Neuroprotective metabolites via fungal biotransformation of a novel sapogenin, cyclocephagenol

Melis Küçüksolak¹, Göklem Üner¹, Petek Ballar Kırımlıbayrak² & Erdal Bedir¹

Cyclocephagenol (1), a novel cycloartane-type sapogenin with tetrahydropyran unit, is only encountered in *Astragalus* species. This rare sapogenin has never been a topic of biological activity or modification studies. The objectives of this study were: (i) to perform microbial transformation studies on cyclocephagenol (1) using *Astragalus* endophyte, *Alternaria eureka* 1E1BL1, followed by isolation and structural characterization of the metabolites; (ii) to investigate neuroprotective activities of the metabolites; (iii) to understand structure–activity relationships towards neuroprotection. The microbial transformation of cyclocephagenol (1) using *Alternaria eureka* resulted in the production of twenty-one (2–22) previously undescribed metabolites. Oxidation, monooxygenation, dehydration, methyl migration, epoxidation, and ring expansion reactions were observed on the triterpenoid skeleton. Structures of the compounds were established by 1D-, 2D-NMR, and HR-MS analyses. The neuroprotective activities of metabolites and parent compound (1) were evaluated against H₂O₂-induced cell injury. The structure–activity relationship (SAR) was established, and the results revealed that 1 and several other metabolites had potent neuroprotective activity. Further studies revealed that selected compounds reduced the amount of ROS and preserved the integrity of the mitochondrial membrane. This is the first report of microbial transformation of cyclocephagenol (1).

Biotransformation is the biochemical reactions performed by living systems or their components (enzymes) to alter molecules. Significant advantages of this methodology are: (i) stereo-, regio- and enantioselective catalysis; (ii) transformation at non-reactive sites of the substrates; (iii) mild condition requirements. In the pharmaceutical industry, microbial biotransformation has been utilized in the enzymatic transformation to synthesize chiral intermediates and end products. Production of cortisone (*Rhizopus nigricans*), hydrocortisone (*Curvularia* sp.) and compactine (*Mucor hiemalis*) are examples of industrial applications of biotransformation where mainly P450 monooxygenases are involved. Additionally, biotransformation of natural products can provide a wide range of structural diversity and improved biological activity.

Research groups have engaged in the discovery of new microorganisms and enzymes to be developed as novel biocatalysts. In this regard, endophytes are powerful organisms because of their capability to produce enzymes necessary for their colonization, and they have been proven to be potent biotransformation systems.

Neurodegeneration refers to the loss of structure/function of neurons leading to neurological diseases including Alzheimer’s and Parkinson’s. The discovery of novel therapeutics against neurodegenerative diseases has been an area of intense research as neurodegenerative diseases are a huge burden on society and the economy. Numerous studies reported that natural products have the potential for the prevention and treatment of neurodegeneration. Astragaloside IV (AST-IV), a cycloartane-type saponin from *Astragalus* species, efficiently attenuated hydrogen peroxide (H₂O₂)-induced neuronal cell death. Moreover, aglycone of AST-IV, viz. cycloastragenol, diminished amyloid-beta mediated neurogenic disfunction.

Herein, based on the potential of cycloartane-type saponins, we also focused on the neuroprotective activity of cyclocephagenol (1), a novel cycloartane-type sapogenin from *Astragalus microcephalus*. As 1 demonstrated significant protection, we further performed a modification study on 1 utilizing microbial transformation and examined the neuroprotective potential of metabolites in H₂O₂-induced injury in SH-SY5Y cells. As a result,...

¹Department of Bioengineering, Faculty of Engineering, İzmir Institute of Technology, Urla, 35430 İzmir, Türkiye. ²Department of Biochemistry, Faculty of Pharmacy, Ege University, Bornova 35100 İzmir, Türkiye. Email: petek.ballar@ege.edu.tr; erdalbedir@iyte.edu.tr
endophytic fungus \textit{Alternaria eureka} 1E1BL1, an endophyte isolated from \textit{Astragalus} plant, provided twenty-one new biotransformation products (2–22) with potent neuroprotective activities.

Results
The neuroprotective activities of cyclocephagenol (1) and cycloastragenol were determined against H$_2$O$_2$-induced SH-SY5Y cell death. Results showed that both cycloastragenol and 1 provided dose-dependent protection against H$_2$O$_2$-induced cell death (Fig. 1). However, the protective activity of 1 started at lower concentrations compared to cycloastragenol (Fig. 1). Based on the potent activity of 1, a biotransformation study on 1 was carried out to develop a molecule library and to investigate structure–activity relationships by \textit{A. eureka} affording notable chemical diversity\textsuperscript{19–23}. Biotransformation of 1 using the endophytic fungus \textit{A. eureka} for 13 days afforded twenty-one metabolites (2–22). The structures of the metabolites are shown in Fig. 2.

Compound 1 is a deglycosylated product of a known cycloartane diglycoside, namely cyclocephaloside I\textsuperscript{24}. Since it has not been previously reported as a new sapogenin, herein, its structural elucidation is discussed. The molecular formula of 1 was determined as C$_{30}$H$_{50}$O$_{5}$ due to the sodium adduct ion peak at m/z 513.35607 [M + Na$^+$] by HR-ESI–MS. The $^1$H-NMR spectrum showed characteristic signals of cyclopropane–methylen protons as an AX system at δ$_{H}$ 0.30 and 0.58 (each d, $J_{AX} = 4.1$ Hz, H-19a and $J_{AX} = 3.1$ Hz H-19b) and seven tertiary methyl groups. Hence, compound 1 was a cycloartane-type triterpenoid, and this inspection was supported by the $^{13}$C-NMR spectral data. The resonances for low-field carbon atoms indicated the presence of four oxymethine carbons (δ$_{C}$ 78.1, C-3; δ$_{C}$ 68.3, C-6; δ$_{C}$ 73.8, C-16; δ$_{C}$ 68.5, C-24) and two oxygenated singlet carbons (δ$_{C}$ 78.8, C-20; δ$_{C}$ 75.1, C-25) substantiated by HSQC. The $^{13}$C and $^1$H-NMR data substantiated with 1D- and 2D-NMR spectra showed that resonances arising from the triterpenoid skeleton were almost superimposable with those of cycloastragenol\textsuperscript{25}, which is one of the major aglycone constituents of \textit{Astragalus} sp., except for the side chain signals. The HMBC experiment suggested that the 24-hydroxy-20,25-epoxy structure was intact in the side chain of 1, as in cyclocephaloside I. The carbon resonances ascribed to the side chain consisting of a doublet (δ$_{C}$ 68.5, C-24), two triplets (δ$_{C}$ 26.4, C-22; 23.8, C-23), two singlets (δ$_{C}$ 78.8, C-20; 75.1, C-25) and three quartets (δ$_{C}$ 28.5, C-21; 28.4, C-26; 27.8; C-27). The HMBC spectrum displayed cross-peaks from H$_{1}$-18 and H$_{2}$-21 to C-17, H-17 and H$_{2}$-21 to C-20, H$_{1}$-26 and H$_{2}$-27 to C-24, and H$_{1}$-26 and H$_{2}$-27 to C-25 confirm this proposal. Thus, the structure of 1 was elucidated as 20,25-epoxy-3β,6α,16β,24α-tetrahydroxycycloartane, which is the aglycone of cyclocephaloside I\textsuperscript{24}. Hence, 1 was named as cyclocephagenol.

Oxygenation at C-7, C-11 and C-12. The HR-ESI–MS data of compounds 2, 6 and 13 supported a molecular formula of C$_{30}$H$_{50}$O$_{6}$ implying a monooxygenation of 1 due to a + 16 amu difference, while compounds 16 and 18 displayed a 32 amu increase over 1, suggesting dihydroxy analogs.

The $^1$H-NMR spectra of 2 and 6 displayed a new oxymethine signal (δ$_{H}$ 4.24 and 4.27, respectively). The $^{13}$C-NMR spectra of both compounds exhibited a significant down-field shift for neighbor carbon signals when compared to that of 1, proposing a monooxygenation at the C-12 position: C-11 (δ$_{C}$ 36.4) and C-13 (δ$_{C}$ 52.3) signals underwent a significant down-field shift (ca. 10.2 and 5.7 ppm, respectively) for 2 and a down-field shift for C-7, C-11 and C-12.
for C-17 and C-18 signals (ca. 9.2 and 7 ppm, respectively) for 6. In the COSY spectra of both metabolites, the correlations between H-12 and H2-11 were readily noted. In addition, H-12 of 6 coupled with the exchangeable hydroxy proton \(\delta_H 5.38, C-12(OH)\) to give a doublet of doublets of doublets type resonance (ddd, \(J = 9.1, 5.9, 2.6 \text{ Hz}\)). This statement was verified with the HMBC experiment. The hydroxy group at C-12 was determined to be \(\beta\)-oriented for 2 and \(\alpha\)-oriented for 6 based on NOESY correlations\(^\text{26}\). Based on this data, the structure of 2 and 6 were identified as 20,25-epoxy-3\(\beta\),6\(\alpha\),12\(\beta\),16\(\beta\),24\(\alpha\)-pentahydroxycycloartane and 20,25-epoxy-3\(\beta\),6\(\alpha\),12\(\alpha\),16\(\beta\),24\(\alpha\)-pentahydroxycycloartane, respectively.

For metabolite 13, as in the case of 2 and 6, an additional oxymethine signal at \(\delta_H 3.87\) (dd, \(J = 8.7, 2.5 \text{ Hz}\)) was observed, which correlated with a resonance at \(\delta_C 65.4\) in the HSQC spectrum. Furthermore, the 1H-NMR spectrum of 13 revealed that one of the 9,19-cyclopropane ring signals (\(\delta_H 1.10, H-19a\)) underwent a significant downfield shift compared to 1, indicating hydroxylation at the C-11 position as described previously\(^\text{23,26}\). The orientation of C-11(OH) was found to be \(\beta\) based on NOESY cross-peak between H-11 (\(\delta_H 3.87\)) and \(\alpha\)-oriented H3-30 (\(\delta_H 0.84\)). Hence, the structure of 13 was established as 20,25-epoxy-3\(\beta\),6\(\alpha\),11\(\beta\),16\(\beta\),24\(\alpha\)-pentahydroxycycloartane.

When 1H- and 13C-NMR spectra of 16 were inspected in detail, two additional low-field protons (\(\delta_H 3.35\) and 3.93) were observed in the low-field region and two extra down-field carbon signals at \(\delta_C 81.0\) and 83.4 were also noted. The 2D-NMR spectra were examined in detail to deduce hydroxylation locations. Key long-range correlations from H3-18 (\(\delta_H 1.35\)) and H-17 (\(\delta_H 2.32\)) to 83.4 ppm substantiated that one of the hydroxy groups was located at C-12 based on the HMBC spectrum. The second hydroxy group was located at C-11 (\(\delta_C 81.0\)) based on the COSY correlation of H-11 (\(\delta_H 3.35\)) with H-12 (\(\delta_H 3.93\)) together with the \(J_{H-C}\) correlations from H-19 (\(\delta_H 0.57\) and 0.83) and H-12 (\(\delta_H 3.93\)) to C-1. The relative configurations at C-11 and C-12 were determined based on the 2D-NOESY data. The orientation of C-12(OH) was found to be \(\beta\) based on NOESY cross-peak between H-12 (\(\delta_H 3.93\)) and \(\alpha\)-oriented H3-30. The strong correlation of H-11 with one of the \(\beta\)-oriented C-19 protons (\(\delta_H 0.83\)) revealed that the hydroxy group at C-11 was \(\alpha\)-oriented. Based on this evidence, the structure of compound 16 was elucidated as 20,25-epoxy-3\(\beta\),6\(\alpha\),11\(\alpha\),12\(\beta\),16\(\beta\),24\(\alpha\)-hexahydroxycycloartane.

Compound 18 was the dihydroxylated derivative of 1, and the first hydroxylation was located at C-12 based on the \(J_{H-C}\) correlations from the characteristic H-17 (\(\delta_H 2.53\)) and H3-18 (\(\delta_H 1.88\)) resonances to the new signal at \(\delta_C 71.4\) (C-12) in the HMBC spectrum. The 13C-NMR spectrum of 18 displayed that C-6 (\(\delta_C 73.2\)) and C-8 (\(\delta_C 52.4\)) signals underwent a significant down-field shift (ca. 4.9 and 5.3 ppm, respectively) when compared to 1. Together with the spin system starting from H-5 [H-5 (\(\delta_H 1.80\) d, \(J = 9.3 \text{ Hz}\)) \(\rightarrow\) H-6 (\(\delta_H 3.74\) s) \(\rightarrow\) H-7 (\(\delta_H 3.71\) s) \(\rightarrow\) H-8 (\(\delta_H 2.40\) d, \(J = 6.9 \text{ Hz}\))] in the COSY spectrum, the location of the second hydroxylation was deduced to be C-7. Long-range correlations from H-6 and H-8 to C-7 (\(\delta_C 75.7\)) in the HMBC spectrum verified the location of the transformation\(^\text{22,23}\). From a detailed inspection of the 2D-NOESY spectrum, the

Figure 2. Biotransformation products of cyclocephagenol (1) by Alternaria eureka 1E1BL1.
correlations of H-12 with α-oriented H3-30 (δH 1.03)/H-17 (δH 2.53) and cross-peaks between H-7 and α-oriented H3-30/H-5 (δH 1.80) disclosed the configurations of hydroxy groups. Consequently, metabolite 18 was elucidated as 20,25-epoxy-3β,6α,7β,12β,16β,24α-hexahydroxy-cycloartane.

Additional oxidation at C-3, C-12 and C-16. All keto products isolated within the scope of this study also had oxymethines as in the abovementioned compounds. 1H-, 13C-NMR and HMBC correlations were inspected to assign locations of oxidation and oxygenation. In the 1H- and 13C-NMR spectra of oxidation products, the absence of low-field oxymethine signals and the observation of keto carbonyl carbons around 210–220 ppm suggested the oxidation of secondary alcohols. In the HMBC spectrum, H2-28, H2-29, H1-1 and H2-2 displayed cross-peaks with the carbonyl signal at 210–220 ppm region substantiating the existence of the carbonyl group at C-3 while the long-range correlations from H-17 and H2-15 to a carbon resonance at ca. 210–220 ppm confirmed the location of the carbonyl group to be at C-16.20,23,26.

Thus, the assignment of oxidation locations for compounds 3 (3-oxo), 4 (16-oxo) and 5 (3,16-dioxo) was readily inferred based on the abovementioned evidence. Additionally, a new proton signal (δH 4.18 for 3; δH 4.15 for 4; δH 4.14 for 5) in the 1H-NMR spectra of 3–5 and a new carbon resonance (δC 71.6 for 3; δC 71.5 for 4; δC 71.6 for 5) in the 13C-NMR spectrum suggested a new hydroxy group in the structure. Examination of 2D-NMR spectra of 3–5 verified monoxygenation at C-12 and the relative configuration was established as β-orientated. Thus, metabolites 3, 4 and 5 were determined as 20,25-epoxy-6α,12β,16β,24α-tetrahydroxycycloartan-3-one, 20,25-epoxy-3β,6α,12α,24α-tetrahydroxycycloarten-16-one and 20,25-epoxy-6α,12β,24α-trihydroxycycloarten-3,16-dione, respectively.

Like metabolites 3–5, after positions of oxidation were determined unambiguously (C-16 for 7, C-3 and C-16 for 8), 1H- and 13C-NMR spectra of 7 and 8 were further examined. A new proton signal (δH 4.19 for 7; δH 4.15 for 8) and a new carbon signal (δC 72.7 for 7; δC 73.7 for 8) suggested that a monooxygenation reaction took place. The 2D-NMR spectra of 7 and 8 implied the location of monoxygenation at C-12 as in metabolites 3–5; however, 2D-NOESY data revealed that the hydroxy group at C-12 was α-orientated in 7 and 8. Therefore, metabolites 7 and 8 were deduced to be 20,25-epoxy-3β,6α,12α,24α-tetrahydroxycycloarten-16-one and 20,25-epoxy-6α,12β,24α-trihydroxycycloarten-3,16-dione, respectively.

Compound 9 gave a major ion peak at m/z 527.33662 ([M + Na]+, calcd for C30H46NaO7, 527.33486). When the 1H-NMR spectrum of 9 was inspected, the characteristic signals belonging to H-3, H-6, H-16 and H-24 oxymethine protons were observed readily, suggesting that oxidation occurred in a new oxymethine carbon. In the HMBC spectrum, H2-11 (δH 1.97 and 2.61), H2-18 (δH 1.72) and H-17 (δH 2.36) showed cross-peaks with a carbon resonating at δC 212.0, confirming the location of the carbonyl group to be at C-16. Based on these results, the structure of 9 was elucidated as 20,25-epoxy-3β,6α,16β,24α-tetrahydroxy-cycloarten-12-one.

When the 13C-NMR and HMBC spectra of 10 and 11 were examined, signals at δC 210–220 range suggested two keto carbonyl groups in the structure of both compounds. The HMBC correlations from H2-11 (δH 1.96 and 2.64) and H2-18 (δH 1.73) to δC 211.3 for 10 and H2-11 (δH 2.09 and 2.83) and H1-18 (δH 2.06) to δC 210.2 for 11 confirmed the location of the first keto group to be at C-12. Moreover, the low-field signal of H-3 in 10 was absent in the 1H-NMR spectrum, whereas the H-16 signal disappeared in that of 11. The carbon signal δC 216.1 had long-range correlations with H2-28 and H2-29, readily assigned to C-3 in the HMBC spectrum of 10, while the HMBC correlations of H-17 (δC 3.08) and H1-15 (δH 2.18 and 2.52) with the carbonyl carbon at δH 214.4 confirmed the oxidation at C-16 to establish the structure of 11. Hence, the structure of 10 was deduced as 20,25-epoxy-6α,16β,24α-tetrahydroxy-cycloarten-3,12-dione, and the structure of 11 was established as 20,25-epoxy-3β,6α,24α-trihydroxy-cycloarten-12,16-dione.

The metabolite 12 gave a molecular formula of C30H44O6, based on the HR-ESI–MS data (m/z 523.30530 ([M + Na]+), calcd for C30H44NaO6, 523.30536). The absence of low-field oxymethine signals due to H-3 and H-16 in the 1H-NMR spectrum of 12 and observation of two keto carbonyl carbons in the 13C-NMR and HMBC spectra suggested that C-3 (δC 215.7) and C-16 (δC 214.1) secondary alcohols had been oxidized, as in 5. Also, the signal at δH 2.096 suggested an additional keto carbonyl group in structure, which showed cross-peaks with H2-11 (δH 2.05 and 2.83), H1-17 (δH 3.07) and H-18 (δH 2.06) in the HMBC spectrum, revealing the oxidation at C-12. Based on these results, the structure of 12 was elucidated as 20,25-epoxy-6α,24α-dihydroxy-cycloarten-3,12,16-trione.

Compounds 14 and 15 possessing similar oxidation patterns with metabolites 3 and 5, respectively, were determined as 11β-hydroxycyclocephenogol derivatives by comparing 1D- and 2D-NMR spectra of 14 and 15 with 13. Consequently, the structures of 14 and 15 were determined as 20,25-epoxy-6α,11β,16β,24α-tetrahydroxy-cycloarten-3-one and 20,25-epoxy-6α,11β,24α-trihydroxy-cycloarten-3,16-dione, respectively.

The HR-ESI–MS spectrum of 17 showed a major ion peak at m/z 541.31528 [M + Na]+ (C30H48NaO6). The oxymethine proton at C-3 was lost in the 1H-NMR spectrum. A detailed inspection of 13C-NMR and HMBC spectra suggested that the C-3 (δC 216.3) secondary alcohol had been oxidized and the signal at δC 211.4 suggested an additional keto carbonyl group in 17, as in 10. Unlike metabolite 10, a broad singlet observed at δH 3.71 displaying a long-distance correlation with the C-12 (δC 211.4) suggested an additional oxymethine group. With these findings, a monooxygenation at C-11 was suggested. The configuration of C-11(OH) was deduced based on the 2D-NOESY data. The correlation of H-11 with α-orientated H3-30 (δH 0.66) revealed that the configuration of the OH group at C-11 was β-orientated. Thus, the structure of 17 was determined to be 20,25-epoxy-6α,11β,16β,24α-tetrahydroxy-cycloarten-3,12-dione.

Ring cleavage and ring expansion products. In the 1H-NMR spectra of metabolites 19, 20, 21 and 22, the characteristic 9,19-cyclopropane ring signals were absent, suggesting a ring cleavage reaction in the triterpenoid skeleton.
The metabolite 19 gave a molecular formula of C₃₀H₄₆O₆ based on the HR-ESI–MS data (m/z 529.35144 [M + Na]+, calcld for C₃₀H₄₆NaO₆, 529.35051) indicating a 16 amu increase over 1, suggesting being a monohydroxy derivative. An additional oxymethylene group at δc 68.5 and two olefinic carbon signals (δc 134.5, 132.7) were observed in the low field of the ¹³C-NMR spectrum. In the HSQC spectrum, double bond carbons showed no correlation with any proton, denoted a tetrasubstituted olefinic system. Based on these findings, the structure of 19 was proposed to have a C-9(10) double bond with a primary alcohol substitution at C-11 based on our previous biotransformation studies, and this assumption was confirmed by the HMBC experiment. In conclusion, metabolite 19 was determined to be 20,25-epoxy-3β,6α,16β,24α-pentahydroxy-ranunculan-9(10)-ene.

The HMBC spectra of 20, 21 and 22 displayed a cross-peak between H-3 (δH 3.82) and C-10 (δc 88.7), indicative of an oxygen bridge between C-3, which correlated with H1-28 and H2-29, and C-10. Based on this evidence and previous biotransformation studies, ring B was inferred as a seven-membered ring system. Also, olefinic carbon resonances were detected in the ¹³C-NMR spectra. The nature of double bonds was determined by examining the correlation of olefinic carbons with protons in the HSQC spectrum.

The molecular formula of 20 was established as C₃₀H₄₈O₆ by HR-ESI–MS analysis (m/z 527.33874 [M + Na]+, calcld for C₃₀H₄₈NaO₆, 527.33486). In the ¹³C-NMR spectrum, two additional oxymethine carbons at δc 80.2 and 88.7 and two olefinic carbon resonances at δc 126.7 and 139.5 were detected. From the HSQC spectrum, the nature of the double bond was determined as a tetra-substituted olefinic system. The δc 126.7 and 139.5 resonances were assigned to C-9 and C-8, respectively, based on the long-distance correlations in the HMBC spectrum (C-9 to H-12; C-8 to H-12 and H-30). The resonance observed at δc 4.39 suggested an additional oxymethine group corresponding to carbon at δc 80.2 in the HSQC spectrum. A detailed inspection of the ¹H- and ¹³C-NMR spectra showed down-field shifts for H-6 and C-6 signals (ca. 0.64 and 8.6 ppm, respectively) when compared to that of 1; therefore, a hydroxylation at C-7 was suggested. The COSY and HSQC spectra revealed a spin system of H-5 (δH 1.47) → H-6 (δH 4.42) → H-7 (δH 4.39), justifying this assignment. In the 2D-NOESY spectrum, the orientation of C-7(OH) was found to be β based on NOESY cross-peaks between H-7 (δH 4.39) and the α-oriented H-30 and H-5. Consequently, the structure of 20 was established as 3β,10β;20,25-diepoxy-6α,7β,16β,24α-tetrahydroxy-9,10-seco-cycloartan-8-ene.

Using the HSQC and HSQC-TOCSY spectra, the δc 70.8 signal was noted. In the COSY and HSQC-TOCSY spectra, the δH 4.73 proton coupled with an oxymethine carbon at δc 80.2 in the HSQC spectrum. A detailed inspection of the 1H- and 13C-NMR spectra showed down-field shifts for H-6 and C-6 signals (ca. 0.64 and 8.6 ppm, respectively) when compared to that of 1; therefore, a hydroxylation at C-7 was suggested. The COSY and HSQC spectra revealed a spin system of H-5 (δH 1.47) → H-6 (δH 4.42) → H-7 (δH 4.39), justifying this assignment. Consequently, the structure of 20 was established as 3β,10β;20,25-diepoxy-6α,7β,16β,24α-tetrahydroxy-9,10-seco-cycloartan-8-ene.

Neuroprotective activity of biotransformation products. The neuroprotective activities of isolated metabolites (except for 22 due to its scarce amount) were determined against H₂O₂-induced SH-SY5Y cell death. Compared to the control group, the compounds except 2, 3, 4, 6, 7, 9, 10, 13, 14, 17, and 20 did not exhibit promising neuroprotective activity (Table 1).

In addition to their protective effect against oxidative injury in SH-SY5Y, the selected compounds were also screened for their effect against 6-OHDA induced neurotoxicity in vitro Parkinson’s disease model. Despite the lower potency of compounds in this model compared to the H₂O₂-induced neurotoxicity model, the tested metabolites still showed a statistically significant protective effect, suggesting that they may act as general protective agents against neurotoxicity mediated by a different mechanism of action (Fig. 5).
Selected metabolites prevent H2O2-induced mitochondria damage. Mitochondrial dysfunction is one of the most emerging pathological processes in neurodegenerative diseases. Since mitochondrial membrane potential was reported as an indicator to detect mitochondrial dysfunction, we next evaluated the effect of H2O2 and four selected compounds on mitochondrial membrane potential by using Mitotracker Red. As expected, the fluorescence intensity significantly decreased after treatment with H2O2 treatment, which suggested that H2O2 could induce mitochondrial dysfunction. All selected compounds efficiently protected cells from H2O2-mediated mitochondrial damage (Fig. 4a,b).

**Discussion**

In recent years, endophytic fungi have received great attention as a whole-cell catalyst because of their capability to produce enzymes necessary for their colonization. It is anticipated that the endophytic biocatalysts will match chemical reactions even as powerful as conventional chemical methods in near future. Additionally, studies on fungal biotransformation of plant secondary metabolites with the plant's own endophytes are very promising. Herein, we report the isolation of 12 metabolites from an endophytic fungus that efficiently protects cells from H2O2-mediated mitochondrial damage.

**Table 1.** Neuroprotective activity of metabolites against H2O2 toxicity. Data are presented as means ± S.D (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 significant difference from H2O2-treated cells.

| Metabolite | Concentration | Cell viability (%) | Metabolite | Concentration | Cell viability (%) |
|------------|---------------|--------------------|------------|---------------|--------------------|
| 2          | 1 nM          | 56.2 ± 1.8***      | 12         | 1 nM          | 36.2 ± 1.9         |
|            | 10 nM         | 61.4 ± 3.7***      |            | 10 nM         | 46.2 ± 1.8*        |
|            | 100 nM        | 61.1 ± 0.7***      |            | 100 nM        | 49.0 ± 0.2**       |
|            | 1000 nM       | 58.1 ± 5.9***      |            | 1000 nM       | 42.4 ± 2.0         |
| 3          | 1 nM          | 46.8 ± 6.0         | 13         | 1 nM          | 51.5 ± 7.9         |
|            | 10 nM         | 46.7 ± 2.1         |            | 10 nM         | 56.4 ± 3.0*        |
|            | 100 nM        | 50.9 ± 0.2**       |            | 100 nM        | 58.1 ± 12.1*       |
|            | 1000 nM       | 65.9 ± 2.3***      |            | 1000 nM       | 66.4 ± 9.3**       |
| 4          | 1 nM          | 34.1 ± 0.4         | 14         | 1 nM          | 60.8 ± 4.4****     |
|            | 10 nM         | 59.7 ± 3.5***      |            | 10 nM         | 56.5 ± 4.2****     |
|            | 100 nM        | 56.3 ± 3.3***      |            | 100 nM        | 39.9 ± 2.7         |
|            | 1000 nM       | 53.6 ± 5.9***      |            | 1000 nM       | 34.1 ± 1.3         |
| 5          | 1 nM          | 26.9 ± 2.5         | 15         | 1 nM          | 37.0 ± 3.0         |
|            | 10 nM         | 25.1 ± 3.6         |            | 10 nM         | 41.2 ± 2.6         |
|            | 100 nM        | 43.8 ± 1.3         |            | 100 nM        | 43.5 ± 5.3*        |
|            | 1000 nM       | 42.5 ± 5.7         |            | 1000 nM       | 37.0 ± 0.5         |
| 6          | 1 nM          | 45.7 ± 2.4         | 16         | 1 nM          | 42.0 ± 2.3         |
|            | 10 nM         | 46.0 ± 5.9         |            | 10 nM         | 46.5 ± 7.2         |
|            | 100 nM        | 54.8 ± 5.8***      |            | 100 nM        | 42.7 ± 3.1         |
|            | 1000 nM       | 52.1 ± 1.3*        |            | 1000 nM       | 41.4 ± 6.6         |
| 7          | 1 nM          | 42.6 ± 3.9         | 17         | 1 nM          | 41.9 ± 1.4         |
|            | 10 nM         | 45.9 ± 5.6         |            | 10 nM         | 42.7 ± 2.3         |
|            | 100 nM        | 55.6 ± 13.3*       |            | 100 nM        | 62.8 ± 7.9***      |
|            | 1000 nM       | 49.0 ± 3.9         |            | 1000 nM       | 72.5 ± 13.3***     |
| 8          | 1 nM          | 36.6 ± 11.0        | 18         | 1 nM          | 44.0 ± 0.7         |
|            | 10 nM         | 40.3 ± 1.5         |            | 10 nM         | 42.0 ± 0.8         |
|            | 100 nM        | 36.6 ± 2.7         |            | 100 nM        | 44.4 ± 0.9         |
|            | 1000 nM       | 40.2 ± 12.9        |            | 1000 nM       | 38.1 ± 4.8         |
| 9          | 1 nM          | 44.2 ± 2.3         | 19         | 1 nM          | 38.0 ± 1.9         |
|            | 10 nM         | 46.2 ± 3.2         |            | 10 nM         | 40.5 ± 7.1         |
|            | 100 nM        | 51.4 ± 4.8*        |            | 100 nM        | 39.9 ± 4.7         |
|            | 1000 nM       | 70.5 ± 0.8***      |            | 1000 nM       | 49.1 ± 3.6         |
| 10         | 1 nM          | 51.1 ± 8.5*        | 20         | 1 nM          | 56.8 ± 1.9**       |
|            | 10 nM         | 58.5 ± 3.4***      |            | 10 nM         | 58.3 ± 6.3**       |
|            | 100 nM        | 74.7 ± 3.3****     |            | 100 nM        | 70.6 ± 6.9***      |
|            | 1000 nM       | 68.1 ± 4.8****     |            | 1000 nM       | 64.8 ± 6.0****     |
| 11         | 1 nM          | 38.2 ± 0.7         | 21         | 1 nM          | 47.0 ± 1.6**       |
|            | 10 nM         | 43.9 ± 6.2         |            | 10 nM         | 44.7 ± 2.4*        |
|            | 100 nM        | 46.9 ± 9.5         |            | 100 nM        | 38.7 ± 3.1         |
|            | 1000 nM       | 54.3 ± 3.7**       |            | 1000 nM       | 35.7 ± 1.4         |
| H2O2       | 70 µM         | 37.9 ± 2.0         |            |            |                    |
limited. Our previous studies demonstrated that *Alternaria eureka* expresses P450 monoxygenase enzymes and effectively catalyzes various modifications on triterpenoid and steroid structures \(^{19–23}\). In this study, the biocatalysis of cyclocephagenol (1) by *A. eureka* yielded twenty-one metabolites. *A. eureka* was found to be capable of modifications including monoxygenation, dehydration, methyl migration, epoxidation, and ring expansion, resulting in the formation of metabolites that would be difficult or impossible to prepare by conventional synthetic methods. Although fungal biotransformation has been used in the modification of natural products for a long time, demonstration of endophytic fungi's utilization in biotransformation is essential for the field not only for utilizing endophytes as potent catalysis systems but also for proving the potential of the plant's own microbiota for transformation studies.

The regioselective hydroxylation at C-12 position was the most prevalent reaction in the cycloartane skeleton. We suggest that *A. eureka* first catalyzes α- or β-hydroxylation at C-12, then performs further modifications. The other monoxygenation locations were identified as C-7 and C-11. Although hydroxylation at C-7 has been encountered with steroidal sapogenins \(^{19,21–23}\), this modification is reported for the first time on the triterpenoid framework by *A. eureka*.

One of the most interesting biotransformation was the dihydroxylation at C-11 and C-12. Previously, 11α-hydroxy,12β-acetoxy steroids were obtained from the gorgonian *Isis hippuris* \(^{32}\). This is the first report of C-11 and C-12 dihydroxylated products obtained via microbial biotransformation. Additionally, 3(10)-epoxy formation and ring expansion modifications have been reported in the previous studies \(^{20,29,33}\), whereas the 6,8-diene system is being reported for the first time in triterpenoid chemistry.

**Figure 3.** Selected metabolites abbreviated H\(_2\)O\(_2\)-induced oxidative stress in SH-SY5Y cells. Data are presented as means ± S.D. (n = 3).
Natural products have the potential for the prevention and treatment of neurodegeneration. In recent years, cycloartane-type saponins, especially astragaloside IV and cycloastragenol, have been reported as a new class of neuroprotective agents. Based on the promising bioactivity of cycloartane-types saponins, we first evaluated the neuroprotective activity of cyclocephagenol (1) against H$_2$O$_2$-induced SH-SY5Y cell death. Compared to cycloastragenol, the neuroprotective activity of 1 started at lower concentrations. Since the neuroprotective activity of cyclocephagenol was noteworthy, a biotransformation study on 1 was carried out to develop a molecule library and to investigate structure–activity relationships by A. eureka.

Among the oxygenated metabolites, 2 and 6 possessing hydroxy group at position 12 (12β and 12α, respectively) showed potent neuroprotective activity. While the most active concentration of 2 was 10 nM, the neuroprotection by 6 was observed at a higher concentration (100 nM). Also, the metabolite 13 possessing hydroxy group at position 11 demonstrated potent neuroprotective activity at higher concentrations (100 and 1000 nM). On the other hand, a dihydroxy group in ring C (16: 11α,12β-dihydroxy) led to a loss of neuroprotection suggesting that the hydroxylation pattern was critical for the activity.

The presence of ketone functionality at C-3 or C-16 (3: 3-oxo-12β-hydroxy, 4: 16-oxo-12β-hydroxy, 7: 16-oxo-12α-hydroxy) had no detrimental effect on neuroprotection compared to metabolite 2 and 6. However, the co-presence of ketone group at C-3 and C-16 (5: 3,16-dioxo-12β-hydroxy, 8: 3,16-dioxo-12α-hydroxy) diminished neuroprotective activity. Moreover, the oxidation at C-12 (9: 12-oxo, 1000 nM) improved neuroprotective activity whereas additional oxidation at C-16 (11: 12,16-dioxo) lessened neuroprotective activity. This finding suggested that the co-presence of ketone group at C-12 and C-16 affects the biological activity negatively. In contrast to...
metabolite 3 (3-oxo-12β-hydroxy), oxidation at position 3 improved neuroprotective activity in 14 (3-oxo,11β-hydroxy) and 10 (3,12-dioxo). Although metabolite 16 (11α,12β-dihydroxy) was not active, activity in 17 (3,12-dioxo, 11β-hydroxy) indicated that oxidation in C-12 was important for biological activity.

Compound 20 was one of the most potent compounds, while neuroprotection was considerably decreased in 21, a dehydration product of 20, revealing that conformational flexibility in the B ring was also crucial.

As a result of SAR studies, we conclude that i) monoxygenation at positions 11 (13) and 12 (2 and 6) is significant bioactivity; ii) oxidation at C-12 (9, 10, 17) improves neuroprotective activity; iii) further increase of hydrophobicity (5, 8, 11, 12) and hydrophilicity (16, 18) diminishes bioactivity; iv) 3(10)-epoxy-9,10-seco-cycloartane products formed by ring expansion and epoxidation reactions could be potential neuroprotective agents as long as they have conformational flexibility. Further studies revealed that selected compounds reduced the amount of ROS and preserved the integrity of the mitochondrial membrane.

Conclusion
Collectively, the biocatalyst potential of A. eureka, which plays an important role in expanding our cycloartane molecule library, has been demonstrated once again by producing 21 new metabolites. In addition to chemical diversity, biotransformation provided several novel compounds having potent protective activity against H_2O_2- and 6-OHDA-induced neurotoxicity. Further studies are warranted to establish a mechanism of action of the bioactive metabolites.

Methods
General experimental procedures. The spectroscopic (NMR and HR-ESI–MS) and chromatographic procedures were described previously.  

Microorganism and starting compound. Cyclocephagenol (1) and cycloastagenol were provided by Bionorm Natural Products, Ltd. (Izmir, Turkey). The fungal endophyte used in this study was isolated from leaves of Astragalus angustifolius and the original strain (Deposit number: 20131E1BL1) was banked at the Bedir Laboratory. Before biotransformation process, the stock culture of A. eureka was pre-cultivated on PDA in Petri dishes for 10 days at 25 °C.

Microbial biotransformation procedures. A one-stage microbial biotransformation process was carried out at a preparative scale using a biotransformation medium. A preparative scale biotransformation study was performed utilizing 1850 mg of 1 with A. eureka for 13 days (25 °C and 180 rpm).

Extraction and isolation. For termination biotransformation, the mycelia were filtered, and the broth was extracted with ethyl acetate (EtOAc) (3x). The EtOAc phase was evaporated by using a rotary evaporator. Compounds 2-22 were isolated from the EtOAc extract (3.05 g). The EtOAc extract was first applied on a reversed-phase column (RP-C18, 80 g) and eluted by MeOH:H_2O (25:75, 35:65, 50:50, 50:55, 70:30, 80:20, 90:10, 100:0) to obtain 12 main fractions (A-I). Fraction B (19.3 mg) was submitted to silica gel column chromatography (10 g) eluting with CHCl_3:MeOH (90:10) to afford 2 mg of 18 (yield: 0.11%). Fraction D (175.3 mg) was applied to a silica gel column (52 g) using CHCl_3:MeOH gradient (95:5, 93:7) to yield 2 (16.9 mg, yield: 0.91%) and 7 (11.8 mg, yield: 0.65%). Fraction E (301.2 mg) was subjected to a silica gel column (52 g) using a CHCl_3:MeOH gradient (93:7, 92:8, 90:10) to give 3 (18.2 mg, yield: 0.98%) and four fractions (E1-4). Fraction E1 (6.0 mg) was further purified on a silica gel column (10 g) and eluted with n-hexane:EtOAc:MeOH (10:1:1) to give 2.8 mg of 14 (yield: 0.15%). To isolate metabolite 9 (8 mg, yield: 0.43%), fraction E2 (27.8 mg) was further purified on a silica gel column (15 g) using n-hexane:EtOAc:MeOH (10:1:1). Fraction E3 (50.1 mg) was subjected to a silica gel column (15 g) using CHCl_3:MeOH (95:5) to afford 15 mg of 13 (yield: 0.18%). Fraction E4 (21.7 mg) was subjected to a silica gel column (15 g), using CHCl_3:MeOH (95:5) for elution, to give 16 (4 mg, yield: 0.22%). Fraction F (192.9 mg) was further purified on a silica gel column (50 g) using CHCl_3:MeOH (97:3, 95:5, 93:7) for elution, to give metabolites 10 (6.3 mg, yield: 0.34%), 19 (31.1 mg, yield: 1.68%) and one impure fraction (F1). Fraction G (9 mg, yield: 0.43%), fraction E2 (27.8 mg) was further purified by a silica gel column (10 g) with the solvent system n-hexane:EtOAc:MeOH (10:10:1), to provide 3.6 mg of 15 (yield: 0.19%). Fraction G (98.7 mg) was submitted to a silica gel column (50 g) using mixtures of CHCl_3:MeOH (95:5, 94:6, 93:7) to give 12 (3.5 mg, yield: 0.19%) and two fractions (G1-2). To isolate metabolite 11 (1.4 mg, yield: 0.075%), fraction G1 (23.3 mg) was subjected to a silica gel column (10 g) using n-hexane:EtOAc:MeOH (10:10:1) to provide 4.6 mg of 19 (yield: 0.25%). Fraction H (275.1 mg) was submitted to a silica gel column (50 g) and eluted with CHCl_3:MeOH gradient (95:5, 93:7, 92:8, 90:10) to afford two fractions (H1-2). Fraction H1 (18.5 mg) was purified by a silica gel column (10 g) and eluted with n-hexane:EtOAc:MeOH (10:10:1), to give 5.4 mg of 5 (yield: 0.29%). Fraction H2 (138.2 mg) was applied to VLC packed with reversed-phase silica gel (RP-C18, 30 g), using ACN:H_2O gradient (25:75, 30:70, 40:60, 50:50), to afford 6 (95.4 mg, yield: 5.16%). Fraction I (86.9 mg) was subjected to a silica gel column (50 g) using CHCl_3:MeOH solvent system (95:5) to give 7 (16.9 mg, yield: 0.91%) and one impure fraction (I1). Fraction I1 (12.9 mg) was further purified on a silica gel column (10 g) and was eluted with a CHCl_3:MeOH gradient (99:1, 98:2) to give 1.1 mg of 22 (yield: 0.06%) and fraction I1a. To purify metabolite 8 (2.4 mg, yield: 0.13%), fraction I1a (7.4 mg) was subjected to silica gel column chromatography (10 g) with the solvent system n-hexane:EtOAc:MeOH (10:10:1). Fraction J (126.5 mg) was submitted to a silica gel column (50 g), eluted with CHCl_3:MeOH (95:5) to give fraction J1 (5 mg). Fraction K (56.7 mg) was subjected to silica gel column chromatography (10 g) to yield 20 (16.3 mg, yield: 0.88%) one impure fraction (K1) after elution with DCM:MeOH gradient (96:4, 95:5, 94:6). Fraction K1 (9.6 mg) was chromatographed on a silica
| Position | 1<sup>st</sup> δ<sub>H</sub> (f Hz) | 2<sup>nd</sup> δ<sub>H</sub> (f Hz) | 3<sup>rd</sup> δ<sub>H</sub> (f Hz) | 4<sup>th</sup> δ<sub>H</sub> (f Hz) | 5<sup>th</sup> δ<sub>H</sub> (f Hz) | 6<sup>th</sup> δ<sub>H</sub> (f Hz) | 7<sup>th</sup> δ<sub>H</sub> (f Hz) |
|----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 1        | 1.20 m, 1.61 m  | 1.37 m, 1.65 m  | 1.40 m, 1.94 m  | 1.27 m, 1.58 m  | 1.29 m, 1.95 dt (14.7, 8.5) | 1.32 m, 1.64 td (12.5, 4.5) | 1.27 m, 1.63 m  |
| 2        | 1.92 m, 2.00 m  | 1.94 m, 2.03 m  | 2.48 m, 2.60 m  | 1.88 m, 2.00 m  | 2.45 m, 2.62 m  | 1.94 m (2H) | 1.98 m (2H) |
| 3        | 3.64 dd (8.6, 2.7) | 3.66 dd (11.5, 4.5) | 3.60 td (7.9, 1.7) | 3.63 dt (10.7, 5.1) | 3.64 m |
| 4        | 5                | 1.70 m          | 1.74 d (9.0)    | 2.11 d (9.2)    | 1.69 d (9.2)    | 2.13 m          | 1.72 m          |
| 6        | 3.78 t (8.7)    | 3.89 dp (11.7, 3.9, 3.3) | 3.72 m | 3.80 q (7.7)    | 3.68 m | 3.76 dd (9.5, 5.8, 3.0) | 3.75 ddd (12.3, 8.9, 3.4) |
| 7        | 1.62 m, 1.79 m  | 1.67 m, 1.93 m  | 1.55 m, 1.77 m  | 1.54 m, 1.74 m  | 1.54 d (13.3), 1.66 dt (12.0, 3.6) | 1.79 td (8.0, 3.5) (2H) | 1.66 m (2H) |
| 8        | 1.94 m          | 2.25 m          | 2.05 dd (11.5, 4.8) | 1.96 d (5.7)    | 1.85 m          | 1.86 m          | 1.76 m          |

Table 2. <sup>1</sup>H NMR spectroscopic data of compounds 1–7 (400 MHz). <sup>4</sup> In C<sub>5</sub>D<sub>5</sub>N.

Structural characterization. Metabolite 1: <sup>1</sup>H-NMR (C<sub>5</sub>D<sub>5</sub>N, 400 MHz): see Table 2; <sup>13</sup>C-NMR (C<sub>5</sub>D<sub>5</sub>N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 513.35607 (C<sub>52</sub>H<sub>42</sub>NaO<sub>6</sub>, calcld. 513.35559).

Metabolite 2: <sup>1</sup>H-NMR (C<sub>5</sub>D<sub>5</sub>N, 400 MHz): see Table 2; <sup>13</sup>C-NMR (C<sub>5</sub>D<sub>5</sub>N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 529.35165 (C<sub>51</sub>H<sub>42</sub>NaO<sub>6</sub>, calcld. 529.35031).

Metabolite 3: <sup>1</sup>H-NMR (C<sub>5</sub>D<sub>5</sub>N, 400 MHz): see Table 2; <sup>13</sup>C-NMR (C<sub>5</sub>D<sub>5</sub>N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 527.33685 (C<sub>51</sub>H<sub>42</sub>NaO<sub>6</sub>, calcld. 527.33486).

Metabolite 4: <sup>1</sup>H-NMR (C<sub>5</sub>D<sub>5</sub>N, 400 MHz): see Table 2; <sup>13</sup>C-NMR (C<sub>5</sub>D<sub>5</sub>N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 527.33596 (C<sub>51</sub>H<sub>42</sub>NaO<sub>6</sub>, calcld. 527.33486).

Metabolite 5: <sup>1</sup>H-NMR (C<sub>5</sub>D<sub>5</sub>N, 400 MHz): see Table 2; <sup>13</sup>C-NMR (C<sub>5</sub>D<sub>5</sub>N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 525.31989 (C<sub>50</sub>H<sub>40</sub>NaO<sub>6</sub>, calcld. 525.31921).

Metabolite 6: <sup>1</sup>H-NMR (C<sub>5</sub>D<sub>5</sub>N, 400 MHz): see Table 2; <sup>13</sup>C-NMR (C<sub>5</sub>D<sub>5</sub>N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 529.35093 (C<sub>52</sub>H<sub>42</sub>NaO<sub>6</sub>, calcld. 529.35051).

Metabolite 7: <sup>1</sup>H-NMR (C<sub>5</sub>D<sub>5</sub>N, 400 MHz): see Table 2; <sup>13</sup>C-NMR (C<sub>5</sub>D<sub>5</sub>N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 527.33594 (C<sub>51</sub>H<sub>42</sub>NaO<sub>6</sub>, calcld. 527.33486).

Metabolite 8: <sup>1</sup>H-NMR (C<sub>5</sub>D<sub>5</sub>N, 400 MHz): see Table 3; <sup>13</sup>C-NMR (C<sub>5</sub>D<sub>5</sub>N, 100 MHz): see Table 5; HR-ESI–MS (negative ion mode): m/z 547.3276 (C<sub>51</sub>H<sub>40</sub>O<sub>6</sub>, calcld. 547.32709).
ESI–MS (positive ion mode):

\[ m/z \] 525.31890  (C30H46NaO6, calcd. 525.31921).

Metabolite 11: 1H-NMR (C5D5N, 400 MHz): see Table 3; 13C-NMR (C5D5N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 525.32125 (C30H46NaO6, calcd. 525.31921).

Metabolite 12: 1H-NMR (CD3OD and a drop of C5D5N, 400 MHz): see Table 4; 13C-NMR (CD3OD and a drop of C5D5N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 523.30530 (C30H46NaO6, calcd. 523.30536).

Metabolite 13: 1H-NMR (CD3OD and a drop of C5D5N, 400 MHz): see Table 3; 13C-NMR (CD3OD and a drop of C5D5N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 529.35090 (C30H48NaO6, calcd. 529.35051).

Metabolite 14: 1H-NMR (C5D5N, 400 MHz): see Table 3; 13C-NMR (C5D5N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 527.33574 (C30H48NaO6, calcd. 527.33486).

Metabolite 15: 1H-NMR (C5D5N, 500 MHz): see Table 4; 13C-NMR (C5D5N, 125 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 525.3170 (C30H48NaO6, calcd. 525.31921).

Metabolite 16: 1H-NMR (CD3OD and a drop of C5D5N, 400 MHz): see Table 4; 13C-NMR (CD3OD and a drop of C5D5N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 545.34597 (C30H50NaO7, calcd. 545.34542).

Metabolite 17: 1H-NMR (C5D5N, 400 MHz): see Table 4; 13C-NMR (C5D5N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 541.31528 (C30H48NaO7, calcd. 541.31412).

Table 3. 1H NMR spectroscopic data of compounds 8–14 (400 MHz). 4In C5D5N. 8In CDCl3. 9In CD3OD and a drop of C5D5N.
| Position | $\delta_H (J$ in Hz)) | $\delta_H (J$ in Hz)) | $\delta_H (J$ in Hz)) | $\delta_H (J$ in Hz)) | $\delta_H (J$ in Hz)) | $\delta_H (J$ in Hz)) | $\delta_H (J$ in Hz)) | $\delta_H (J$ in Hz)) |
|----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 1        | 1.28 m, 1.96 m  | 1.24 m, 1.80 m  | 1.34 m, 1.61 m  | 1.36 m, 2.91 d (9.1) | 1.35 m, 1.58 m | 1.39 m, 1.64 m | 1.53 m, 1.72 m | 1.53 m, 1.72 m |
| 2        | 1.70 m (2H)    | 2.54 t (6.6) (2H) | 1.88 m, 1.97 m  | 1.85 m, 2.02 m  | 1.68 m, 1.93 m | 1.65 m, 1.81 m | 1.68 m, 1.84 m | 1.68 m, 1.84 m |
| 3        | 3.26 m         | 3.61 m          | 3.73 m          | 3.82 dd (5,6,1,7) | 3.78 m          | 3.79 m |
| 4        | 1.54 d (9.8)   | 1.86 d (9.9)    | 1.80 d (9.3)    | 2.72 d (7.2)    | 1.47 d (12.0)    | 1.96 m          | 1.94 m |
| 5        | 3.42 m         | 3.65 m          | 3.74 m          | 4.11 t (8.4)    | 4.42 m          | 6.88 m          | 6.09 m |
| 6        | 1.42 m (2H)    | 1.34 m, 1.52 m  | 3.71 m          | 1.70 d (11.8), 2.02 m  | 4.39 m          | 5.98 m          | 6.05 d (10.4) |
| 7        | 1.53 m         | 2.23 m          | 1.80 m          | 2.06 d (6.9)    | 2.05 m          | 2.17 m |
| 8        | 4.34 m         | 3.35 brs        | 3.71 brs        | 1.67 m, 2.43 d (5,6) | 3.32 m          | 1.74 m, 2.14 m | 2.65 m, 2.91 m | 4.73 m |
| 9        | 2.48 dd (14.6, 3.1), 2.84 m | 3.93 brs | 4.21 m | 1.85 m, 2.78 d (13.9) | 1.77 m, 2.16 m | 4.45 m  | 4.36 d (6.7) |
| 10       | 1.60 s         | 1.35 s          | 1.88 s          | 1.61 s          | 1.40 s          | 1.78 s |
| 11       | 0.58 d (4.3), 1.80 d (4.2) | 0.57 d (4.2), 0.83 m | 0.51 d (4.8), 1.47 d (5.1) | 0.40 d (4.5), 0.92 d (4.4) | 3.91 m, 3.97 m | 1.91 m, 3.17 m | 2.42 m, 2.54 m | 2.95 d (11.8), 3.13 m |
| 12       | 1.49 s         | 1.65 s          | 1.62 s          | 2.02 s          | 1.55 s          | 1.51 s          | 2.01 s          | 1.99 s |
| 13       | 1.33 m, 2.31 m | 1.88 d (12.9), 2.94 d (7.6) | 1.42 m, 2.16 m | 2.92 m, 2.63 m | 1.22 d (14.0), 3.26 d (13.9) | 1.26 m, 3.23 t (13.7, 4.4) | 2.38 m, 2.67 m | 2.33 m, 2.68 m |
| 14       | 1.86 m, 2.17 m | 1.64 m, 2.17 t (13.5) | 1.72 m, 2.18 m | 1.90 m, 2.29 m | 1.83 m, 2.11 m | 1.86 m, 2.16 m | 1.99 m, 2.32 m | 1.98 m, 2.32 m |
| 15       | 3.64 brs       | 3.45 brs        | 3.51 brs        | 3.70 brs        | 3.65 brs        | 3.65 brs        | 3.75 brs        | 3.78 brs |
| 16       | 1.42 s         | 1.28 s          | 1.37 s          | 1.44 s          | 1.42 s          | 1.42 s          | 1.48 s |
| 17       | 1.28 s         | 1.21 s          | 1.19 s          | 1.58 s          | 1.29 s          | 1.31 s          | 1.65 s          | 1.70 s |
| 18       | 1.83 s         | 1.31 s          | 1.38 s          | 1.86 s          | 1.77 s          | 1.66 s          | 0.96 s          | 1.16 s |
| 19       | 1.55 s         | 0.97 s          | 1.24 s          | 1.32 s          | 1.08 s          | 1.31 s          | 1.08 s          | 0.97 s |
| 20       | 1.00 s         | 1.11 s          | 0.66 s          | 1.03 s          | 0.81 s          | 0.92 s          | 0.94 s          | 0.94 s |

Table 4. $^1$H NMR spectroscopic data of compounds 15–22 (400 MHz and *500 MHz). $^*1$C$_2$D$_2$N. $^*2$In CD$_3$OD and a drop of C$_2$D$_2$N.

Metabolite 18: $^1$H-NMR (C$_2$D$_2$N, 400 MHz): see Table 4; $^{13}$C-NMR (C$_2$D$_2$N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 545.34775 (C$_{30}$H$_{50}$NaO$_7$, calcd. 545.34542).

Metabolite 19: $^1$H-NMR (C$_2$D$_2$N, 400 MHz): see Table 4; $^{13}$C-NMR (C$_2$D$_2$N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 529.35144 (C$_{30}$H$_{48}$NaO$_6$, calcd. 529.35051).

Metabolite 20: $^1$H-NMR (C$_2$D$_2$N, 400 MHz): see Table 4; $^{13}$C-NMR (C$_2$D$_2$N, 100 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 527.33874 (C$_{29}$H$_{46}$NaO$_6$, calcd. 527.33486).

Metabolite 21: $^1$H-NMR (C$_2$D$_2$N, 500 MHz): see Table 4; $^{13}$C-NMR (C$_2$D$_2$N, 125 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 509.32640 (C$_{29}$H$_{46}$NaO$_6$, calcd. 509.32492).

Metabolite 22: $^1$H-NMR (C$_2$D$_2$N, 500 MHz): see Table 4; $^{13}$C-NMR (C$_2$D$_2$N, 125 MHz): see Table 5; HR-ESI–MS (positive ion mode): m/z 525.3166 (C$_{29}$H$_{46}$NaO$_6$, calcd. 525.31921).

**Biological activities.** Determination of cell viability. SH-SYSY cell line was maintained in high-glucose Dulbecco’s modified Eagle medium (DMEM) containing 10% FBS at 37 °C, and 5% CO$_2$. The cells were homogeneously seeded in 96 well plate (20,000 cells/well) and incubated for 24 h. Following 2 h incubation with compounds or vehicle (DMSO), the cells were treated with 70 μM H$_2$O$_2$. For the 6-OHDA mediated toxicity experiments, cells were treated with 50 μM 6-OHDA after 8 h of treatment with the compounds. For both experiments, cell viability was determined after 24 h via the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Briefly, the cells were incubated with MTT (0.5 mg/ml final concentration) for 4 h. Then, all the media was pulled out, and DMSO was added to wells. Photometric absorbance was measured at a wavelength of 590/690 nm by using Varioscan flash spectrophotometer by Thermo Scientific. The statistical significance of
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Table 5. \(^{13}\)C NMR spectroscopic data of compounds 1–22 (100 MHz and \(*^{125}\) MHz). \(^{1}\)In \(\text{C}_2\text{D}_5\text{N}. \(^{2}\)In \(\text{CDCl}_3. \(^{3}\)In \(\text{CD}_3\text{OD}\) and a drop of \(\text{C}_2\text{D}_5\text{N}. \(^{4}\)Overlapped signals. \(^{5}\)Not detected.

differences between compounds and \(\text{H}_2\text{O}_2\) or \(\text{6-OHDA}\) treatments were assessed by one-way ANOVA using GraphPad Prism software.

**Determination of ROS levels.** SH-SY5Y cells were seeded onto the 6 well plates and were treated with either compounds or the vesicle for 2 h. Then, cells were treated with \(70 \mu\text{M H}_2\text{O}_2\). Following procedures were performed as described previously\(^{34}\).

**Determination of mitochondrial membrane potential.** SH-SY5Y cells were seeded onto the coverslip. After 2 h pretreatment with 100 nM of each compound, cells were exposed to \(70 \mu\text{M H}_2\text{O}_2\). Following 24 h incubation, cells were treated with 100 nM MitoTracker® Red FM (Thermo Fisher Scientific, US) for 30 min at 37 °C. Then cells were washed with PBS. After mounting, cells were immediately observed using a fluorescence microscope (Olympus IX70).

All photographs were taken under the same conditions, and the fluorescence intensity of the mitochondria relative to the cell volume was calculated in 30 cells using the ImageJ software.

**Data availability**

The data that support the findings of this study are available from the corresponding authors (EB and PBK) upon reasonable request.

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Author contributions
Conceptualization: E.B. and M.K.; Methodology: P.B.K. and E.B.; Formal analysis and investigation: M.K. and G.Ü.; Writing—original draft preparation: M.K. and G.Ü.; Writing—review and editing: M.K., G.Ü., P.B.K. and E.B.; Funding acquisition: P.B.K.; Resources: P.B.K. and E.B.; Supervision: P.B.K. and E.B.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to P.B.K. or E.B.

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