and collected by filtration, washed well with H_2O, dried, and recrystallized from EtOH to give compound **14** (0.180 g, 95%) as buff crystals: mp 261−263 °C. ^{1}H NMR (DMSO-d_6) δ 1.40 (t, 3H, J = 7.3 Hz, CH_3), 3.31 (q, 2H, J = 7.3 Hz, CH_2), 7.67−7.73 (m, 5H$_{arom}$). ^{13}C NMR (DMSO-d_6) δ 14.2 (CH_3), 26.5 (CH_2), 75.0 (C-8), 91.1 (C-6), 110.4 (CN), 114.2 (CN), 128.6, 129.5, 130.2, 132.5 (Ar−C), 147.4 (C-2), 150.4 (C-7), 151.8 (CO), 157.1 (C-8a). IR (KBr) ν 3050 (arom CH), 2980, 2930 (aliph CH), 2210 (CN), 1675 (CO) cm^{-1}. MS: m/z (%) = 357 (M^+, 30). Anal. Calcd for $C_{16}H_{10}ClN_5OS$ (355.80): C, 54.01; H,

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2.83; Cl, 9.96; N, 19.68; S, 9.01. Found: C, 53.93; H, 3.03; Cl, 9.85; N, 19.79; S, 8.92.

*7-Azido-2-ethylthio-1,5-dihydro-5-oxo-1-phenyl-1,2,4-triazolo-[1,5-a]pyridine-6,8-dicarbonitrile (**15**).* NaN_3 (0.055 g, 0.846 mmol) was added to a solution of chloro compound **14** (0.150 g, 0.422 mmol) in DMF (5 mL). The reaction mixture was stirred for 4 h at room temperature (25 °C), and then it was poured into H_2O. The precipitated solid product was collected by filtration, washed well with H_2O, dried, and recrystallized from MeOH to give compound **15** (0.110 g, 72%) as colorless crystals: mp 190−191 °C (dec.). ^{1}H NMR (DMSO-d_6) δ 1.40 (t, 3H, J = 7.16 Hz, CH_3), 3.30 (q, 2H, J = 7.28 Hz, CH_2), 7.68 (m, 5H$_{arom}$). ^{13}C NMR (DMSO-d_6) δ 14.1 (CH_3), 26.4 (CH_2), 67.4 (C-8), 82.4 (C-6), 109.4 (CN), 113.1 (CN), 128.5, 129.6, 129.9, 132.2 (Ar−C), 147.3 (C-2), 152.7 (C-7), 153.7 (CO), 156.5 (C-8a). IR (KBr) ν 3050 (arom CH), 2985, 2930 (aliph CH), 2130 (N_3), 2210 (CN), 1683 (CO) cm^{-1}. MS: m/z (%) = 362 (M^+, 9). Anal. Calcd for $C_{16}H_{10}N_8OS$ (362.37): C, 53.03; H, 2.78; N, 30.92; S, 8.85. Found: C, 53.24; H, 2.89; N, 31.05; S, 8.97.

*7-Alkylamino-2-ethylthio-6-[(hydroxyimino)-methyl]-5-oxo-1-phenyl-1,5-dihydro-1,2,4-triazolo[1,5-a]pyridine-8-carbonitriles **17a,b**: General Procedure.* A mixture of compound **8** (0.150 g, 0.40 mmol) and alkylamines **16a,b** (0.116 g, 1.6 mmol) in DMF (5 mL) was refluxed for 2 h until TLC showed the disappearance of the starting compounds. After cooling to room temperature, the mixture was evaporated to dryness in vacuum. The remaining oily residue was triturated with H_2O. The precipitated solid product was collected by filtration, washed with water, dried, and finally recrystallized from MeOH to give compounds **17a,b**.

*7-Butylamino-2-ethylthio-6-[(hydroxyimino)-methyl]-5-oxo-1-phenyl-1,5-dihydro-1,2,4-triazolo[1,5-a]pyridine-8-carbonitrile (**17a**).* Yield: 0.145 g (88%) as yellow crystals: mp 242−244 °C. ^{1}H NMR (DMSO-d_6) δ 0.85 (t, 3H, J = 7.3 Hz, CH_3), 1.29 (m, 2H, CH_2), 1.34 (t, 3H, J = 7.3 Hz, CH_3), 1.52 (m, 2H, CH_2), 3.20 (q, 2H, J = 7.3 Hz, CH_2), 3.52 (q, 2H, J = 7.3 Hz, CH_2), 7.56−7.67 (m, 5H$_{arom}$), 8.47 (s, 1H, CH═NOH), 9.06 (t, 1H, J = 5 Hz, NH), 10.78 (s, 1H, OH). ^{13}C NMR (DMSO-d_6) δ 13.6 (CH_3), 14.4 (CH_3), 19.3 (CH_2), 26.2 (CH_2), 31.8 (CH_2), 44.3 (CH_2), 58.1 (C-8), 90.2 (C-6), 114.4 (CN), 128.9, 129.6, 130.8, 131.5 (Ar−C), 147.3 (oxime-carbon), 147.8 (C-2), 153.6 (CO), 153.9 (C-8a), 154.2 (C-7). IR (KBr) ν 3400 (OH), 3200 (NH), 3056 (arom CH), 2960 (aliph CH), 2210 (CN), 1650 (CO) cm^{-1}. MS: m/z (%) = 411 (M^+ + 1, 12), 410 (M^+, 12). Anal. Calcd for $C_{20}H_{22}N_6O_2S$ (410.49): C, 58.52; H, 5.40; N, 20.47; S, 7.81. Found: C, 58.66; H, 5.29; N, 20.33; S, 7.94.

*7-Isobutylamino-2-ethylthio-6-[(hydroxyimino)methyl]-5-oxo-1-phenyl-1,5-dihydro-1,2,4-triazolo[1,5-a]pyridine-8-carbonitrile (**17b**).* Yield: (0.160 g, 97%) as yellow crystals: mp 222−224 °C. ^{1}H NMR (DMSO-d_6) δ 0.92 (d, 6H, J = 7 Hz, 2CH_3), 1.39 (t, 3H, J = 7 Hz, CH_3), 1.81 (m, 1H, H$_{aliph}$), 3.26 (q, 2H, J = 7 Hz, CH_2), 3.38 (t, 2H, J = 7 Hz, CH_2), 7.65 (m, 5H$_{arom}$), 8.54 (s, 1H, CH═NOH), 9.17 (t, 1H, J = 5 Hz, NH,), 10.82 (s, 1H, OH). ^{13}C NMR (DMSO-d_6) δ 14.2 (CH_3), 19.4 (2CH_3), 26.3 (CH_2), 28.8 (aliph CH), 52.8 (CH_2), 58.0 (C-8), 90.2 (C-6), 114.3 (CN), 128.9, 129.6, 130.7, 131.5 (Ar−C), 147.3 (oxime-carbon), 147.7 (C-2), 153.6 (CO), 154.0 (C-8a), 154.2 (C-7). IR (KBr) ν 3350 (OH), 3312 (NH), 3050 (arom CH), 2944, 2912, 2880 (aliph CH), 2210 (CN), 1650 (CO) cm^{-1}. Anal. Calcd for $C_{20}H_{22}N_6O_2S$ (410.49): C, 58.52; H, 5.40; N, 20.47; S, 7.81. Found: C, 58.46; H, 5.56; N, 20.62; S, 7.64.

*7-Alkylamino-2-ethylthio-5-oxo-1-phenyl-1,5-dihydro-1,2,4-triazolo[1,5-a]pyridine-6,8-dicarbonitriles **19a,b**: General Procedure.* A solution of compounds **17a,b** (0.20 g, 0.487 mmol) in DMF (5 mL) was refluxed for 15 h (TLC control). After concentration and cooling to room temperature, H_2O was added and the resulting solid product was collected by filtration, washed with H_2O, dried, and recrystallized from MeOH to give compounds **19a,b**.

*7-Butylamino-2-ethylthio-5-oxo-1-phenyl-1,5-dihydro-1,2,4-triazolo[1,5-a]pyridine-6,8-dicarbonitrile (**19a**).* Yield: (0.180 g, 94%) as brownish crystals: mp 244−246 °C. ^{1}H NMR (DMSO-d_6) δ 0.87 (t, 3H, J = 7.3 Hz, CH_3), 1.28 (m, 2H, CH_2), 1.35 (t, 3H, J = 7.3 Hz, CH_3), 1.55 (m, 2H, CH_2), 3.23 (q, 2H, J = 7.3 Hz, CH_2,), 3.57 (q, 2H, J = 7.3 Hz, CH_2), 7.11 (t, 1H, J = 5 Hz, NH), 7.68 (m, 5H$_{arom}$). ^{13}C NMR (DMSO-d_6) δ 13.4 (CH_3), 14.2 (CH_3), 18.1 (CH_2), 26.2 (CH_2), 30.6 (CH_2), 42.8 (CH_2), 59.8 (C-6), 71.8 (C-8), 111.4 (CN), 115.9 (CN), 128.8, 129.7, 130.2, 131.8 (Ar−C), 147.9 (C-2), 153.8 (CO), 154.1 (C-8a), 158.9 (C-7). IR (KBr) ν 3312 (NH), 3050 (arom CH), 2960, 2928, 2864 (aliph CH), 2210 (CN), 1654 (CO) cm^{-1}. MS: m/z (%) = 393 (M^+ + 1, 17), 392 (M^+, 64). Anal. Calcd for $C_{20}H_{20}N_6OS$ (392.48): C, 61.20; H, 5.14; N, 21.41; S, 8.17. Found: C, 61.13; H, 5.31; N, 21.51; S, 8.29.

*7-Isobutylamino-2-ethylthio-5-oxo-1-phenyl-1,5-dihydro-1,2,4-triazolo[1,5-a]pyridine-6,8-dicarbonitrile (**19b**).* Yield: (0.160 g, 84%) as brownish crystals: mp 286−287 °C. ^{1}H NMR (DMSO-d_6) δ 0.87 (d, 6H, J = 7 Hz, 2CH_3), 1.38 (t, 3H, J = 7 Hz, CH_3), 1.92 (m, 1H, H$_{aliph}$), 3.23 (q, 2H, J = 7 Hz, CH_2), 3.40 (t, 2H, J = 7 Hz, CH_2), 7.13 (t, 1H, J = 5 Hz, NH), 7.68 (m, 5H $_{arom}$). ^{13}C NMR (DMSO-d_6) δ 14.2 (CH_3), 19.5 (2CH_3), 26.2 (CH_2), 28.8 (aliph CH), 52.7 (CH_2), 59.8 (C-6), 71.8 (C-8), 111.4 (CN), 115.8 (CN), 128.8, 129.8, 130.2, 131.8 (Ar−C), 147.9 (C-2), 153.8 (CO), 154.0 (C-8a), 158.9 (C-7). IR (KBr) ν 3328 (NH), 3050 (arom CH), 2944 (aliph CH), 2210 (CN), 1664 (CO) cm^{-1}. MS: m/z (%) = 393 (M^+ + 1, 80), 392 (M^+, 71); Anal. Calcd for $C_{20}H_{20}N_6OS$ (392.48): C, 61.20; H, 5.14; N, 21.41; S, 8.17. Found: C, 61.34; H, 5.05; N, 21.58; S, 8.02.

4.2. Biology. *Caenorhabditis elegans Strains Used, Maintenance and Culture Conditions.* Strains were maintained and manipulated under standard conditions.[42,43] The strain used in this study was Bristol N2 (wild type). To obtain age-synchronized nematodes, self-fertilizing hermaphrodites (3 days old) were left to lay eggs and afterward removed to set the eggs in synchrony. After hatching and reaching adulthood, adult worms were transferred to new plates every day during the egg-laying period to obtain large amounts of age-synchronous worms and young adult worms were transferred to NGM agar plates containing 40 μM 5-fluorodesoxyuridine (Sigma-Aldrich, Germany)[42] to prevent eggs from hatching.[39]

Lifespan Assay. The lifespan assay was conducted at 20 °C as described previously.[43,44] The worms were observed and counted daily in the absence (control) or presence of different concentrations of compounds **5**, **8**, **13**, and **17b** (50 nM, 500 nM, 5 μM) and 67 μg/mL of proanthocyanidin (as a positive control antioxidant) to determine whether they were dead or alive. They were prodded gently with the tip of a platinum thin wire, and when they no longer responded, they were considered to be dead. Dead worms were removed from the plates. The number of worms for each analysis was approximately 100. For all lifespans, experiments and assays were repeated at least three times.

Thermotolerance Assay. Thermotolerance assay was performed with hermaphrodites on adult day 7, after the majority of egg-laying had ceased. Animals were transferred onto 3 cm NGM agar plates supplemented as indicated and then incubated at 35 °C for 16 h[45] for N2 strain in the presence and absence of different concentrations of compounds **5**, **8**, **13**, and **17b**.

Survival was scored as the number of animals responsive to gentle touch as a fraction of the original number of animals on the plate. Animals that had died from desiccation on the sides of the plate were censored.

Oxidative Stress Resistance Assay. Paraquat assay was performed with 30 worms per trial at 20 °C. To assay paraquat sensitivity, seven-day-old adult worms[46] were placed overnight on agar plates containing 40 μM 5′-fluorodeoxyuridine (FUDR), and 40 mM paraquat[39] in the presence and absence of different concentrations of compounds **5**, **8**, **13**, and **17b**. Viability was assayed over a 20 h period.

Isolation of C. elegans Protein Extracts. A large number of worms was incubated with different concentrations compounds **5**, **8**, **13**, and **17b** and 67 μg/mL proanthocyanidin or without treatment (control) for 7 days in the presence of 40 μM FUDR to prevent production of progeny. Subsequent to the incubation times, worms were harvested from plates using M9 buffer and washed free of bacteria by sucrose flotation and suspended in a 2-fold volume of homogenization buffer (0.05 M Tris-HCl, pH 7.9, 25% glycerol, 0.1 mM EDTA, 0.32 M NH_4SO_4) containing a protease inhibitor tablet (Roche, Germany). The lysates were homogenized on ice using Polytron homogenizer. The homogenates were centrifuged at 12000 rpm for 5 min and the supernatants assayed for protein concentration (Pierce, Rockford, USA).

ELISA of N-ε-(Carboxymethyl)lysine-modified proteins. *N*-ε-(Carboxymethyl)lysine (CML) concentrations were determined by a competitive enzyme-linked immunosorbent assay (Roche Diagnostics, Germany) using streptavidin-coated microtiter plates as described.[40,47] Biotinylated bovine serum albumin, which was glycated with glucose over 3 weeks, bound to a mouse monoclonal antibody specific for the CML epitope, which was labeled with horseradish peroxidase. ABTS: 2,2-azino-di-[3-ethylbenzthiazolinesulfonate]diammonium salt (ABTS) plus sodium perborate served as a substrate for the indicator enzyme. ABTS is a trademark of a member of the Roche group. CML-modified proteins from the sample compete for the antibody binding site. As a standard, the monomeric epitope *N*-carboxy-methylamino-caproate was used, yielding results as ng of CML. The limit of detection was 5 mg CML/mL.

Lipid Peroxidation. Lipid peroxidation was determined by measuring malondialdehyde (MDA), an index of lipid peroxidation according to the method used in previous work[4,5,11] using 1,1′,3,3′-tetramethoxypropane as standard. In brief, 8.1% SDS was added to the worm lysate (prepared as described above) and incubated for 10 min at room temperature, followed by boiling with 20% acetic acid and 0.6% thiobarbituric acid for 60 min in a water bath. On cooling, a mixture of butanol:pyridine (15:1 v/v) was added and centrifuged at 1200 rpm for 5 min. Absorbance of the upper colored layer was measured at 532 nm, and the concentration of MDA was expressed in terms of nmol/g protein. Butylhydroxy toluene (0.01%) was added to each assay mixture in order to prevent undesirable auto-oxidation of the sample during the assay.

Statistical Analysis. Statistical analysis was performed using SPSS software for calculation of Kaplan−Meier survival curves and comparison of lifespan. *P*-Value calculations comparing different lifespans were made using the Kruskal−Wallis test. Mean and maximum lifespan values and Kaplan−Meier curves were the result of data pulled from three different experiments performed independently at different time-points. Individual differences between the various treatments were then identified using Dunn's test (post hoc test). A value of $P < 0.05$ was considered as statistically significant.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ROS; reactive oxygen species; AGE; advanced glycation end products; *C. elegans*; *Caenorhabditis elegans*

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