2. EXPERIMENTAL DETAILS

2.1 Test Substances

The test substances for this study were NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid. See Appendix 3 for complete nomenclature and chemical structures.

2.2 Analytical Reference Substances

The test substances also served as the analytical reference substances. In addition, isotopically labeled analogs of the test compounds were used as internal standards. See Appendix 3 for complete nomenclature and chemical structures of the test and reference substances. The test and reference substances were stored in a freezer until used to make fortification and calibration solutions. The stock solutions were stored in a freezer at - 18°C to - 25°C and the calibration and fortification solutions were stored at less than 4°C when not in use.

2.3 Test System (Untreated Control Soil)

The test system was comprised of subsamples of untreated control soil from an agricultural site in Mississippi. The soil was taken from the 0 to 6 inch depth. This soil was obtained as part of Bayer CropScience study, Terrestrial Field Dissipation of NNI-0001 in Mississippi Soil, 2003, study number 03EFAMY002. The soil was classified on the chain of custody form as "Series name: Bosket sandy loam". The soil characterization report from A & L Great Lakes Laboratories for the 0 to 6 inch layer of soil from this location gave the soil classification as "silt loam" with a pH of 5.4 and 0.66 percent organic material.[4] The untreated control soil was stored at ambient temperature.

2.4 Method Summary

20 gram subsamples of the homogenized control soil were weighed into 100 mL beakers. Fortified samples were prepared by adding the appropriate amounts of a mixed standard for each fortification level (0.5ng/g (ppb) and 5 ng/g). Control and fortified soil samples were extracted with 40 mL of a mixture of acetonitrile/water/acetic acid (500/500/1; v/v/v). Soil and solvent were stirred and heated in the microwave for six minutes at 250 watts at a maximum temperature of 50°C. After extraction, an appropriate amount of labeled internal standard solution was added to each sample. Approximately 10mL of the sample extract was centrifuged to separate out the solids. Supernatant was transferred into an HPLC vial for analysis by electrospray LC/MS/MS. A flow diagram of the method is given in Appendix 4.

2.5 Instrumentation used

- Microwave, Milestone, Ethos E with Model 320 controller, fiber optic temperature probe and flat tray carousel/rotor
- Sciex API 3000 LC/MS/MS System (Applied Biosystems)
- Shimadzu LC-10AD VP HPLC Pumps (2) with 250µL High Pressure Mixer and Shimadzu SCL-10A VP Pump Controller
- Gilson 215 Autosampler

2.6 Liquid Chromatographic Conditions

Column: Superspher 100 RP-18
Length 75 mm, 4 mm i.d.
Particle size 4 µm, pore size 100 nm
Cat. No.: 1.50980.0001
Merck, distributed in the US by VWR

Column oven temperature: Ambient

Inline filter: Upchurch, ultra-low volume, pre-column filter, Cat. No. A-318, with A-102x, 0.5µm frits.

Mobile phase: A: water / acetonitrile / acetic acid (900/100/1; v/v/v)
B: acetonitrile / acetic acid (1000/1; v/v)

Flow rate (column): 1.0 mL/min
Flow rate (interface): 1.0 mL/min, no split

Table 1: HPLC Gradient Parameters

Time [min]	0	5	6	9	10	13
% A	60	40	10	10	60	60
% B	40	60	90	90	40	40

Injection volume: 50 µL
Run time: About 14 minutes between injections.

Table 2: Divert Valve Timing

Time [min]	Setting
0 to 1.00	Stream to waste
1.10 to 7.0	Stream to interface
7.1 to end	Stream to waste

Retention times:

NNI-0001:	approx. 4.9 min
NNI-0001-des-iodo:	approx. 3.9 min
NNI-0001-3-OH:	approx. 3.8 min
NNI-0001-3-OH-hydroxyperfluoroalkyl:	approx. 1.9 min
NNI-0001-benzoic-acid:	approx. 2.6 min

2.7 Mass Spectrometric Parameters Used

Table 3: MS/MS Operating Parameters

Nebulizer Gas Setting	15
Curtain Gas Setting	9
Collision Gas Setting	5
Turbo Gas [L/min]	8.5
Turbo Gas Temperature [°C]	500
Resolution of Q1 and Q3	Unit (~0.7 amu)

Compound dependent:	NNI-0001	NNI-0001-d6	NNI-0001-des-iodo	NNI-0001-des-iodo-d6	NNI-0001-3-OH	NNI-0001-3-OH-d6
Q1 Mass [amu]	681	687	555.1	561.1	571.1	577.1
Q3 Mass [amu]	254	259	254	259	296	296
Dwell [msec]	250	250	250	250	250	250
Ionization Mode	ESP-	ESP-	ESP-	ESP-	ESP-	ESP-
IS [V]	-4300	-4300	-4300	-4300	-4300	-4300
EP [V]	-10	-10	-10	-10	-10	-10
DP [V]	-16*	-16*	-41	-41	-51	-51
FP [V]	-104*	-104*	-190	-190	-220	-220
CE [V]	-32	-32	-30	-30	-22	-22
CXP [V]	-24	-24	-22	-22	-24	-24

*The DP and FP voltages are those used in the method trials. The correct method values should have been DP = -46 and FP = -210. The values used provided adequate NNI-0001 sensitivity. The values from the method as written may provide somewhat better sensitivity.

Table 3: MS/MS Operating Parameters (continued)

	NNI-0001-3-OH-hydroxyper-fluoroalkyl	NNI-0001-3-OH-hydroxyper-fluoroalkyl-d6	NNI-0001-benzoic-acid	NNI-0001-benzoic-acid-d3
Q1 Mass [amu]	569.1	575.1	711	714
Q3 Mass [amu]	296	296	304	307
Dwell [msec]	250	250	250	250
Ionization Mode	ESP-	ESP-	ESP-	ESP-
IS [V]	-4300	-4300	-4300	-4300
EP [V]	-10	-10	-10	-10
DP [V]	-46	-46	-36	-36
FP [V]	-190	-190	-180	-180
CE [V]	-24	-24	-32	-32
CXP [V]	-22	-22	-26	-26

IS: Ion Spray Voltage DP: Declustering Potential FP: Focusing Potential
EP: Entrance Potential CE: Collision Energy CXP: Collision Cell Exit Potential
ESP-: electrospray, negative ion mode, i.e. production of negative ions

2.8 Minor Modification Not Affecting Results

The amount of solution taken for centrifuging was 10mL instead of 1.5mL. This was done because of the centrifuge rotor used and the size of the containers required by the difference in the centrifuge used. This is considered a normal substitution of similar lab equipment and should have no effect on the results obtained using the method.

2.9 Calculations

Quantitation of the validation results were performed using the method as written by running standards and generating a calibration curve based on analyte and labeled internal standard responses. For calculation of the concentrations linear regression calibration curves were used. These curves were calculated using the instrument quantitation software.

The linear equation used may be expressed as:

$$y = \text{Intercept} + \text{Slope} \cdot x$$

To provide better fit near the limit of detection, the linear regression was forced through zero, making the intercept value zero.

Labeled internal standards were used, thus:

$$y = \frac{Area_{Standard}}{Area_{Internal\ Standard}} = Int\ Ratio \quad and \quad x = \frac{Conc_{Standard}}{Conc_{IS}} = Conc_{ratio}$$

Int. Ratio	*intensity ratio*
$Conc_{Standard}$	*concentration of standard solution [ng/mL]*
$Conc_{IS}$	*concentration of internal standard solution [ng/mL]*
$Conc_{ratio}$	*concentration ratio*

The concentrations of the compounds NNI-0001, NNI-0001-des-iodo, NNI-0001-3-OH, NNI-0001-3-OH-hydroxyperfluoroalkyl and NNI-0001-benzoic acid contents in the wet soil were calculated as follows:

$$Dilution_{Factor} = \frac{Volume_{Extraction}}{Weight}$$

$$Conc_{Analyte} = \frac{Int.\ Ratio - Intercept}{Slope}, \quad Int.\ Ratio = \frac{Area_{Analyte}}{Area_{Internal\ Standard}}$$

$$Conc_{Soil\ Wet} = Conc_{Analyte} \times Dilution_{Factor} \times Conc_{IS}$$

$Volume_{Extraction}$	*volume of the extraction solvent [mL]*
Weight	*weight of the soil (sediment) sample [g]*
Intercept	*intercept of the linear regression curve, forced to zero in this case*
Slope	*slope of the linear regression curve*
$Area_{Analyte}$	*area of the analyte in the sample solution*
$Conc_{Soil\ Wet}$	*concentration of the analyte in wet soil [ng/g]*

The recovery was calculated according to the following equation:

$$Recovery = \frac{Conc_{Soil\ Wet} \times 100\%}{Conc_{Soil\ Spiked}}$$

$Conc_{Soil\ Spiked}$	*concentration of the reference substance spiked [ng/g]*

Example calculation for a 5 ng/g or ppb recovery of NNI-0001 fortified in Mississippi soil. (Sample: RAAMX006-113, from the second validation trial, recovery 4.75 ng/g or ppb)

$$\text{Dilution}_{\text{Factor}} = \frac{40\ \text{mL}}{20\,\text{g}} = 2.0\,\frac{\text{mL}}{\text{g}}$$

$$\text{Int. Ratio} = \frac{51840}{359200} = 0.14432$$

$$\text{Conc}_{\text{Analyte}} = \frac{0.14432 - 0}{1.2164} = 0.118645$$

$$\text{Conc}_{\text{Soil Wet}} = 0.118645 \times 2.0\,\frac{\text{mL}}{\text{g}} \times 20\,\frac{\text{ng}}{\text{mL}} = 4.7458\,\frac{\text{ng}}{\text{g}} = 4.75 \text{ ng/g to three places.}$$

$$\text{Recovery} = \frac{4.7458\ \frac{\text{ng}}{\text{g}} \times 100\%}{5\ \frac{\text{ng}}{\text{g}}} = 94.92\% = 94.9\% \text{ to three places.}$$

Remark: Calculations were performed using the instrument software *Analyst (version 1.2)*. The example calculation is done using the area values reported by the instrument. The instrument software carries additional figures not shown in the intermediate results. The instrument software calculated a recovery of 4.75ng/g and a percent recovery of 94.9% (rounded to three figures) for this sample, the same as the example calculation in this case.

5. DISCUSSION

The first independent laboratory trial followed the method as written except for instrument specific parameters and a minor method modification noted above. Some of the individual recoveries for NNI-0001 and several of the other analytes were below 70%. The reason for the low recoveries was hypothesized to be due to insufficient adsorption of the microwave energy into the samples.

The number of samples to be run in each trial being greater than the ten positions in the microwave carousel necessitated that they be split into two batches. The number of samples did not divide equally, so for the first batch the rotor was filled with nine soil samples and a reagent blank. For the second batch the rotor only contained four soils and a reagent blank. While not otherwise affecting the method, a temperature probe had been placed in the UTC sample or a reagent blank during each of the two batches of microwave extractions. It was found that the final temperature reached in the two batches was different. The full load only reached about 39°C, while the partial load reached about 47°C in the same 3 minute 250 watt extraction period. All the recoveries in the less-than-full load were greater than 73% while all the less than 70% recoveries were in the full load. These data are consistent with the smaller load receiving more energy per sample.

From our communication with the method development lab after the first trial, we learned that the development lab intended that all positions in the rotor be filled, but this instruction was inadvertently left out of the method. We also learned that the type of stir bar used had been found to affect the amount of energy adsorbed by the samples and that a particular "dumb-bell" shaped stir bar was always used. This bar is more massive than the ones used in the first trial and may absorb and transfer more of the microwave energy to the samples. We obtained these type stir bars for use in our second trial. It may be that this change alone, along with running a full load to provide consistency, would have been sufficient modification to obtain good recovery. However, after discussion with the development laboratory it was also decided to increase the extraction time from three minutes to six minutes to get more energy into the samples. This modification required use of an oven with temperature control to control the final temperature to 50°C so that the extraction solvent would not boil over. Without temperature control, the samples would have to be monitored closely and the extraction time or energy input adjusted, if need be, to prevent solvent boil over.

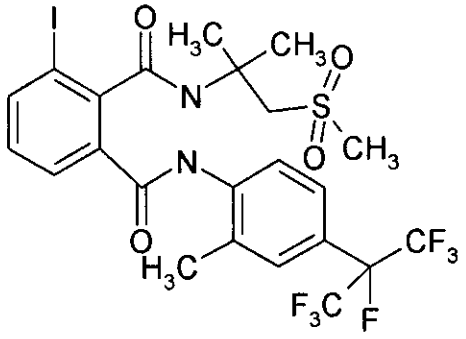
Individual recoveries and summary results for both the first and second IL trials are found in Tables 4 and 5. Additional details for trial 2 are found in the Results Tables in Appendix 5. Data from the second trial demonstrate the successful independent laboratory validation of the analytical method.

6. RECOMMENDATIONS AND CONCLUSSIONS

It is recommended that the method be revised to address the issue of consistent microwave energy absorption required to obtain consistent good recoveries. Our conclusion is that this may be achieved possibly in one of two ways. One way is to specify the specific type of stir bar, a particular "dumb-bell" shape and size that appears to affect energy absorbed into the samples. This should be combined with a statement that all the positions in the ten position rotor should be filled. The second way to achieve good recoveries would be based on the modification used here in the second and successful trial, in which the microwave energy is controlled by use of a temperature probe. This probe is an accessory for the Ethos microwave oven used in the method. It is proposed that this modification would make the stir bar type and the number of samples run at one time in the oven less critical and ensure good microwave energy absorption and good recoveries. The method is considered applicable as an enforcement method provided that the issues identified in this ILV are adequately addressed in a revision of the method.

Appendix 3 Identity and Purity of the Test and Reference Materials Used

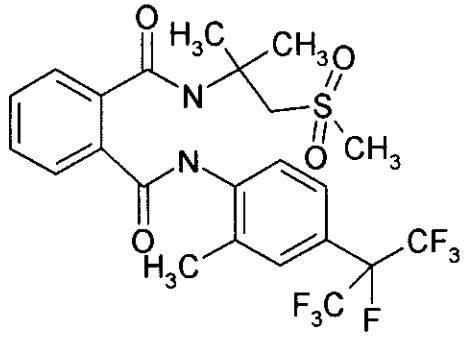
NNI-0001:
Structural formula:



Common name:	not available
Code name:	NNI-0001
Chemical code:	AE 1302996
Chemical name:	3-Iodo-N^2-(2-methanesulfonyl-1.1-dimethyl-ethyl)- N^1-[2-methyl-4-(1,2,2,2-tetrafluoro-1-trifluoromethyl-ethyl)-phenyl]-phthalamide
Empirical formula:	C23 H22 F7 I N2 O4 S
Molecular weight:	682.4 g/mol

Batch, purity and expiry date of material used in this study: K-1155, 98.5%, 10/21/2006

NNI-0001-des-iodo:
Structural formula:

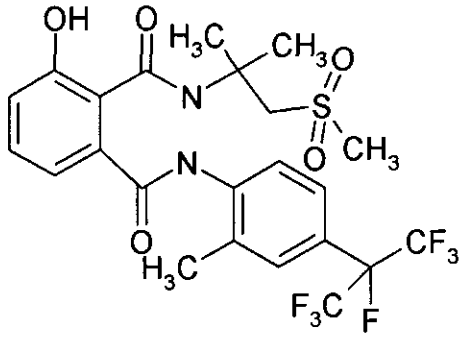


Common Name:	not available
Code name:	NNI-0001-des-iodo
Chemical codes:	AE 1303002, A-1
Chemical name:	N^2-(1,1-dimethyl-2-methylsulfonylethyl)- N^1-{2-methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]phenyl}-phthalamide
Empirical formula:	C23 H23 F7 N2 O4 S
Molecular weight:	556.5 g/mol

Batch, purity and expiry date of material used in this study: K-1303, 99.3%, 10/22/2004
Reanalysis: K-1303, 99.2, 12/28/2006

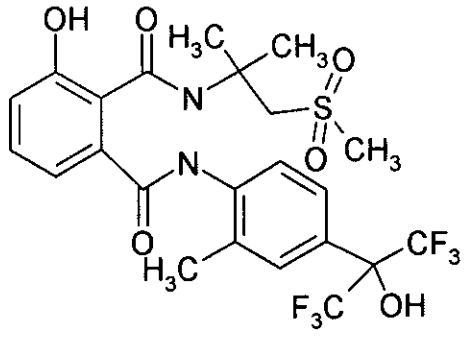
NNI-0001-3-OH:

Structural formula:



Common Name: not available
Code name: NNI-0001-3-OH
Chemical codes: AE 1423032, A-2
Chemical name: 3-hydroxy-N^2-(2-mesyl-1,1-dimethyl-ethyl)-N^1-{2-methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]phenyl}-phthalamide
Empirical formula: C23 H23 F7 N2 O5 S
Molecular weight: 572.5 g/mol
Batch, purity and expiry date of material used in this study: K-1301, 97.5%, 11/12/2004
Reanalysis: K-1301, 97.5%, 1/11/2010

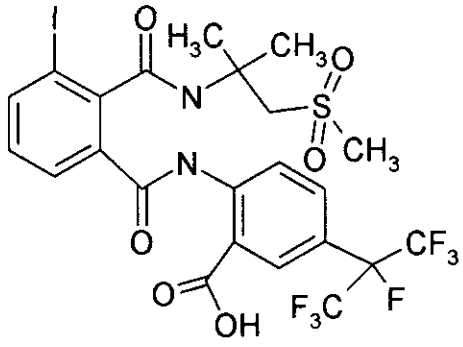
NNI-0001-3-OH-hydroxyfluoralkyl:
Structural formula:



Common name: not available
Code name: NNI-0001-3-OH-hydroxyperfluoroalkyl
Chemical codes: AE 1423031, A10, dihydroxy NNI-0001
Chemical name: 3-hydroxy-N^2-(2-mesyl-1,1-dimethyl-ethyl)-N^1-{2-methyl-4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl}-phthalamide
Empirical formula: C23 H24 F6 N2 O6 S
Molecular weight: 570.5 g/mol
Batch, purity and expiry date of material used in this study: K-1302, 98.1%, 3/29/2004
Reanalysis: K-1302, 98.1%, 9/15/2009

NNI-0001- benzoic acid:

Structural formula:

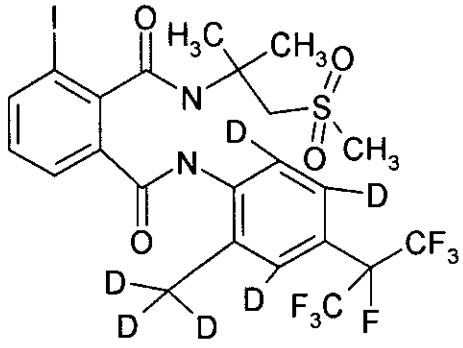


Common name:	not available
Code name:	NNI-0001-benzoic-acid
Chemical codes:	AE 1651796, A-18
Chemical name:	2-({2-[(1,1-dimethyl-2-mesylethyl)carbamoyl]-3-iodo-benzoyl}amino)-5-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]benzoic acid
Empirical formula:	C23 H20 F7 I N2 O6 S
Molecular weight:	712.4 g/mol

Batch, purity and expiry date of material used in this study: K-1164, 98.4 %, 5/25/08

NNI-0001-d6 (used as internal standard for the active analyte NNI-0001):

Structural formula:

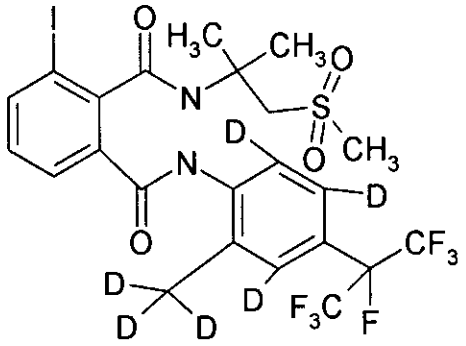


Code name:	NNI-0001-d6
Chemical name:	3-iodo-N^2-(2-mesyl-1,1-dimethyl-ethyl)- N^1-[2-(methyl-D_3)-4-(1,2,2,2-tetrafluoro-1-trifluoromethyl-ethyl)-phenyl-D_3]-phthalamide
Empirical formula:	C23 H16 D6 F7 I N2 O4 S
Molecular weight:	688.4 g/mol

Batch, purity and expiry date of material used in this study: K-1176, 99.9%, 7/6/2007

NNI-0001-des-iodo-d6 (internal standard for the metabolite NNI-0001-des-iodo):

Structural formula:

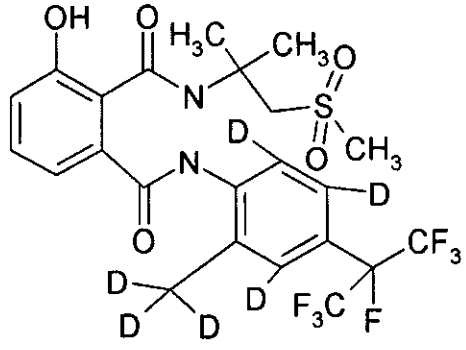


Code name:	NNI-0001-des-iodo-d6
Chemical code:	D6-A-1
Chemical name:	N^2-(1,1-dimethyl-2-methylsulfonylethyl)- N^1-{2-[D_3]methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]phenyl][3,5,6-D_3]phenyl}-phthalamide
Empirical formula:	C23 H17 D6 F7 N2 O4 S
Molecular weight:	562.5 g/mol

Batch, purity and expiry date of material used in this study: K-1279, 97.9%, 6/28/2008

NNI-0001-3-OH-d6 (used as internal standard for the metabolite NNI-0001-3-OH):

Structural formula:

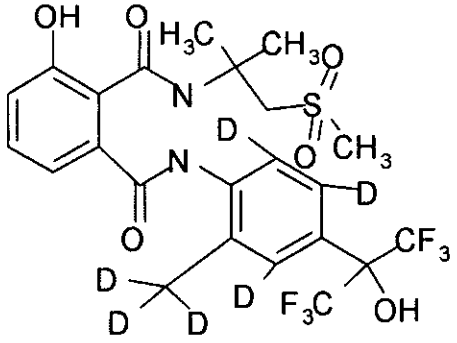


Code name:	NNI-0001-3-OH-d6
Chemical code:	D6-A-2
Chemical name:	N^2-(1,1-dimethyl-2-methylsulfonylethyl)-3-hydroxy- N^1-{2-[D_3]methyl-4- [1,2,2,2-tetrafluoro-1-(trifluoromethyl)-ethyl]- 3,5,6-D_3]phenyl}-phthalamide
Empirical formula:	C23 H17 D6 F7 N2 O5 S
Molecular weight:	578.5 g/mol

Batch, purity and expiry date of material used in this study: K-1280, 98.3%, 6/28/2008

NNI-0001-3-OH-hydroxyperfluoroalkyl-d6
(used as internal standard for the metabolite NNI-0001-3-OH-hydroxyperfluoroalkyl):

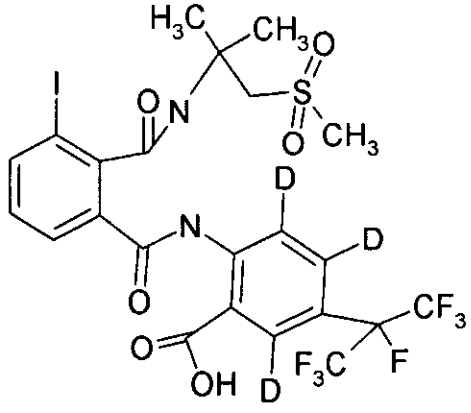
Structural formula:



Code name:	NNI-0001-3-OH-hydroxyperfluoroalkyl-d6
Chemical code:	D6-A-10
Chemical name:	N^2-(1,1-dimethyl-2-methylsulfonylethyl)-3-hydroxy- N^1-{4-[1-hydroxy -2,2,2-trifluoro-1-(trifluoromethyl)-ethyl]- 2-[D_3]-methyl[3,5,6-D_3]phenyl}phthalamide
Empirical formula:	C23 H18 D6 F6 N2 O6 S
Molecular weight:	576.6 g/mol

Batch, purity and expiry date of material used in this study: K-1281, 98.5%, 6/28/2008

NNI-0001- benzoic acid-d3
(used as internal standard for the metabolite NNI-0001-benzoic-acid):
Structural formula



Code name:	NNI-0001-benzoic-acid-d3
Chemical code:	D3-A-18
Chemical name:	2-{[2-{[(1,1-dimethyl-2-methylsulfonylethyl)amino]carbonyl}-3-iodophenyl)carbonyl]amino}-5-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl][3,4,6-D_3]benzoic acid
Empirical formula:	C23 H20 D3F7 I N2 O6 S
Molecular weight:	715.37 g/mol

Batch, purity and expiry date of material used in this study: 4FH6801S, 93.7%, 4/20/10

Appendix 4 Flow Diagram of the Method