Supporting Information

Sulfur Analogues of Tyrosine in the Development of Triazene Hybrid Compounds: A New Strategy against Melanoma

Margarida Granada[1,*], Eduarda Mendes[1,2*], Maria Jesus Perry[1,2**], Maria João Penetra[1], Maria Manuela Gaspar[1,2], Jacinta O. Pinho[1,2], Sofia Serra[1], Catarina Teixeira António[1], Ana Francisco[1,2]

[1]*Faculty of Pharmacy, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal.*

[2]*Research Institute for Medicines (iMed.ULisboa), Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal.*

*Margarida Granada and Eduarda Mendes contributed equally

** Corresponding author (mjprocha@ff.ul.pt)

Table of Contents

Synthesis and characterization

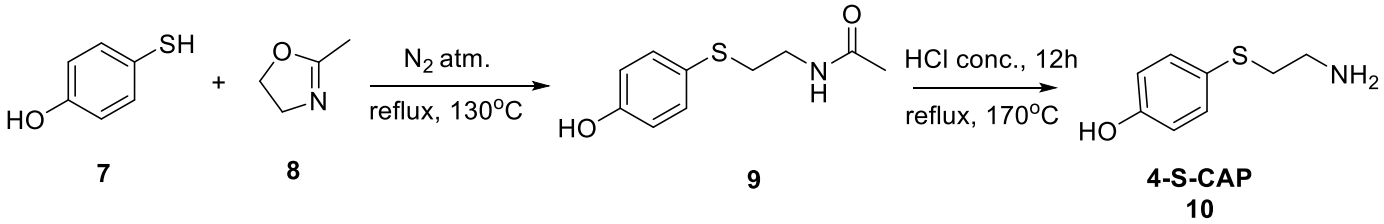
Materials and general information

All the triazenes used in this study should be considered as mutagenic and/or carcinogenic and appropriate care should be taken in order to handle them safely.

Melting points were determined using a Kofler camera Bock-Monoscop "M" and were not corrected. IR spectra were recorded as KBr discs using a Perkin Elmer 1310 spectrophotometer. ^{1}H and ^{13}C-NMR spectra were recorded in $CDCl_3$, MeOD or DMSO-d_6 using either a Brucker AM 400 WB spectrometer or a Bruker Ultra-Shield 300 MHz spectrometer; Chemical shifts, δ, are reported as p.p.m. from Me_4Si, and coupling constants, J, in Hz. Mass spectra were recorded in a Waters Micromass Quattro micro API Benchtop Mass Spectrometer**.** All chemicals were reagent grade except those for kinetic studies and HPLC, which were of analytical or LiChrosolv® (Merck) grade. 1-Aryl-3-methyltriazenes were synthesized by previously published methods. Mushroom tyrosinase (5350 U mg^{-1}) was purchased from Sigma-Aldrich (EC number 1.14.18.1).

General procedures for the synthesis of hybrid compounds 11a-e

4-S-CAP synthesis (**10**)



7 **8** **9** **4-S-CAP 10**

To obtain **10**, 2-methyl-2-oxazoline (16.9 mL, 0.197 mol) **8** was added to 4-mercaptophenol **7** (24.9 g, 0.197 mol) and the reaction mixture was refluxed (130 °C) for 2 h under nitrogen atmosphere. The reaction was monitored by TLC. Then, the reaction mixture was cooled to 0°C. The formation of a white precipitate **9** was observed, which was collected by vacuum filtration and recrystallized from EtOH. Compound **9** (*N*-acetyl-HOPAES) (10.7 g, 50.5 mmol) was refluxed (170 °C) in concentrated HCl (20 mL) under a nitrogen atmosphere for 12 h. The reaction was monitored by TLC. Upon completion, the reaction mixture was cooled to room temperature, diluted with water (20 mL) and extracted with ethyl ether (2 x 15 mL). The organic phases were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. A solid compound was obtained which was recrystallized in hot EtOH / H_2O, to give pure 4-S-CAP (**10**).

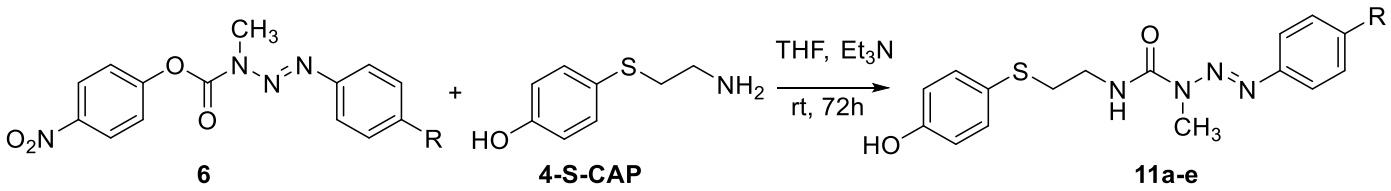
N-acetyl-HOPAES (**9**)

^{1}H-NMR (400 MHz, D_2O) δ: 1.77 (3H, s, COCH_3); 2.88 (2H, t, J= 6.3 Hz, NHCH_2); 3.24 (2H, m, J= 6.3 Hz, SCH_2); 6.76 – 7.30 (4H, AA'XX', J=8.1 Hz, *Ar*OH).

4-((2-aminoethyl)thio)phenol **10** (**4-S-CAP**)

^{1}H-NMR (400MHz, D_2O) δ: 3.06 (4H, s, SCH_2CH_2NH); 6.85 – 7.40 (4H, AA'XX', J=8,6 Hz, *Ar*OH). ^{13}C-DEPT-NMR δ (100MHz, D_2O): 36.34 (S*C*H_2); 41.82 (NH*C*H_2); 120.27 ((*C*H)$_2$ArOH); 138.39 ((*C*H)$_2$ArS).

Synthesis of hybrid compounds **11a-e**



To obtain hybrid compounds **11**, the corresponding pure carbamate (**6**) were synthesized by previously published methods [1]. To a solution in THF (10 mL) of the corresponding carbamate **6a-e** (0.425 mmol) was added 4-S-CAP (0.15 g, 0.863 mmol) and triethylamine (180 µL, 1.3 mmol). The reaction mixture was stirred at room temperature for 72 h, and monitored by TLC. After, the solvent was evaporated under reduced pressure and the crude purified by column chromatography (CH_2Cl_2/MeOH (95:5)). The isolated compounds were recrystallized from MeOH/*n*-hexane.

*3-[4-(hydroxyphenyl)thioethyl-aminocarbonyl]-1-(4-bromophenyl)-3-methyltriazene (**11a**)*

Yield 80%; yellow crystals; mp 167-170 ºC; ν_{max}/cm^{-1} 1671 (C=O); 3244 (NH); 3408 (OH); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.93 (2H, t, J=6,6 Hz, SCH_2); 3.30 (3H, s, NCH_3); 3.41 (2H, t, J=6,6 Hz, NHCH$_2$); 6.66 – 7.21 (4H, AA'XX', J=8,7 Hz, *Ar*OH); 7.38 – 7.49 (4H, AA'BB', J=8,8 Hz, *Ar*R); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 28.62 (N*C*H$_3$); 36.08 (S*C*H$_2$); 40.16 (N*C*H$_2$); 116.61 (*Ar*OH); 122.67 (*Ar*R); 123.82 (*Ar*OH) 123.85 (*Ar*R); 132.68 (*Ar*R); 134.74 (*Ar*OH); 148.44 (*Ar*R); 155.59 (*Ar*OH); 157,42 (*C*=O); ESI MS (m/z) [M+H]$^+$ Calcd for $C_{16}H_{17}BrN_4O_2S$ 408. Found ESI$^+$-MS: *m/z* (%): 446/448 [M+K]$^+$.

*3-[4-(hydroxyphenyl)thioethyl-aminocarbonyl]-1-(4-tolyl)-3-methyltriazene (**11b**)*

Yield 53%; yellow crystals; mp 151-154 ºC; ν_{max}/cm^{-1} 1668 (C=O); 3171 (NH); 3377 (OH).; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.24 (3H, s, R(CH_3)); 2.86 (2H, t, J=6,5 Hz, SCH_2); 3.25 (3H, s, NCH_3); 3.37 (2H, t, J=6,5 Hz, NHCH_2); 6.60 – 7.09 (4H, AA'XX', J=8,6 Hz, *Ar*OH); 7.15 – 7.32 (4H, AA'BB', J=8,2 Hz, *Ar*R); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 22.13 (R(*C*H$_3$)); 29.10 (N*C*H$_3$); 36.95 (S*C*H$_2$); 40.24 (N*C*H$_2$); 117.34 (*Ar*OH); 122.76 (*Ar*R); 124.22 (*Ar*OH); 130.93 (*Ar*OH); 135.44 (*Ar*R); 139.91 (*Ar*R); 156.29 (*Ar*OH); 157.97 (*Ar*R); 158.02 (*C*=O); ESI MS (m/z) $[M+H]^+$ Calcd for $C_{17}H_{20}N_4O_2S$ 344. Found ESI$^+$-MS: *m/z* 383 $[M+K]^+$.

*3-[4-(hydroxyphenyl)thioethyl-aminocarbonyl]-1-(4-cyanophenyl)-3-methyltriazene (**11c**)*

Yield 72%; yellow crystals; mp 168-170 ºC; ν_{max}/cm^{-1}: 1676 (C=O); 2230 (R(CN); 3235 (NH); 3381 (OH); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.77 (2H, t, J=6,2 Hz, SCH_2); 3.18 (3H, s, NCH_3); 3.28 (2H, t, J=6,4 Hz, NHCH_2); 6.47 – 7.04 (4H, AA'XX', J=7,6 Hz, *Ar*OH); 7.45 – 7.50 (4H, AA'BB', J=8,0 Hz, *Ar*R); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 28.92 (N*C*H$_3$); 35.85 (S*C*H2); 40.26 (N*C*H$_2$); 111.50 (*Ar*R); 116.48 (*Ar*OH); 118.92 (R(*C*N)); 122.98 (*Ar*R); 123.72 (*Ar*OH); 133.65 (*Ar*R); 134.30 (*Ar*OH); 152.56 (*Ar*R); 155.02 (*Ar*OH); 157.31 (*C*=O); ESI MS (m/z) $[M-H]^-$ Calcd for $C_{17}H_{17}N_5O_2S$ 355. Found ESI$^-$-MS: *m/z* (%):354 $[M-H]^-$.

*3-[4-(hydroxyphenyl)thioethyl-aminocarbonyl]-1-(4-ethoxycarbonylphenyl)-3-methyltriazene (**11d**)*

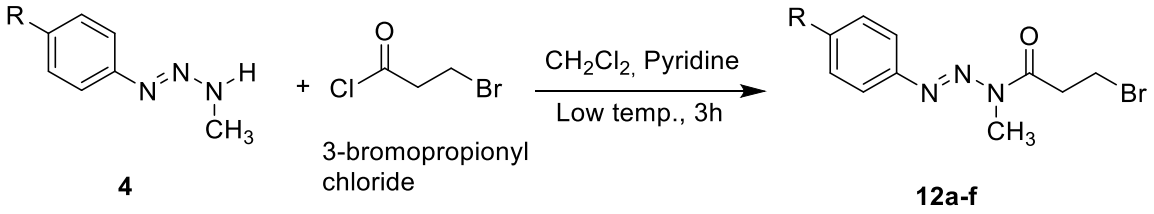
Yield 63%; yellow crystals; mp 120-122 ºC; ν_{max}/cm^{-1}) 1676 (C=O); 1713 (R(C=O)); 3211 (NH); 3392 (OH).; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.28 (3H, t, J = 7,1 Hz, R(CH_3)); 2.89 (2H, t, J=6,3 Hz, SCH_2); 3.30 (3H, s, NCH_3); 3.41 (2H, t, J=6,3 Hz, NHCH_2); 4.26 (2H, q, J = 7,1 Hz, (R(CH_2)); 6.62 – 7.18 (4H, AA'XX', J=8,5 Hz, *Ar*OH); 7.46 – 7.98 (4H, AA'XX', J=8,5 Hz, *Ar*R).); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.34 (R(*C*H$_3$)); 30.98 (N*C*H$_3$); 35.86 (S*C*H$_2$); 39.61 (N*C*H$_2$); 61.38 (R(*C*H$_2$)); 116.33 (*Ar*OH); 121.68 (*Ar*R); 123.26 (*Ar*OH); 129.93 (*Ar*R); 130.75 (*Ar*R); 134.16 (*Ar*OH); 152.36 (*Ar*R); 154.63 (*Ar*OH); 156.84 (*C*=O); 166.49 (*C*=O); ESI MS (m/z) $[M-H]^-$ Calcd for $C_{19}H_{22}N_4O_4S$ 402. Found ESI$^+$-MS: *m/z* (%):425 $[M+Na]^+$.

*3-[4-(hydroxyphenyl)thioethyl-aminocarbonyl]-1-(4-acetylphenyl)-3-methyltriazene (**11e**)*

Yield 54%; yellow crystals; mp 161-164 ºC; ν_{max}/cm^{-1} 1666 (C=O); 3210 (NH); 3408 (OH); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.10 (2H, t, J= 6,2 Hz, SCH_2); 2.94 (2H, t, J=6,2 Hz, NHCH_2); 2.56 (3H, s, R(CH_3)); 3.36 (3H, s, NCH_3); 6.66 – 7.22 (4H, AA'XX', J= 8,5 Hz, *Ar*OH); 7.54 – 7.95 (4H, AA'XX', J= 8,4 Hz, *Ar*R); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 26.63 (R(*C*H$_3$)); 28.59 (N*C*H$_3$); 35.80 (S*C*H$_2$); 39.53 (N*C*H$_2$); 116.21 (*Ar*OH); 121.89 (*Ar*R); 123.02 (*Ar*OH); 129.61 (ArR); 134.11 (*Ar*OH); 136.31 (*Ar*R); 152.44 (*Ar*R); 154.61 (*Ar*OH); 156.90 (*C*=O); 198.90 (R(*C*=O)).; ESI MS (m/z) $[M+H]^+$ Calcd for $C_{18}H_{20}N_4O_3S$ 372. Found ESI$^+$-MS: *m/z* (%):411 $[M+K]^+$.

General procedures for the synthesis of hybrid compounds 13a-f

Synthesis of intermediates **12a-f**



To a solution of 1.17 mmol of the corresponding MMT (**4**) in CH_2Cl_2 (5 mL) was added pyridine 1.17 mmol (94 µL) and 3-bromopropionyl chloride 1.41 mmol (140 µL). The reaction mixture was stirred for approximately 3 h in an ice bath. The progress of the reaction was monitored by TLC. Upon completion, the solvent was evaporated under reduced pressure and the resulting crude was purified by column chromatography using dichloromethane and *n*-hexane as eluents (10:90) to (50:50).

*3-[1-bromopropanoyl]-1-(4-bromophenyl)-3-methyltriazene (**12a**)*

Yield 35%; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 3.45 (3H, s, NCH_3), 3.53 (2H, td, J = 6,86 ; 0,70 Hz, CH_2CH_2Br), 3.75 (2H, t, J = 6,74 Hz, CH_2CH_2Br), 7.46-7.60 (4H, AA'BB', J = 8,87 Hz, *Ar*R).

*3-[1-bromopropanoyl]-1-(4-toluyl)-3-methyltriazene (**12b**)*

Yield 18%; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.54 (3H, s, R = CH_3), 3.56 (1.37 H, t, J = 6,82 Hz, CH_2CH_2Br), 3.57 (3H, s, NCH_3), 3.68 (0.55H, t, J = 6,94 Hz, CH_2CH_2Br), 3.88 (0.5H, t, J = 6,89 Hz, CH_2CH_2Br), 4.06 (1.49H, t, J = 6,79 Hz, CH_2CH_2Br), 7.38 (2H, d, J = 7,55 Hz, *Ar*R), 7.64 (2H, d, J = 8,31 Hz, *Ar*R).

*3-[1-bromopropanoyl]-1-(4-cyanophenyl)-3-methyltriazene (**12c**)*

Yield 22; %^{1}H-NMR (300 MHz, $CDCl_3$) δ: 3.44 (3H, s, NCH_3), 3.97 (1H, t, J = 6,6 Hz, CH_2CH_2Br), 3.81 (1H, td, J = 6,80 ; 6,5; 0,9 Hz, CH_2CH_2Br), 3.66 (1H, td, J = 6,80 ; 6,3; 0,9 Hz, CH_2CH_2Br), 3.54 (1H, t, J = 6,6 Hz, CH_2CH_2Br), 7.93 (2H, m, *Ar*R), 7.87 (m, 2H, *Ar*R).

*3-[1-bromopropanoyl]-1-(4-ethoxycarbonylphenyl)-3-methyltriazene (**12d**)*

Yield 15; %^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.38 (3H, t, J=7,2 Hz, CH_3), 3.44 (3H, s, NCH_3), 3.98 (0.6H, t, J = 6,6 Hz, CH_2CH_2Br), 3.82 (1.5H, td, J = 6,90 ; 6,5 ; 0,9 Hz, CH_2CH_2Br), 3.65 (1.5H, td, J = 6,80; 6,3 ; 0,9 Hz, CH_2CH_2Br), 3.53 (0.6H, t, J = 6,6 Hz, CH_2CH_2Br), 4.37 (2H, q, J=7,2 Hz, OCH_2) ; 8.13 (m, 2H, *Ar*R), 7.76 (m, 2H, *Ar*R).

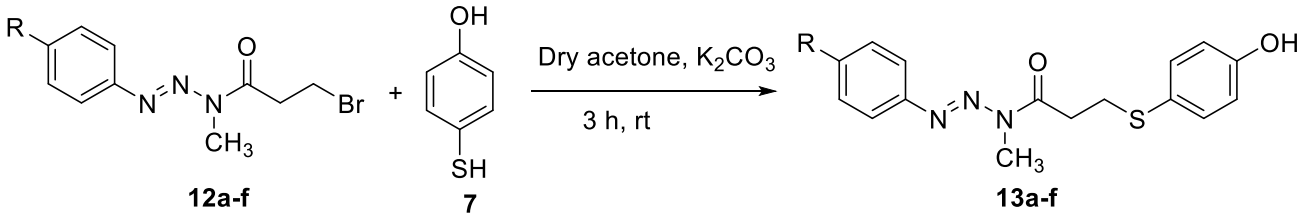
*3-[1-bromopropanoyl]-1-(4-acetylphenyl)-3-methyltriazene (**12e**)*

Yield 41%; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.62 (3*H*, s), 3.44 (3H, s, NC*H*$_3$), 3.97 (0.5H, t, *J* = 6,6 Hz, C*H*$_2$C*H*$_2$Br), 3.82 (1,5H, td, *J* = 6,80 ; 6,3 ; 0,9 Hz, C*H*$_2$C*H*$_2$Br), 3.65 (1.5H, td, *J* = 6,80 ; 6,3; 0,9 Hz, C*H*$_2$C*H*$_2$Br), 3.53 (0,5H, t, *J* = 6.6 Hz, C*H*$_2$C*H*$_2$Br), 8.12 (m, 2H, *Ar*R), 7.77 (m, 2H, *Ar*R).

*3-[1-bromopropanoyl]-1-(4-chlorophenyl)-3-methyltriazene (**12f**)*

Yield 24%; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 3.35 (0.60 H, t, *J* = 6,86 Hz, C*H*$_2$C*H*$_2$Br), 3.37 (3H, s, NC*H*$_3$), 3.46 (1.5H, t, *J* = 6,90 Hz, C*H*$_2$C*H*$_2$Br), 3.67 (1.26 H, td, *J* = 6,79 ; 0,6 Hz, C*H*$_2$C*H*$_2$Br), 3.85 (0.7 H, t, *J* = 6,6 Hz, C*H*$_2$C*H*$_2$Br), 7.33 (2H, m, *Ar*R), 7.47 (2H, m, *Ar*R).

Synthesis of hybrid compounds **13a-f**



12a-f **7** **13a-f**

To a solution of 4-mercaptophenol **7**, 0.64 mmol (80 mg) in dry acetone (5 mL) was added 1.28 mmol (175 mg) of K_2CO_3 and the reaction mixture was stirred at room temperature for 15 minutes. A solution of the corresponding compound **12a-f** (0.32 mmol) in 5 mL of dry acetone was thereafter slowly added to the reaction mixture and stirred for 3 h (protected from sunlight) at room temperature. Upon completion, the solvent was evaporated. The desired product (**13**) was purified by column chromatography (Table S1).

*3-[4-(hydroxyphenyl)thioethyl-carboxamide]-1-(4-bromophenyl)-3-methyltriazene (**13a**)*

Yield 74%; mp 154-157 ºC. ^{1}H-NMR (300 Hz, Acetone-d_6) δ: 3.16-3.22 (4H, m, COC*H*$_2$C*H*$_2$S), 3.36 (3H, s, NC*H*$_3$), 6.81-7.36 (4H, AA'XX', *J* = 8,73 Hz, *Ar*OH), 7.44-7.68 (AA'BB', 4H, *J* = 8,86 Hz, R*Ar*), 8.59 (s, 1H, O*H*). ^{13}C-NMR (75 Hz, Acetone-d_6) δ: 27.98 (N*C*H$_3$), 32.07 (CO*C*H$_2$CH$_2$S), 35.10 (COCH$_2$*C*H$_2$S), 117.15 (*Ar*OH), 123.11 (*Ar*OH), 124.72 (*Ar*R), 125.01 (*Ar*R), 133.36 (*Ar*R), 135.36 (*Ar*OH), 149.06 (*Ar*R), 158.35 (*Ar*OH), 174.34 (N*C*O). ESI MS (m/z) $[M-H]^-$ Calcd for $C_{16}H_{16}BrN_3O_2S$ 393. Found ESI$^-$-MS: *m/z* (%): 392/394 $[M-H]^-$.

*3-[4-(hydroxyphenyl)thioethyl-carboxamide]-1-(4-tolyl)-3-methyltriazene (**13b**)*

Yield 45%; mp 144-148 ºC. ^{1}H-NMR (300 Hz, Acetone-d_6) δ: 2.37 (3H, s, R = C*H*$_3$), 3.13-3.22 (4H, m, COC*H*$_2$C*H*$_2$S), 3.34 (3H, s, NC*H*$_3$), 6.81-7.37 (4H, AA'XX', *J* = 8,71 Hz, *Ar*OH), 7.29-7.46 (4H, AA'BB', *J* = 8,19 Hz, *Ar*R), 8,62 (s, 1H, O*H*). ^{13}C-NMR (75 Hz, Acetone-d_6) δ: 21.22 (R = *C*H$_3$), 27.63 (N*C*H$_3$), 32.02 (CO*C*H$_2$CH$_2$S), 35.08 (COCH$_2$*C*H$_2$S), 117.02 (*Ar*OH), 122.71 (*Ar*OH), 124.95 (*Ar*R), 130.69 (*Ar*R), 135.23 (*Ar*OH), 140.01 (*Ar*R), 147.63 (*Ar*R), 158.21 (*Ar*OH), 174.14 (N*C*O). ESI MS (m/z) [M-H]$^-$ Calcd for $C_{17}H_{19}N_3O_2S$ 329. Found ESI$^-$-MS: *m/z* (%): 328 [M-H]$^-$.

*3-[4-(hydroxyphenyl)thioethyl-carboxamide]-1-(4-cyanophenyl)-3-methyltriazene (**13c**)*

Yield 54%; mp 179-181 ºC. ^{1}H-NMR (300 Hz, Acetone-d_6) δ: 3.28-3.14 (4H, m, COC*H*$_2$C*H*$_2$S), 3.39 (3H, s, NC*H*$_3$), 6.82 (2H, m, *ArH*), 7.33 (2H, m, *ArH*), 7.67 (2H, m, *ArH*), 7.88 (2H, dt, *J* = 8,7; 2,1; 1,8 Hz, Ar*H*), 8.55 (1H, s, O*H*). ^{13}C-NMR (75 Hz, Acetone-d_6) δ: 28.19 (N*C*H$_3$), 31.92 (CO*C*H$_2$CH$_2$S), 34.97 (COCH$_2$*C*H$_2$S), 112.79 (*Ar*R), 117.06 (*Ar*OH), 119.10 (*CN*), 123.63 (*Ar*R), 124.87 (*Ar*OH), 134.36 (*Ar*R), 135.26 (*Ar*R), 135.23 (*Ar*OH), 152.85 (*Ar*R), 158.24 (*Ar*OH), 174.42 (N*C*O). ESI MS (m/z) [M-H]$^-$ Calcd for $C_{17}H_{16}N_4O_2S$ 340. Found ESI$^-$-MS: *m/z* (%):339 [M-H]$^-$.

*3-[4-(hydroxyphenyl)thioethyl-carboxamide]-1-(4-ethoxycarbonylphenyl)-3-methyltriazene (**13d**)*

Yield 41%; mp 142-145 ºC. ^{1}H-NMR (300 Hz, Acetone-d_6) δ: 1.39 (3H, t, *J*=7,2 Hz, C*H*$_3$), 3.28-3.14 (2H, m, COC*H*$_2$C*H*$_2$S), 3.39 (3H, s, NC*H*$_3$), 4.38 (2H, q, *J*=7,2 Hz, C*H*$_2$), 6.82 (2H, m, *ArH*), 7.33 (2H, m, *ArH*), 7.62 (2H, m, *ArH*), 8.12 (dt, 2H, *J*= 8,7; 2,1Hz, Ar*H*), 8.75 (s, O*H*). ^{13}C-NMR (75 Hz, Acetone-d_6) δ: 14.61 (*C*H$_3$), 28.06 (N*C*H$_3$), 31.92 (CO*C*H$_2$CH$_2$S), 35.02 (COCH$_2$*C*H$_2$S), 61.68 (*C*H$_2$), 117.05 (*Ar*OH), 122.83 (*Ar*R), 124.79 (*Ar*OH), 124.87 (*Ar*OH), 131.42 (*Ar*R), 131.47 (*Ar*R), 135.26 (*Ar*OH), 153.11 (*Ar*R), 158.28 (*Ar*OH), 166.20 (*C*O), 174.41 (N*C*O). ESI MS (m/z) [M-H]$^-$ Calcd for $C_{19}H_{21}N_3O_4S$ 387. Found ESI$^-$-MS: *m/z* (%):386 [M-H]$^-$.

*3-[4-(hydroxyphenyl)thioethyl-carboxamide]-1-(4-acetylphenyl)-3-methyltriazene (**13e**)*

Yield 47%; mp 137-140 ºC. ^{1}H-NMR (300 Hz, Acetone-d_6) δ: 2.63 (3H, s, C*H*$_3$), 3.27-3.13 (4H, m, COC*H*$_2$C*H*$_2$S), 3.38 (3H, s, NC*H*$_3$), 6.83 (2H, m, *ArH*), 7.33 (2H, m, *ArH*), 7.60 (2H, m, *ArH*), 8.09 (2H, dt, *J*= 8,7; 2,1Hz, Ar*H*), 8.70 (s, O*H*). ^{13}C-NMR (75 Hz, Acetone-d_6) δ: 28.06 (N*C*H$_3$), 26.81 (*C*H$_3$), 31.89 (CO*C*H$_2$CH$_2$S), 34.97 (COCH$_2$*C*H$_2$S), 117.22 (*Ar*OH), 122.73 (*Ar*R), 124.74 (*ArOH*), 130.41 (*Ar*R), 135.29 (*Ar*OH),), 137.90 (*Ar*H), 152.82 (*Ar*H), 158.24 (*Ar*OH), 174.35 (N*C*O), 197.24 (*C*O). ESI MS (m/z) [M-H]$^-$ Calcd for $C_{18}H_{19}N_3O_3S$ 357. Found ESI$^-$-MS: *m/z* (%):356 [M-H]$^-$.

*3-[4-(hydroxyphenyl)thioethyl-carboxamide]-1-(4-chlorophenyl)-3-methyltriazene (**13f**)*

Yield 47%; mp 159-160 ºC. ^{1}H-NMR (300 Hz, Acetone-d_6) δ: 3.08-3.21 (4H, m, COC*H*$_2$C*H*$_2$S), 3.33 (3H, s, NC*H*$_3$), 6.75-7.27 (4H, AA'XX', *J* = 8,7 Hz, *Ar*OH), 7.46 (4H, s , *Ar*R). ^{13}C-NMR (75 Hz, Acetone-d_6) δ: 26.79 (N*C*H$_3$), 31.09 (CO*C*H$_2$CH$_2$S), 34.04 (COCH$_2$*C*H$_2$S), 115.93 (*Ar*OH), 123.32 (*Ar*OH), 123.64 (*Ar*R), 129.24 (*Ar*R), 134.10 (*Ar*R), 134.35 (*Ar*OH), 147.50 (*Ar*R), 157.37 (*Ar*OH), 173.77 (N*C*O). ESI MS (m/z) [M-H]$^-$ Calcd for $C_{16}H_{16}ClN_3O_2S$ 349. Found ESI$^-$-MS: *m/z* (%):348 [M-H]$^-$.

Table S1. Solvents for chromatography purification (Chrom) and for recrystallization (Recryst) of the compounds **13a-e**.

Comp.	**Chrom.**	**Recryst.**
13a	30:70 DCM/*n*-hexane	---
13b	DCM	DCM/Petroleum ether
13c	DCM	DCM/Petroleum ether
13d	99:1 DCM:MeOH	DCM/Petroleum ether
13e	DCM	DCM/Petroleum ether

HPLC analysis

The analytical high-performance liquid chromatography (HPLC) system comprised a Merck Hitachi L-7110 pump with an L-7400 UV detector, a manual sample injection module equipped with a 20 µL loop, a Merck Lichrospher 100 RP18 125 mm 4.6 mm (5 µm) column equipped with a Merck Lichrocart pre-column (Merck, Germany). In all studies, the HPLC conditions were: detector wavelength 300 nm; mobile phase, acetonitrile/water, flow rate 1.0 mL/min. (Table S2 and S3)

Table S2. Maximum UV absorption wavelength - λ_{max} values (nm) for compounds **11** and **13**, in ACN.

Comp.	**λ_{max}**	**Comp.**	**λ_{max}**
11a	299	**13a**	294
11b	297	**13b**	292
11c	304	**13c**	296
11d	305	**13d**	302
11e	307	**13e**	301

Table S3. HPLC elution conditions for the mobile phase in kinetic assays for compounds **11a-e** and **13a-e**.

Compound	**Mobile phase**	**Compound**	**Mobile Phase**
11a	50:50 ACN/H_2O	**13a**	55:45 ACN/H_2O
11b	50:50 ACN/H_2O	**13b**	55:45 ACN/H_2O
11c	40:60 ACN/H_2O	**13c**	45:55 ACN/H_2O
11d	50:50 ACN/H_2O	**13d**	50:50 ACN/H_2O
11e	40:60 ACN/H_2O	**13e**	55:45 ACN/H_2O

Stability studies

Phosphate buffered saline

A 30 μL aliquot of a 10^{-2} M stock solution of compounds **11a-e** and **13a-e** in ACN was added to 10 mL of PBS (0.01 M, pH=7.4) thermostated at 37ºC. At different times, several aliquots of the reaction mixture were taken and analysed by HPLC at λ = 300 nm with an isocratic elution ACN:H_2O. (Table S3).

Human plasma (80% v/v)

The human plasma was collected from several healthy donors in sodium heparin and stored at -70ºC until required. A mixture of 2 mL of human plasma and 0.5 mL of PBS (0.01 M, pH=7.4) was thermostated at 37ºC. To this mixture were added 10 μL of a 10^{-2} M stock solution of compound **11a-e** and **13a-e** in ACN. Several aliquots (200 μL) of the reaction mixture were taken at different times and added to eppendorfs already containing 400 μL of cold ACN. The eppendorfs were centrifuged at 14000 rpm for 5 min and the supernatant was analysed by HPLC at λ = 300 nm with an isocratic elution ACN:H_2O. (Table S3)

Mushroom tyrosinase

Tyrosinase (Sigma, UK) was received as a lyophilised powder. The enzyme was dissolved in PBS (pH 7.4) to produce a stock solution of 10 000 U/mL. Aliquots of the stock solution (89.4 μL, 900 units / 29.8 μL, 300 units) were stored at -20ºC until used. Depending on the enzyme units needed for each assay, the aliquots were added to a solution of 2.4 mL of PBS (0.01 M, pH=7.4) and 0.6 mL of DMSO at 37ºC. To this mixture were added 10 μL of a 10^{-2} M stock solution of compounds **11a-e** and **13a-b** in ACN. Several aliquots (200 μL) of the reaction mixture were collected at selected times and added to eppendorfs with 400 μL of cold ACN. The Eppendorf's were centrifuged at 14000 rpm for 5 min and the supernatant was analysed by HPLC at λ = 300 nm with an isocratic elution ACN:H_2O. (Table S3)

Evaluation of hybrids as tyrosinase substrates

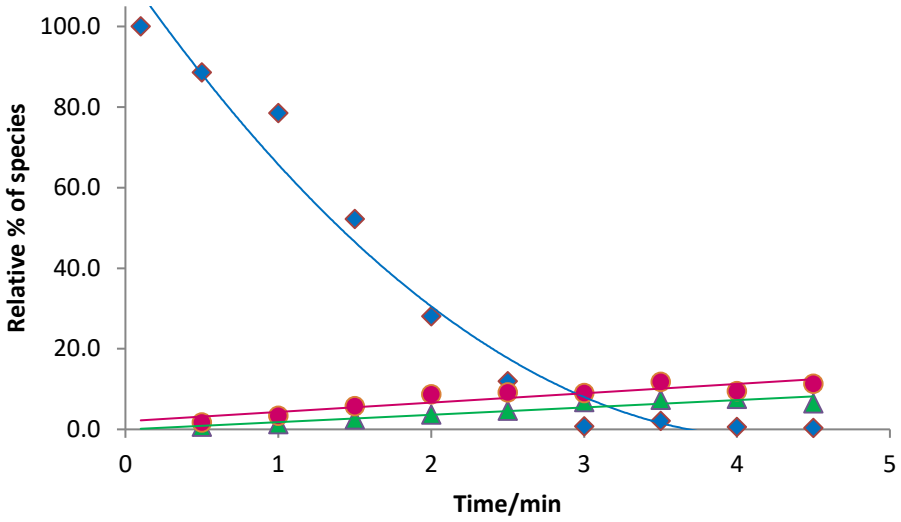
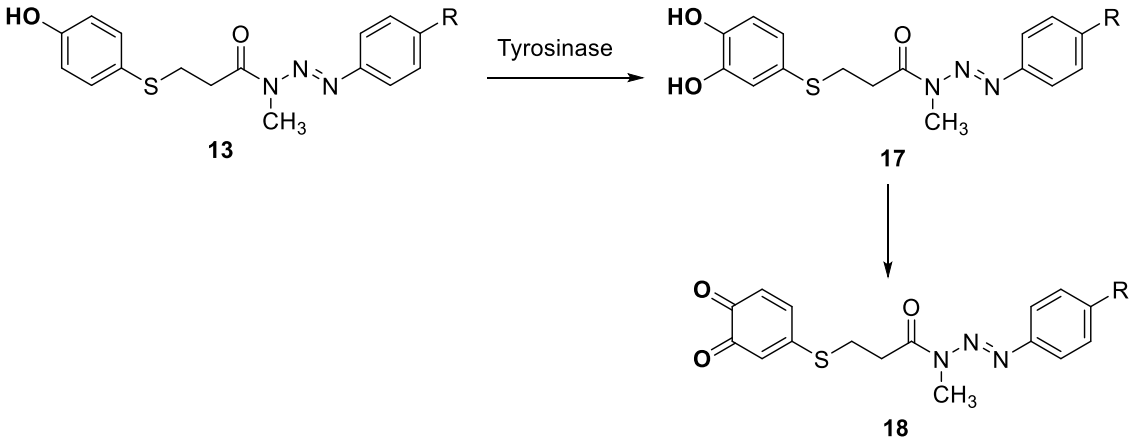


Figure S1 – Time-course curves for the decay of hybrid compound **11c** (♦)**,** monomethyltriazene **4c** (▲) and the respective aniline **14c** (●) in the presence of the enzyme tyrosinase (100 U/mL).



Scheme S1. Degradation of hybrid compounds **13** in the presence of tyrosinase.

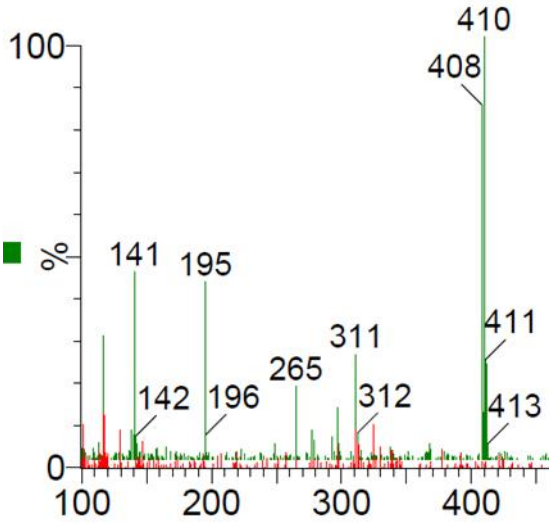


Figure S2. Mass spectra of the peak with Rt =5,17 min for the incubation of compound **13a** in the presence of tyrosinase.

General procedure for the determination of K_m and V_{max}

The K_m and V_{max} with mushroom tyrosinase were determined by measuring the initial rate of conversion of hybrid compounds **11b-d** into the oxidation products at the wavelength previously selected, using a range of prodrug concentrations (0–0.4 mM) in the presence of the enzyme ($5x10^{-6}$ mM). K_m and V_{max} were calculated through a non-linear Michaelis–Menten regression with the Graphpad Prism program. The k_{cat} was calculated from the V_{max} by dividing it by the amount of enzyme (M) in the reaction mixture. *Km* and *Vmax* values were determined by HPLC method as described previously. The tyrosinase concentration was calculated taking the value of Mr as 120,000 Da. Protein content was determined by Bradford's method using bovine serum albumin as standard. Each substrate was incubated with 8.0 units of mushroom tyrosinase in a water bath at 37ºC at six different compound concentrations (0 and 0.4 mM), following simple saturation kinetics. Initial rates were determined by the slope of the straight line of the initial reaction for decrease of the compounds **11b-d** concentration versus time. Values are the mean of triplicates. Data were fit using a simple Michaelis Menten equation to yield K_m and V_{max} values. Linear regression analysis was performed using GraphPad Prism 5, GraphPad Software Inc.

Table S4. Kinetic parameters for the oxidation of triazene derivatives **11b-d** by mushroom tyrosinase, compared with similar (previous) compounds

Compound	K_m (mM)	V_{max} ($x10^{-5}$ mM/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	**Ref.**
11b	0.12±0.01	0.14±0.01	468.54	3904.5 $x10^3$	
11c	0.064±0.01	0.058±0.001	194.11	3032.9 $x10^3$	
11d	0.082±0.01	0.061±0.01	204.15	2489.6 $x10^3$	
L-tyrosine	0.27±0.01	1800±5	7.9	29259	[2]
Tyramine	0.51±0.02	590±30	25.9	51000	[3,4]
Dopamine	2.2±0.1	10000±520	439	200000	[3,4]
1 (X=H; R=CN)	0.31±0.08	3000±500	0,6	1935	[3]
1 (X=OH ; R=EtOCO)	0.71±0.40	31000±14000	6	8450	[3]
2 (n=4, R=CN)	0.86±0.22		0.009	1.39	[5]
2 (n=4; R=MeOCO)	1.08±0.25		0.013	13.88	[5]
3 (R=CN)	0.20±0.09	0.5±0.05	0.030	151	[6]
3 (R=MeOCO)	0.22±0.04	1.0±0.04	0.050	247	[6]
10 4-**S-CAP**	0.117	3.9	--	--	[7]

Values reported are the average ± one standard deviation for the three independent determinations.

Apparent partition coefficients

The apparent partition coefficients (log P_{exp}) were determined at room temperature using 1-octanol–phosphate buffer (pH 7.4). Each phase was mutually saturated before the experiment. The volumes of each phase were chosen so that solute concentrations in the aqueous phase after distribution were readily measurable. The compounds were dissolved in 1-octanol, and the 1-octanol–phosphate buffer mixtures were shaken for 30 min to reach an equilibrium distribution. Each phase was analysed separately by HPLC, but the organic phase was diluted in acetonitrile (1:10) before HPLC analysis. Partition coefficients, P_{exp}, were calculated from the ratio of the peak area in octanol to the peak area in the buffer.

Molecular Properties Scores

Table S5. Molecular properties for compounds **11** and **13**. [8]

Compound	MW	LogP (calc)*	HBA	HBD	TPSA	Rotatable Bonds (n)
11a	409.31	4.31	6	2	77.29	6
11b	344.44	3.88	6	2	77.29	6
11c	355.42	3.54	7	2	101.08	6
11d	402.48	3.88	8	2	103.6	9
11e	372.45	3.51	7	2	94.36	7
13a	394.29	5.06	5	1	65.27	6
13b	329.43	4.43	5	1	65.27	6
13c	340.41	4.15	6	1	89.06	6
13d	387.46	4.68	7	1	91.57	9
13e	357.44	3.99	6	1	82.34	7
13f	349.84	4.71	5	1	65.27	6

*Calculated values: VCCLAB, Virtual Computational Chemistry Laboratory, http://www.vcclab.org, 2005.

General procedure for the cytotoxicity screening assays

Cell lines culture conditions

The human melanoma cell line MNT-1 (kindly offered by Dr. José Ramalho, Faculdade de Ciências Médicas, Lisbon, Portugal), the murine melanoma B16F10 cell line (ATCC#CRL-6475) and the non-melanoma cell line (the human keratinocyte cell line) HaCaT, (CLS, 300493), were cultured in Dulbecco's modified Eagle's medium (DMEM) with high-glucose (4500 mg/L), supplemented with 10% fetal bovine serum (FBS) and 50 U/mL penicillin, 50 μg/mL streptomycin (hereafter complete medium), at 37ºC in a 5% CO_2 humidified atmosphere.

Cell proliferation and viability assays

The viability of melanoma and non-melanoma cells in the presence of the synthesized compounds was performed by the MTS assay according to the manufacturer's instructions. **[**9**]**

Cells were seeded in 96-well plates (200 μL; 1.5 x 10^3 cells/mL) for 24 h. The culture medium was removed and the compounds at concentrations ranging from to 20 to 150 μM prepared in complete medium were added. Cells were allowed to proliferate for 72 h at 37ºC in a humidified CO_2 controlled atmosphere. After that, the media was removed and replaced with incomplete fresh media. Subsequently, 20 μL of MTS were added to each well and incubated for 2 h under the same culture conditions as above mentioned. The absorbance was measured at 490 nm in a BioRad microplate reader Model 680 (Bio-Rad, Hercules). Control assays were conducted in the absence of compounds and in the presence of similar amounts of organic solvent used in the highest concentration tested. All the tests were performed with six samples for each concentration under study.

The IC_{50} (50% inhibitory concentration) was defined as the concentration of the compound that inhibited cell proliferation by 50% calculated by linear regression analysis.

Hepatotoxicity studies

We performed the spectrophotometric quantification of the GSH (free thiol) using Ellman's reagent. This reagent is composed of 5,50-dithiobis(2-nitrobenzoic acid) (DTNB), which reacts with free thiolates, forming a disulfide with liberation of the dianion, 2-nitro-5-thiobenzoic acid (TNB) (Figure S3). TNB exists at neutral pH as an intensely coloured dianion (TNB^{2-}), with a λ_{max} = 412 nm. [10]

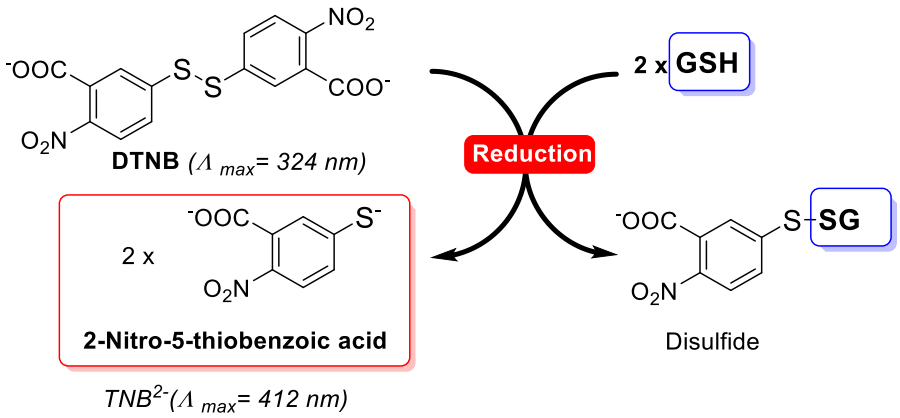


Figure S3. Mechanism of the nucleophilic substitution reaction between DTNB and GSH (thiol) showing the generation of the chromophoric product TNB^{2-}.

In these assays, the percentage of GSH depletion (GSH depletion (%)) observed is related with the non-depleted GSH by the equation 1:

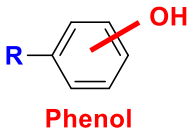
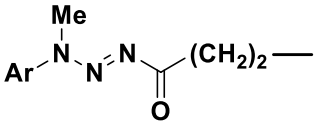
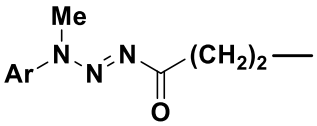
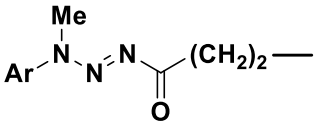
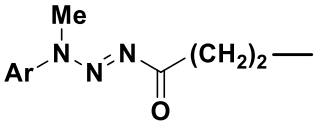
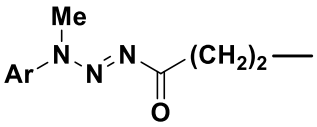
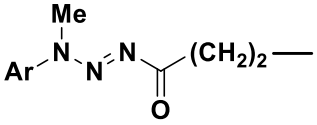
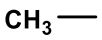
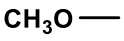
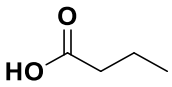
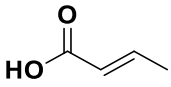
$$\text{GSH}_{\text{deplection}}\ (\%) = \left(\frac{\text{Total GSH (n)} - \text{Non deplected GSH (n)}}{\text{Total GSH (n)}} \times 100 \right) \times 2^{*} \qquad \textbf{(Equation 1)}$$

*- The GSH concentration used in these assays was twice as the concentration of triazene hybrids

Incubation mixture contained 881 µL of phosphate buffer (0.1M, pH 7.4, DETAPAC (Diethylenetriaminepentaacetic acid), 1mM, 50 µL of rat liver microsomes solution (20 mg/mL), 20 µL of GSH solution (10 mM), 32.5 µL of NADPH solution A (31 mM $NADP^{+}$, 66 mM Glucose-6-phosphate and 66 mM $MgCl_2$ in H_2O), 6.5 µL of NADPH solution B (40 U/mL Glucose-6-phosphate dehydrogenase in 5 mM sodium citrate) and 10 µL of a 10^{-2} M stock solution of compound **11c** in a final volume of 1 mL. The mixture was gently mixed at 37ºC. Then, three aliquots of 250 µL were taken at different times (30, 60, 180 min) and added to eppendorfs with 25 µL of trichloroacetic acid (30% w/v). Reaction mixture was centrifuged at 14000 rpm for 5 min. The GSH levels of a 100 µL aliquot of the supernatant was determined by the addition of 875 µL of Tris/HCl buffer (0.1 M, pH 8.9) and 25 µL of DTNB (5,5'-dithio-bis-[2-nitrobenzoic acid) solution (2 mg/mL). The absorbance of the solution was measured at 412 nm.

We observed that compounds **2 CARB-TZ** promote an increase of GSH depletion (%), reaching values of depletion between 35% and 64% in 180 minutes incubation assays.

Table S6. Results for the metabolism of phenolic compounds by rat liver microsomal preparations translated into GSH depletion levels

Compound	R—		**% GSH depletion**	**Ref.**
2 CARB-TZ (Ar = *p*-MeOCO-Ph)		3-hydroxy	45.7±5.0	[5]
2 CARB-TZ (Ar = *p*-CN-Ph)		3-hydroxy	63.5±5.0	[5]
2 CARB-TZ (Ar = *p*-MeOCO-Ph)		4-hydroxy	39.3±1.0	[5]
2 CARB-TZ (Ar = *p*-MeCO-Ph)		4-hydroxy	34.6±8.6	[5]
2 CARB-TZ (Ar = *p*-CN-Ph)		4-hydroxy	39.6±8.6	[5]
2 CARB-TZ (Ar = *p*-NH_2CO-Ph)		4-hydroxy	43.6±2.0	[5]
3-HT		3-hydroxy	51±4	[11]
3-HAP		3-hydroxy	48±4	[11]
4-HT		4-hydroxy	60±7	[11]
4-HA		4-hydroxy	44±3	[11]
4-HPP		4-hydroxy	56±4	[11]
4-HCA		4-hydroxy	92±5	[12]

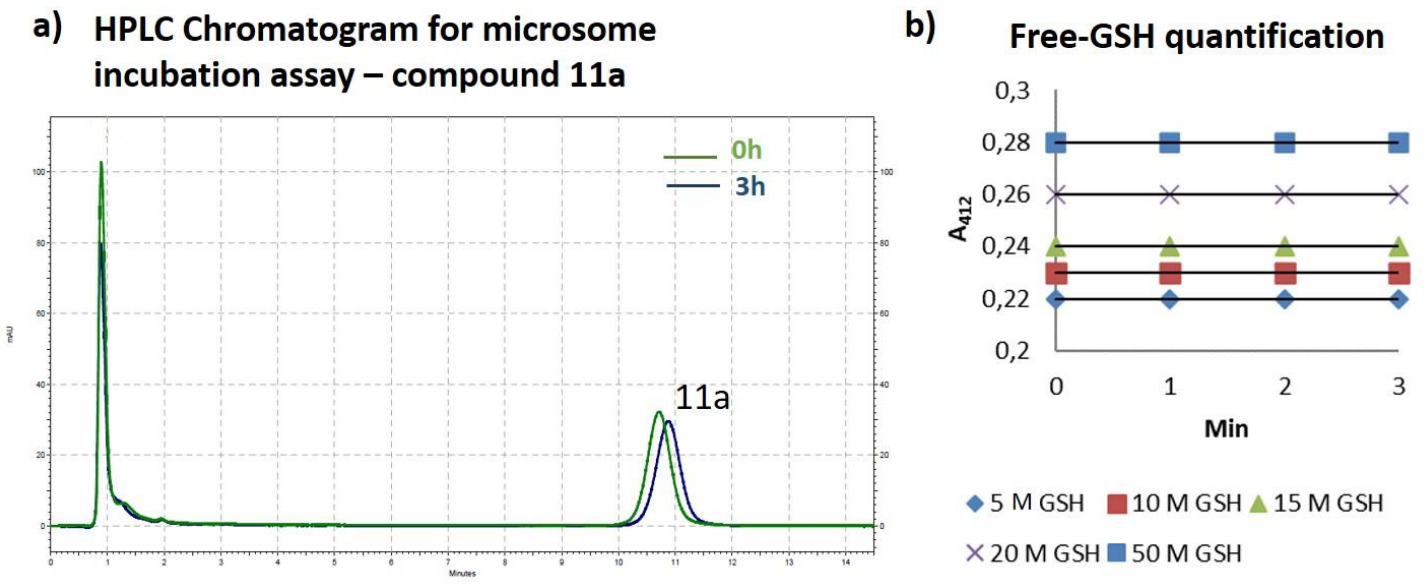


Figure S4- Toxicity assessment of compound **11a**: **a**) HPLC study for the incubation of **11a** with mice microsomes. **b**) *In vitro* spectrophotometric quantification of free glutathione after incubation of compound **11a** with microsomes.

***In vivo* toxicity profile in BALB/c mice**

In vivo safety assessment of **11d**

Healthy BALB/c mice received three i.v. administrations of **11d** at a dose of 12 mg/kg of body weight. The control group was constituted by a group of mice that received the vehicle for HM solubilization. Two days after the last administration, organs of interest and blood were collected. Tissue indexes and γ-GT levels from plasma samples were determined. Results are expressed as mean ± s.d. (n = 5).

All animal experiments were conducted according to the animal welfare organ of the Faculty of Pharmacy, Universidade de Lisboa, approved by the competent national authority Direção-Geral de Alimentação e Veterinária (DGAV) and in accordance with the EU Directive (2010/63/UE) and Portuguese laws (DR 113/2013, 2880/2015 and 260/2016 and 1/2019) for the use and care of animals in research.

Haemolysis assay

The haemolytic activity of HM in free form was determined using EDTA-preserved peripheral human blood, obtained from voluntary donors and used in the same day of experiments [8]. Serum was firstly removed by centrifugation at 1,000 g, for 10 min and, after, erythrocyte suspension was washed three times in PBS, at 1,000 g, for 10 min. **11d** concentrations ranging from 0.2 to 400 μM were distributed in 96-well plates (100 μL/well). Then, 100 μL of erythrocyte suspension was added to all wells, incubated at 37 °C for 1 h, and centrifuged at 800 g for 10 min. Absorbance of supernatants was measured at 550 nm with a reference filter at 620 nm. The percentage of the haemolytic activity for each sample was calculated comparing each individual determination to a positive control, corresponding to 100% haemolysis (erythrocytes in distilled water) and negative control (erythrocytes in PBS) according to equation (2), where AbsS is the average absorbance of the sample, AbsN is the average absorbance of the negative control and AbsP is the average absorbance of the positive control.

$$\text{Haemolysis (\%)} = ((\text{AbsS}-\text{AbsN}))/((\text{AbsP}-\text{AbsN})) \times 100) \quad \textbf{(Equation 2)}$$

References

[1] - V. Capucha, E. Mendes, A.P. Francisco, M.J. Perry, Development of triazene prodrugs for ADEPT strategy: New insights into drug delivery system based on carboxypeptidase G2 activation, Bioorganic Med. Chem. Lett. (2012) 6903-6908. https://doi.org/10.1016/j.bmcl.2012.09.029.

[2] - J.C. Espin, P.A. Garcia-Ruiz, J. Tudela, F. Garcia-Canovas, Study of stereospecificity in mushroom tyrosinase, Biochem. J. 331 (1998) 547–551. https://doi.org/10.1042/bj3310547.

[3] - M.J. Perry, E. Mendes, A.L. Simplício, A. Coelho, R.V. Soares, J. Iley, R. Moreira, A.P. Francisco, Dopamine- and tyramine-based derivatives of triazenes: Activation by tyrosinase and implications for prodrug design, Eur. J. Med. Chem. 44 (2009) 3228-3234. https://doi.org/10.1016/j.ejmech.2009.03.025.

[4] - J.C. Espin, R. Varon, L.G. Fenoll, M.A. Gilabert, P.A. Garcia-Ruiz, J. Tudela, F. Garcia-Canovas, Kinetic characterization of the substrate specificity and mechanism of mushroom tyrosinase, Eur. J. Biochem. 267 (2000) 1270–1279. https://doi.org/10.1046/j.1432-1327.2000.01013.x.

[5] - A. Sousa, F. Santos, M.M. Gaspar, S. Calado, J.D. Pereira, E. Mendes, A.P. Francisco, M.J. Perry, The selective cytotoxicity of new triazene compounds to human melanoma cells, Bioorganic Med. Chem. 25 (2017) 3900–3910. https://doi.org/10.1016/j.bmc.2017.04.049.

[6] - A.S. Monteiro, J. Almeida, G. Cabral, P. Severino, P.A. Videira, A. Sousa, R. Nunes, J.D. Pereira, A.P. Francisco, M.J. Perry, E. Mendes, Synthesis and evaluation of N-acylamino acids derivatives of triazenes. Activation by tyrosinase in human melanoma cell lines, Eur. J. Med. Chem. 70 (2013) 1–9. https://doi.org/10.1016/j.ejmech.2013.09.040.

[7] - Y. Minamitsuji, K. Toyofuku, S. Sugiyama, K. Yamada, K. Jimbow, Sulfur containing tyrosine analogs can cause selective melanocytotoxicity involving tyrosinase-mediated apoptosis, J. Investig. DERMATOLOGY Symp. Proc. 4 (1999) 130–136. https://doi.org/10.1038/sj.jidsp.5640196.

[8] - Molinspiration software - https://www.molinspiration.com/cgi-bin/properties

[9] - Nave, M. et al. Nanoformulations of a potent copper-based aquaporin inhibitor with cytotoxic effect against cancer cells. Nanomedicine 11, 1817–1830 (2016).

[10] - Frederick A. Villamena, Chapter 6 - UV–Vis Absorption and Chemiluminescence Techniques, Editor(s): Frederick A. Villamena, Reactive Species Detection in Biology, Elsevier, 2017, Pages 203-251, https://doi.org/10.1016/B978-0-12-420017-3.00006-2.

[11] - Vad, N. M., Kandala, P. K., Srivastava, S. K., & Moridani, M. Y. (2010). Structure-toxicity relationship of phenolic analogs as anti-melanoma agents: an enzyme directed prodrug approach. Chemico-biological interactions, 183(3), 462–471. https://doi.org/10.1016/j.cbi.2009.11.020