**Supplemental Section:**

**European Journal of Drug Metabolism and Pharmacokinetics**

# "Pharmacokinetics and toxicology of the neuroprotective *e,e,e*-methanofullerene(60)-63-tris malonic acid [$C_3$] in mice and primates"

Joshua I. Hardt, Joel S. Perlmutter, Christopher J. Smith, Kevin L. Quick, Ling Wei, Subhasish K. Chakraborty and Laura L. Dugan

**Synthesis of *e,e,e*-methanofullerene(60)-63-tris malonic acid (*C 3*).**

$C_3$ synthesis requires a two-step process beginning with esterification and followed by conversion to an acid. The $C_3$ ester [*e,e,e*-tris(dimethylmalonyl)fullerene] was prepared by dissolving $C_{60}$ (720 mg, 1.00 mmol) in toluene at a concentration of 1mg/ ml by stirring overnight at room temperature. Dimethyl bromomalalonate (632.4 mg, 2.69 mmol) was added, followed by 1, 8-diazabicyclo (5.4.0) undec-7-ene (DBU, 493 mg, 3.24 mmol). The reaction mixture was stirred for 2 hours, filtered through a silica gel pad and concentrated under rotary vacuum evaporator. The residue was column chromatographed on a 500 ml silica gel (Merck, 280 -400 mesh) column, starting with toluene. Subsequently the column eluted by increasing the amount of *tert* butyl acetate relative to the toluene. The $C_3$ ester fraction eluted in 3% *tert* butyl acetate in toluene was collected. The purity of $C_3$ ester was monitored by TLC and HPLC. Combined $C_3$ ester fractions were re-chromatographed using as solvent 2% *tert* butyl acetate in toluene. The yield of $C_3$ ester (250 mg) was approximately 23% based on $C_{60}$. Galbraith Laboratories (Knoxville, TN, USA) performed carbon-hydrogen analysis. Calculations for $C_{75}H_{18}O_{12}$ x $CH_3C_6H_5$ (1203.10) were C, 81.86; H, 2.18; with analysis giving a composition of C, 81.54, 81.52; H, 2.35, 2.33. Preparation of the acid form began with a solution of water (1.52 mL, 84.4 mmol), methanol (40 mL) and $C_3$ methyl ester (0.845 mmol by UV) in 950 mL of toluene (containing ~5% t-butyl acetate) that was stirred for 1 h under $N_2$. The initially cloudy solution was clear after 1 h. Sodium hydroxide in methanol (16.9 mL of 0.93 M, 16.9 mmol) was then added. After 1.5 h, all color was precipitated, and there was no ester remaining in the toluene solution as assessed by TLC. Next, 100 ml of water was added and stirred for 30 min, which resulted in the color going to the aqueous phase. The toluene layer was removed, and the aqueous layer concentrated *in vacuo* to remove methanol and excess toluene (if any) and then heated for 2 h at 60 ºC under $N_2$ to

complete the hydrolysis. The aqueous layer was chilled to 0-5 ºC, acidified with sulfuric acid (1.10 mL of 3.7 M, 4.07 mmol) and extracted with ethyl acetate (2x80 mL and 1x20 mL). The combined ethyl acetate extracts was washed with 3x80 mL of water that removed some contaminants. Concentrating the ethyl acetate fraction under vacuum at 45ºC gave 798.1 mg (92.1 % yield). Carbon-hydrogen calculations for $C_{69}H_6O_{12}$ x $2H_2O$ x $3CH_3OH$ (1158.95) were C,74.62; H,1.91; with results from analysis by Galbraith laboratories of C,75.15,74.99; H, 1.96,1.94. $^1H$ and $^{13}C$ NMR (Varian Unity 300 Spectrometer), as well as positive and negative ion electrospray mass spectroscopy (Agilent LC/MSD) confirmed chemical structures.

**Preparation of $^{14}C$-$C_3$ carboxyfullerene.**

Unlabeled *$C_3$* with purity greater than 97% was synthesized for these studies using a modification of the method of Lamparth [1]. To generate $^{14}C$-labeled *$C_3$* with exactly one $^{14}C$ per molecule on the extremely stable cyclopropane carbon required modification of the Lamparth procedure as follows. 1-hydroxybenzotriazole (0.50 mmol) in 0.8 ml of methanol was added to malonic acid-2-$^{14}C$ (98.5%, 0.080 mmol, 0.92 mCi) in 10.5 ml of methanol followed by 1,3-diisopropylcarbodiimide (0.077ml, 0.492 mmol) and 4-(dimethylamino) pyridine (0.058 mmol in 0.2 ml of methanol). After 6 days, 20 ml of toluene was added, and the volume was reduced to approximately 5 ml. Pure dimethyl malonate-2-$^{14}C$ was extracted from this liquid by chromatography (1 cm x 10 cm column silica gel) with a gradient of ethyl acetate in toluene (0 to 5%)]. Purified dimethyl malonate-2-$^{14}C$ (~0.081 mmol) and *e,e* -$C_{62}(COOMe)_4$ (0.155 mmol) were combined in 100 ml of 25% dichloromethane (DCM) in toluene. Iodine (0.095 mmol) in 0.5 ml of toluene was added followed by 1, 8-diazobicyclo[5.4.0]undec-7-ene (0.22 mmol). After one hour, the reaction mixture was chromatographed on a silica gel column in 25 %

DCM/toluene. Using a step-wise gradient of toluene from 0 to 10% ethyl acetate, we eluted the mono-, bis, tris- and finally tetra-malonic ester derivatives. The *e,e,e*-$C_{62}{}^{14}C(COOMe)_6$ isomer (*$C_3$*) appeared in the 5% ethyl acetate fraction. Solvents were removed *in vacuo*. Hydrolysis to convert the *$C_3$* malonic ester to the acid was done using sodium methoxide in methanol (0.24 ml of 2.2 M, 0.54 mmol) plus *e,e,e*-$C_{62}{}^{14}C(COOMe)_6$ (0.027 mmol) in 30 ml of toluene at room temperature. Extraction provided 28.0 mg of *e,e,e*-methanofullerene(60)-63-tris malonic acid [14C-$C_3$]. The powder was dissolved in sterile 0.1 N NaOH. The product was characterized by NMR, HPLC analysis and mass spectroscopy.

**Reference:**

[1] Lamparth I, Hirsch A. Water-soluble malonic acid derivatives of C60 with a defined three-dimensional structure. *J. Chem. Soc., Chem. Commun.* 1994:1727-8.