Title

An Analytical Method for the Determination of Residues of BCS-CS55621, and Its Metabolites BCS-CY96288, BCS-CU97237, BCS-DA63612, BCS-CC26101, BCS-CZ38260, BCS-BP32808, and BCS-DH17585 in Water Using LC/MS/MS

-Analytical Method-

Test Substance

Fluoxapiprolin (BCS-CS55621)

Guideline Number

OECD Guidance Document on Pesticide Residue Analytical Methods, Series on Testing and Assessment Document 72 and Series on Pesticides: Document 39, August 2007 (OECD Guideline, ENV/JM/MONO (2007) 17, Aug 13, 2007)

PMRA Residue Chemistry Guidelines, Regulatory Directive 98-02, Section 3, Residue Analytical Method, June 1998

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1.0 SUMMARY

An analytical method was developed to determine the residues of BCS-CS55621 and its metabolites BCS-CY96288, BCS-CU97237, BCS-DA63612, BCS-CC26101, BCS-CZ38260, BCS-BP32808, and BCS-DH17585 in water.

Residues of BCS-CS55621 and its metabolites are diluted from water using a mixture of 1:1 (v/v) acetonitrile/water. Isotopic internal standards are added to the sample. A sample aliquot is analyzed for BCS-CS55621, and its metabolites BCS-CY96288, BCS-CU97237, BCS-DA63612, BCS-CC26101, BCS-CZ38260, BCS-BP32808, and BCS-DH17585 by LC/MS/MS. Quantification is based on a comparison of peak areas with those of known standards.

This method was developed to analyze residues of BCS-CS55621 and its metabolites in water matrix at a target limit of quantification (LOQ) of 0.5 ng/mL, but can be adjusted as required.

2.0 BACKGROUND

Fluoxapiprolin (BCS-CS55621) is a fungicide currently under development by Bayer CropScience. The analytical method presented in this report is designed to measure residues of BCS-CS55621, and its metabolites BCS-CY96288, BCS-CU97237, BCS-DA63612, BCS-CC26101, BCS-CZ38260, BCS-BP32808, and BCS-DH17585 in water using isotopically-labeled internal standards and LC/MS/MS detection.

3.0 APPARATUS

Functional equivalents may be substituted.

- Various general laboratory glassware and utensils.
- LC Column: Phenomenex Luna Omega 1.6 µm C18 100 Å, 100 x 2.1 mm (Part number: 00D-4742-AN) or equivalent
- Optional guard column as needed
- Vortex
- Sonicator
- Mass spectrometer, API 6500 or API 6500+ with ESI interface and mass spectrometric detector, ABSciex, or equivalent

4.0 REAGENTS AND CONSUMABLES

Functional equivalents may be substituted

- Acetonitrile (Optima Grade, Fisher Part No. A996-4)
- Acetic acid, Glacial
- Water (Optima Grade; Fisher Part No. W7-4)
- Methanol (Optima grade Fisher Part No. A456-1)
- 4:1 (v/v) acetonitrile/water. Combine 800 mL acetonitrile and 200 mL water. Mix well.
- 1:1 (v/v) acetonitrile/water. Combine 500 mL acetonitrile and 500 mL water. Mix well.
- 1:9 (v/v) acetonitrile/water. Combine 100 mL acetonitrile and 900 mL water. Mix well.
- 0.5% acetic acid in acetonitrile. Add 5 mL acetic acid to 1000 mL acetonitrile. Mix well.
- 0.5% acetic acid in water. Add 5 mL acetic acid to 1000 mL water. Mix well.
- HPLC vials and caps (2-mL, National Scientific, Part Nos. C4011-5W and C4011-55)
- Disposable transfer pipet (Thermo Part No. HS206373B)
- Pipettors and tips
- Autosampler glass insert

5.0 PREPARATION OF STANDARD SOLUTIONS

The native reference standards used in this method are for BCS-CS55621, and its metabolites BCS-CY96288, BCS-CU97237, BCS-DA63612, BCS-CC26101, BCS-CZ38260, BCS-BP32808, and BCS-DH17585. The isotopic internal standards (IS) standard required for this method are: BCS-CS55621-$^{13}C_3$,$^{15}N_3$, BCS-CY96288-$^{13}C_3$,$^{15}N_3$, BCS-DH34157-$^{13}C_6$, BCS-DA63612-$^{13}C_6$, BCS-CC26101-$^{13}C_2$, BCS-CZ38260-$^{15}N_2$, BCS-BP32808-$^{15}N_2$, and BCS-CU97237-$^{13}C_6$. These standards may be obtained from Bayer CropScience. Additional details about these chemicals are given in Appendix 1.

The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

NOTE: The following procedure is an example description of how these standard solutions may be prepared. Alternate or additional standards of appropriate weight and volume may be prepared as needed.

Volumetric glassware and calibrated pipets should be used in the preparation of all analytical standards. Corrections for standard purities should be applied when expressing standard concentrations.

5.1 Primary Standard Solutions

Native reference standards and internal standard primary solutions are prepared from the reference standards as shown below.

Table 1. Primary reference (native) standard solution preparation

Native Reference Standards	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
BCS-CS55621	~5	50	Acetonitrile	~100
BCS-CY96288	~5	50	Acetonitrile	~100
BCS-CU97237	~5	50	4:1 ACN/water	~100
BCS-DA63612	~5	50	4:1 ACN/water	~100
BCS-CC26101	~5	50	Acetonitrile	~100
BCS-CZ38260	~5	50	Acetonitrile	~100
BCS-BP32808	~5	50	Acetonitrile	~100
BCS-DH17585	~5	50	4:1 ACN/water	~100

Table 2. Primary internal standard solution preparation.

Isotopic Internal Standards	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
BCS-CS55621-$^{13}C_3$,$^{15}N_3$	~2.5	25	Acetonitrile	~100
BCS-CY96288-$^{13}C_3$,$^{15}N_3$	~2.5	25	Acetonitrile	~100
BCS-CU97237-$^{13}C_6$	~2.5	25	4:1 ACN/water	~100
BCS-DA63612-$^{13}C_6$	~2.5	25	4:1 ACN/water	~100
BCS-CC26101-$^{13}C_2$	~2.5	25	Acetonitrile	~100
BCS-CZ38260-$^{15}N_2$	~2.5	25	Acetonitrile	~100
BCS-BP32808-$^{15}N_2$	~2.5	25	Acetonitrile	~100
BCS-DH34157-$^{13}C_6$	~2.5	25	4:1 ACN/water	~100

Note: The concentration of the primary solutions should also be corrected for purity of the standard during the initial preparation. Primary solutions should be stored in a freezer when not in use.

5.2 Secondary Standard Solutions

Mixed secondary reference and internal standard solutions are prepared from the primary standard solutions as shown below. Take the appropriate aliquot of each of the primary standard solutions to give the required mixed secondary standard concentration. All secondary native and internal standards should be stored in a refrigerator when not in use.

Table 3. Secondary mixed reference standard solution preparation.

Compound	Primary Standard Concentration (µg/mL)	Aliquot from Primary Standard (mL)	Final Volume (mL)	Mixed Secondary Standard Final Concentration (ng/mL)	Solvent
BCS-CS55621	~100	~1	100	1000	4:1 ACN/H_2O
BCS-CY96288	~100	~1			
BCS-CU97237	~100	~1			
BCS-DA63612	~100	~1			
BCS-CC26101	~100	~1			
BCS-CZ38260	~100	~1			
BCS-BP32808	~100	~1			
BCS-DH17585	~100	~1			

Additional secondary reference standard solutions are prepared as shown below.

Table 4. Additional mixed native spiking solution preparation.

Concentration of Mixed Native Standard Solution used for dilution (ng/mL)	Aliquot Taken (mL)	Dilution Volume (mL)	Final Concentration of Mixed Native Secondary Standard Solution (ng/mL)	Solvent
1000	10.0	100	100	1:1 ACN/H_2O
100	10.0	100	10	1:1 ACN/H_2O

Table 5. Secondary mixed internal standard solution preparation.

Compound	Primary Standard Concentration (µg/mL)	Aliquot from Primary Standard (mL)	Final Volume (mL)	Mixed Secondary Standard Final Concentration (µg/mL)	Solvent
BCS-CS55621-$^{13}C_3$,$^{15}N_3$	~100	~1	100	1.0	4:1 ACN/H_2O
BCS-CY96288-$^{13}C_3$,$^{15}N_3$	~100	~1			
BCS-CU97237-$^{13}C_6$	~100	~1			
BCS-DA63612-$^{13}C_6$	~100	~1			
BCS-CC26101-$^{13}C_2$	~100	~1			
BCS-CZ38260-$^{15}N_2$	~100	~1			
BCS-BP32808-$^{15}N_2$	~100	~1			
BCS-DH34157-$^{13}C_6$	~100	~1			

Additional internal standard mixture solution is prepared as shown below.

Table 6. Mixed internal standard solution preparation.

Concentration of Mixed Native Standard Solution used for dilution (ng/mL)	Aliquot Taken (mL)	Dilution Volume (mL)	Final Concentration of Mixed Native Secondary Standard Solution (ng/mL)	Solvent
1000	2.0	100	20	1:1 ACN/H_2O
100	2.0	50	4	1:9 ACN/H_2O

5.3 Calibration Standards

Note: Additional standards may be prepared when necessary; however, the concentration of internal standard must remain the same in all calibration standards. Calibration solutions are prepared in 1:9 (v/v) acetonitrile/water. Calibration standards should be stored in a refrigerator when not in use.

Table 7. Calibration Standard Solutions

Native Conc. of Calibration Solution (ng/mL)	IS Conc. in Calibration Solution (ng/mL)	Conc. of Native Standard Solution used for dilution (ng/mL)	Conc. of Internal Standard Solution used for dilution (ng/mL)	Aliquot Native Taken (mL)	Aliquot Internal Standard Taken (mL)	Dilution Volume (mL)
20*	4	1000	1000	1.00	0.20	50
16*	4	1000	1000	0.80	0.20	50
10	4	1000	1000	0.50	0.20	50
8	4	1000	1000	0.40	0.20	50
4	4	1000	1000	0.20	0.20	50
1	4	100	1000	0.50	0.20	50
0.4	4	100	1000	0.20	0.20	50
0.2	4	10	1000	1.00	0.20	50
0.1	4	10	1000	0.50	0.20	50

* The standards are for the extended curve in case to analyze the high residue samples.

6.0 EXTRACTION PROCEDURE

Fortified recovery samples are prepared by spiking a known level of the appropriate standard(s) into a sample. A control sample should also be prepared, using the same samples as the fortified sample, to determine the residue levels, real or apparent, found in this sample and the % recovery calculated as described in Section 8.1.

1. Transfer 1.0 mL, by pipet, water into an autosampler vial.

   Remark: Add the corresponding amount of spiking solution for the fortified recovery sample (e.g. 0.05 mL of a 10 ng/mL analyte mixture at LOQ level at 0.5 ng/mL).

2. Add 0.25 mL 20 ng/mL mixed internal standards in 1:1 v/v acetonitrile:water.
3. Cap the autosampler and mix well for LC/MS/MS analysis.

7.0 ANALYSIS BY LC/MS/MS

7.1 Analytical Procedure

Step 1. Using the recommended procedures listed below; analyze a minimum of five calibration standard solutions (if necessary, additional standard solutions may be added).

Step 2. Analyze an aliquot of each of the analytical samples.

Note: Up to 20 sample analyses can be made after the analysis of the standard solutions.

Step 3. Repeat Step 1.

Step 4. When necessary analyze additional samples and calibration standard solutions. Always finish the procedure with a set of calibration solutions

7.2 HPLC Conditions

Note: The analyst should optimize chromatographic conditions to obtain satisfactory chromatography. As the HPLC column ages, the retention times of the analytes may change.

Mobile Phase A: 0.5% Acetic acid in water
Mobile Phase B: 0.5% Acetic acid in acetonitrile
Oven: 40 ºC
HPLC Column: Phenomenex Luna Omega C18 100 mm x 2.1 mm 1.6 µm particle size
Injection Volume: 20 µL (adjust as needed)
Pumping mode: Binary Flow
Binary flow rate: 0.30 mL/min

Time (min)	Module	Events	Parameters
0.0	Pumps	Pump B Conc	5
0.3	Pumps	Pump B Conc	5
2.5	Pumps	Pump B Conc	80
4.0	Pumps	Total Flow	0.3
4.1	Pumps	Total Flow	0.6
5.8	Pumps	Pump B Conc	100
5.9	Pumps	Pump B Conc	5
6.8	Pumps	Total Flow	0.6
6.9	Pumps	Total Flow	0.3
7.0	Controller	Stop	

7.3 Mass Spectrometer Conditions

Note: The following recommended conditions were used on an API 6500+ instrument but the analyst should optimize the mass spectrometer conditions to obtain satisfactory system response prior to use.

Two product ions per analyte are listed in Table 8, one MRM-transition serving for quantitation and the second MRM-transition to be used, only if required, to confirm the presence of any detected residues.

The detection by MS/MS was performed on a triple-quadrupole tandem mass spectrometer, equipped with a Turbo IonSpray (ESI) interface operated in multiple reaction monitoring (MRM) with unit mass resolution.

Curtain Gas (CUR)	55
Collision Gas (CAD)	8
Ion Source Gas 1 (GS1)	55
Ion Source Gas 2 (GS2)	55
Source Temp. (TEM)	600
Ion Transfer Voltage (IS+)	5500
Ion Transfer Voltage (IS-)	-4500

Table 8. MS/MS Parameters for the determination of BCS-CS55621, and its metabolites BCS-CY96288, BCS-CU97237, BCS-DA63612, BCS-CC26101, BCS-CZ38260, BCS-BP32808, and BCS-DH17585

Analyte Name *	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	DP	EP	CE	CXP	Retention Time (min)
BCS-CS55621-Q	+	650	610.2	30	10	41	9	~3.93
BCS-CS55621-C	+	650	378	30	10	46	9	~3.93
BCS-CS55621-IS	+	656	616.2	30	10	41	9	~3.93
BCS-CY96288-Q	+	666	626.1	30	10	28	9	~3.76
BCS-CY96288-C	+	666	191	30	10	46	9	~3.76
BCS-CS55621-IS	+	672	634.1	30	10	28	9	~3.76
BCS-CU97237-Q	+	442.2	332	30	10	38	9	~2.92
BCS-CU97237-C	+	442.2	208	30	10	38	9	~2.92
BCS-CU97237-IS	+	448	338	30	10	38	9	~2.92
BCS-DA63612-Q	+	456	345.2	30	10	50	9	~2.34
BCS-DA63612-C	+	456	222.1	30	10	37	9	~2.34
BCS-DA63612-IS	+	462	351	30	10	50	9	~2.34

Analyte Name *	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	DP	EP	CE	CXP	Retention Time (min)
BCS-CC26101-Q	-	225	161	-30	-10	-35	-9	~3.20
BCS-CC26101-C	-	225	141	-30	-10	-42	-9	~3.20
BCS-CC26101-IS	-	227	162	-30	-10	-35	-9	~3.20
BCS-CZ38260-Q	-	161	76	-30	-10	-21	-9	~2.74
BCS-CZ38260-IS-Q	-	163	77	-30	-10	-21	-9	~2.74
BCS-CZ38260-C	+	163.1	125	30	10	28	9	~2.74
BCS-CZ38260-C2	+	163.1	97	30	10	32	9	~2.74
BCS-CZ38260-IS-C	+	165	127	30	10	28	9	~2.74
BCS-BP32808-Q	+	169	101	30	10	37	9	~3.31
BCS-BP32808-C	+	169	149	30	10	27	9	~3.31
BCS-BP32808-IS	+	171	101	30	10	37	9	~3.31
BCS-DH17585-Q	+	403	169	30	10	27	9	~3.39
BCS-DH17585-C	+	403	157	30	10	37	9	~3.39
BCS-DH17585-IS	+	409	169	30	10	27	9	~3.39

Note: * Q – quantitative ion transition. C – confirmatory ion transition. IS – internal standard ion transition.

8.0 CALCULATION OF RESULTS

The example calculation displayed below was used by the laboratory developing this method. Alternate calculation procedures appropriate to the reporting requirements may be substituted.

Residue concentrations were determined using calibration curves which were generated after each analysis using Analyst 1.7.1 software using linear regression with 1/x weighting.

The standards were fit to the linear equation:

$$Y = MX + B \text{ with } 1/x \text{ weighting.}$$

where: M is the calibration line slope
B is the calibration line intercept
Y is the native peak area: isotopic peak area ratio

$$Residue\ (ng/mL) = \frac{(Y - B)}{M} X\ D$$

Where

$$Dilution\ Factor\ (D) = \frac{Final\ volume\ (V2)}{Initial\ sample\ volume.\ (V1)}$$

Where: V1 = 1 mL
V2 = 1.25 mL

Analyst software was used to calculate the amount of BCS-CS55621, and its metabolites BCS-CY96288, BCS-CU97237, BCS-DA63612, BCS-CC26101, BCS-CZ38260, BCS-BP32808, and BCS-DH17585 in ng/g for each sample and the percent recovery for the fortified samples.

Residue levels beyond the calibration curve: In some cases, an unknown sample contains residues at a level above the calibration curve. If so, the preferred strategy is to extend the calibration curve to cover the unknown sample. If this is not an option, contact the development laboratory for instructions on how to proceed.

Recovery (fortification) experiments may be performed as needed to monitor method efficiency and reproducibility. Recovery experiments are intended to be used for data collection methods or establishing and validating method efficiency and are prepared by adding a known amount of native standard solution to a sample aliquot and preparing the sample for analysis as described in Section 6.

With each sample set, prepare and analyze an untreated control sample and one or more fortified control samples. Calculate recoveries using the following equation:

$$Recovery\ (\%) = \frac{(R-S)}{T} X\ 100$$

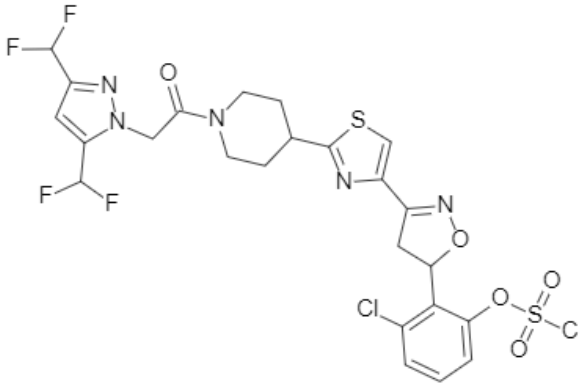
Where: R = ng/g of target analyte found in fortified (recovery) sample
S = ng/g of target analyte found in control sample, real or apparent
T = theoretical ng/mL in fortified sample

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Recovery samples are fortified prior to extraction at the LOQ of 0.5 ng/mL or other appropriate level with fortification solutions. Calculate the final residue for the control (S) and fortified control (R) samples.

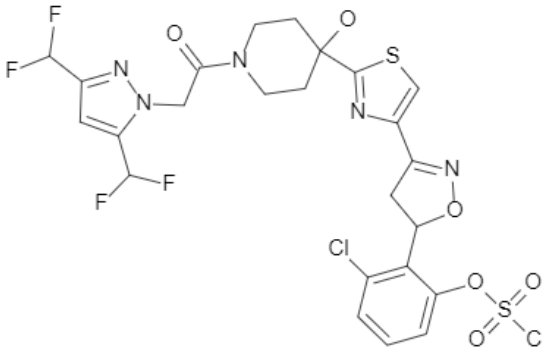
Appendix 1 Reference Substances

The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

Code Name:	BCS-CS55621
Synonyms:	Fluoxapiprolin
IUPAC Name:	2-{3-[2-(1-{[3,5-bis(difluoromethyl)-1H-pyrazol-1-yl] acetyl} piperidin-4-yl)-1,3-thiazol-4-yl]-4,5-dihydro-1,2-oxazol-5-yl}-3-chlorophenylmethanesulfonate
CAS Number:	1360819-11-9
Molecular Formula:	$C_{25}H_{24}ClF_4N_5O_5S_2$
Molecular Weight:	650.06 g/mol
Chemical Structure:	

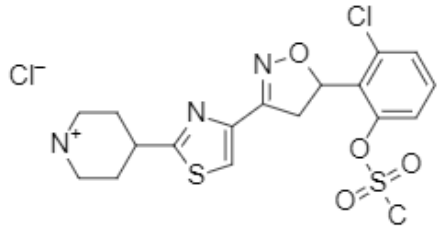


Code Name:	BCS-CY96288
IUPAC Name:	[2-[3-[2-[1-[2-[3,5-bis(difluoromethyl)pyrazol-1-yl]acetyl]-4-hydroxy-4-piperidyl]thiazol-4-yl]-4,5-dihydroisoxazol-5-yl]-3-chloro-phenyl] methanesulfonate
Molecular Formula:	$C_{25}H_{24}ClF_4N_5O_5S_2$
Molecular Weight:	666.06 g/mol
Chemical Structure:	

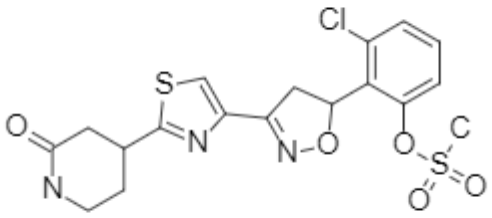


Appendix 1 Continued

Code Name: BCS- CU97237
IUPAC Name: 4-[4-(5-{2-chloro-6-[(methylsulfonyl)oxy]phenyl}-4,5-dihydro-1,2-oxazol-3-yl)-1,3-thiazol-2-yl]piperidinium chloride
Molecular Formula: $C_{18}H_{21}ClN_3O_4S_2Cl$
Molecular Weight: 478.41 g/mol
Chemical Structure:

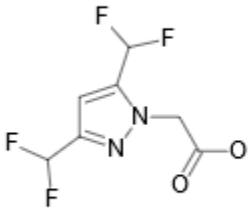


Code Name: BCS-DA63612
IUPAC Name: 3-chloro-2-{3-[2-(2-oxopiperidin-4-yl)-1,3-thiazol-4-yl]-4,5-dihydro-1,2-oxazol-5-yl}phenyl methanesulfonate
Molecular Formula: $C_{18}H_{18}ClN_3O_5S_2$
Molecular Weight: 455.94 g/mol
Chemical Structure:

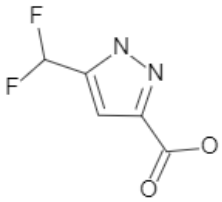


Appendix 1 Continued

Code Name: BCS-CC26101
IUPAC Name: 2-[3,5-bis(difluoromethyl)pyrazol-1-yl]acetic acid
Molecular Formula: $C_7H_6F_4N_2O_2$
Molecular Weight: 226.13 g/mol
Chemical Structure:

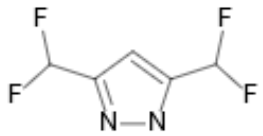


Code Name: BCS-CZ38260
IUPAC Name: 5-(difluoromethyl)-1H-pyrazole-3-carboxylic acid
Molecular Formula: $C_5H_4F_2N_2O_2$
Molecular Weight: 162.09 g/mol
Chemical Structure:

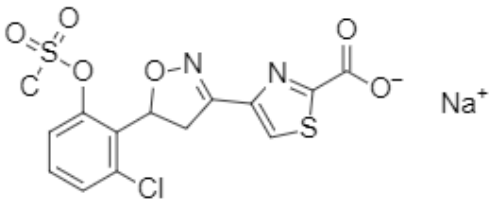


Appendix 1 Continued

Code Name: BCS-BP32808
IUPAC Name: 3,5-bis(difluoromethyl)-1H-pyrazole
Molecular Formula: $C_5H_4F_4N_2$
Molecular Weight: 168.09 g/mol
Chemical Structure:

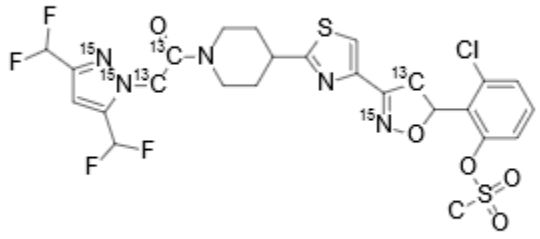


Code Name: BCS-DH17585
IUPAC Name: Sodium 4-[5-(2-chloro-6-methylsulfonyloxy-phenyl)-4,5-dihydroisoxazol-3-yl]thiazole-2-carboxylate
Molecular Formula: $C_{14}H_{10}ClN_2O_6S_2Na$
Molecular Weight: 424.81 g/mol
Chemical Structure:

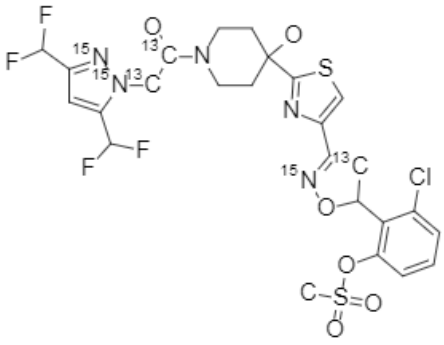


Appendix 1 Continued

Code Name: BCS-CS55621-$^{13}C_3$, $^{15}N_3$
Molecular Formula: $^{13}C_3C_{22}H_{24}ClF_4{}^{15}N_3N_2O_5S_2$
Molecular Weight: 656.02 g/mol
Chemical Structure:

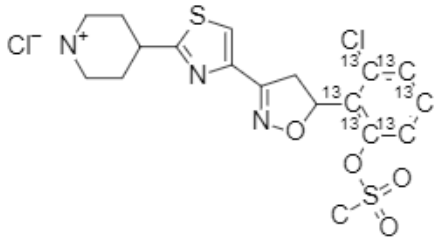


Code Name: BCS- CY96288-$^{13}C_3$, $^{15}N_3$
Molecular Formula: $^{13}C_3C_{22}H_{24}ClF_4{}^{15}N_3N_2O_6S_2$
Molecular Weight: 672.02 g/mol
Chemical Structure:

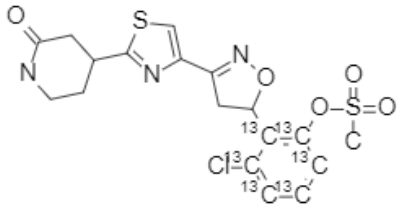


Appendix 1 Continued

Code Name: BCS-CU97237-$^{13}C_6$
Molecular Formula: $^{13}C_6C_{12}H_{21}ClN_3O_4S_2$ Cl
Molecular Weight: 484.37 g/mol
Chemical Structure:

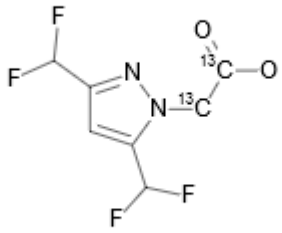


Code Name: BCS-DA63612-$^{13}C_6$
Molecular Formula: $^{13}C_6C_{12}H_{18}ClN_3O_5S_2$
Molecular Weight: 461.89 g/mol
Chemical Structure:

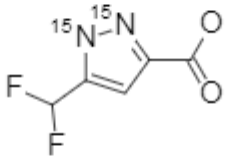


Appendix 1 Continued

Code Name: BCS-CC26101-$^{13}C_2$
Molecular Formula: $^{13}C_2C_5H_6F_4N_2O_2$
Molecular Weight: 228.11 g/mol
Chemical Structure:

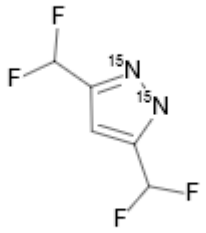


Code Name: BCS CZ38260-$^{15}N_2$
Molecular Formula: $C_5H_4F_2{}^{15}N_2O_2$
Molecular Weight: 164.08 g/mol
Chemical Structure:



Appendix 1 Continued

Code Name: BCS-BP32808-$^{15}N_2$
Molecular Formula: $C_5H_4F_4{}^{15}N_2$
Molecular Weight: 170.08 g/mol
Chemical Structure:



Code Name: BCS-DH34157-$^{13}C_6$
Molecular Formula: $^{13}C_6C_8H_{10}ClN_2O_6S_2.H_4N$
Molecular Weight: 425.82 g/mol
Chemical Structure: