The method consists of sample purification using ENV SPE cartridges conditioned with methanol followed by 1 mM formic acid (aq), elution with 0.02 M ammonium hydroxide in acetonitrile, reconstitution in methanol, addition of 0.02 M formic acid (aq), and filtration through a 0.2 m PTFE filter to prepare for LC/MS/MS analysis. The structures of DPX-HGW86 and its metabolites IN-J9Z38, N-JCZ38, IN-JSE76, IN-K5A77, IN-K5A78, IN-K5A79, and IN-PLT97 can be found in Appendix 2.

3.0 MATERIALS

Equivalent equipment and materials may be substituted unless otherwise specified; note any specifications in the following descriptions before making substitutions. Substitutions should only be made if equivalency/suitability has been verified with acceptable control and fortification recovery data.

3.1 Equipment

EQUIPMENT DESCRIPTION	PRODUCT ID	SUPPLIER
Analytical Balance	Mettler XP205DR Analytical Balance	Mettler Instrument Corp (Hightstown, NJ)
Analytical Evaporator	N-Evap® Model 111 with stainless steel luer fit needles with water bath	Organomation Assoc. (South Berlin, MA)
Sonication	5200 Ultrasonic cleaner	Branson Ultrasonics Corp. (Danbury, CT)
Filtration	Xpertex® syringe filter, 0.2-µm PTFE, 13 mm diam. Cat. No. 9445601 BD™ Syringe, 3 mL, luer-lok™ tip, Cat. No. 14-823-40	PJ Cobert Associates (St. Louis, MO) Fisher Scientific (Fairlawn, NJ)
Solid Phase Extraction	25-mL SPE Reservoir, Cat. No. 944026	P.J. Cobert
Solid Phase Extraction	Visiprep DL™ SPE Manifold, Cat. No. 57250-U	Supelco (Bellefonte, PA)
Solid Phase Extraction	Bond Elut™ ENV SPE Cartridge, 6 mL/500 mg, Cat. No. 12255011, Adapter caps for 6-mL cartridge, Cat. No. 12131001	Varian, Inc. (Palo Alto, CA)
Labware	Corning® 50 mL Disposable Centrifuge Tube, Cat. No. 05-538-55A Kimble® 50-mL conical glass centrifuge tube, Cat. No. 05-538-41A	Fisher Scientific (Fairlawn, NJ)
Pipettes	FisherBrand® Disposable 5 mL Pipettes, Cat. No. 13-678-25D FisherBrand® 9" Disposable Pastuer Pipettes, Cat. No. 13-678-20D FisherBrand® Disposable 2 mL Pipettes, Cat. No. 13-678-25C	Fisher Scientific (Fairlawn, NJ)
Pipettes	100-1000 µL Microman M1000 micropipettor 50-250 µL Microman M250 micropipettor	Gilson (Middleton, WI)
Vortex Mixer	Vortex Genie 2, cup head Cat. No. 58815-234	VWR International (Bridgeport, NJ)

3.1 Equipment (continued)

UPLC/MS/MS System

EQUIPMENT DESCRIPTION	PRODUCT ID	SUPPLIER
UPLC	Waters Acquity System	Waters (Milford, MA)
Autosampler Vials	Target DP Snap-It Vials and caps with T/S/T Septa, Cat. No. 03-395C and 03-396M, respectively	Fisher Scientific (Fairlawn, NJ)
UPLC Column	Synergi Polar RP; 3 mm × 50 mm, 2.5 μm particle size diameter	Phenomenex® (Torrance, CA)
Switching Valve	Valco 6-Port electrically actuated valve, Cat. No. 1384	Valco Instruments, Inc. (Houston, TX)
Triple Quadrupole MS	API 5000 triple quadrupole mass spectrometer using Turbo Ion Spray (TIS) and Analyst version 1.5 software	Applied Biosystems/MDS Sciex (Foster City, CA)

3.2 Reagents and Standards

3.2.1 Reagents

The equivalency/suitability of substituted reagents should be verified.

REAGENTS	PRODUCT DESCRIPTION	PRODUCT ID	SUPPLIER
Formic Acid	99%	A0258465; A0255304	Fisher Scientific (Fairlawn, NJ)
Methanol	Optima®, ACS	091867; 091506; 095121	Fisher Scientific (Fairlawn, NJ)
Acetonitrile	HPLC	B00J0596	Acros (Fairlawn, NJ)
Water	In-house De-Ionized OmniSolv®	NA 49126; 49242; 49203	Labconco (Kansas City, MO) EM Science (Gibbstown, NJ)
Ammonium Hydroxide	Certified, ACS	072478	Fisher Scientific (Fairlawn, NJ)

3.2.2 *Reference Analytical Standards*

Reference analytical standards of the following were used:

COMPOUND	LOT NO.	PURITY (%)
DPX-HGW86	307	99.2
IN-J9Z38	002	96.4
IN-JCZ38	004	87.5
IN-JSE76	004	94.8
IN-K5A77	001	95.3
IN-K5A78	001	96.1
IN-K5A79	002	84.4
IN-PLT97	002	87.0

The standards were synthesized at E. I. du Pont de Nemours and Company, DuPont Agricultural Products, Wilmington, DE. Characterization data are archived by DuPont Agricultural Products, E. I. du Pont de Nemours and Company, Wilmington, DE.

3.3 Safety and Health

No unusually hazardous materials are used in this method. All appropriate material safety data sheets should be read and followed, and proper personal protective equipment should be used.

4.0 METHODS

4.1 Principle of the Analytical Method

Water samples were prepared by placing a 20-mL sample into a 50-mL centrifuge tube, fortifying as necessary, and mixing using a vortex mixer. The samples were purified using ENV SPE cartridges. The cartridges were conditioned with 1 column volume of methanol followed by 1 column volume of 1 mM formic acid (aq), and the eluate was allowed to go to waste. A 25-mL reservoir was attached to the ENV cartridge, an additional 5 mL of 1 mM formic acid (aq) was added, and the eluate was allowed to go to waste. Samples were added to the reservoirs and passed through the cartridges using gravity flow, where DPX-HGW86 and its metabolites were retained. Once the samples were through the cartridges, the reservoirs were removed and the ENV cartridges were washed with 5 mL of HPLC-grade water. Vacuum was applied to remove remaining solution. Analytes were eluted with 3 × 5 mL of 0.02 M ammonium hydroxide in acetonitrile into a glass centrifuge tube using gravity flow. The extracts were evaporated to dryness, reconstituted in 1.0 mL of methanol, and then mixed using a vortex mixer and sonicated. One milliliter of 0.02 M formic acid (aq) was added, and the samples were again mixed using a vortex mixer and sonicated. A portion of the extract was filtered through a 0.2 μm PTFE filter into HPLC vials and analyzed by LC/MS/MS.

4.2 Analytical Procedure

4.2.1 Glassware & Equipment Cleaning Procedures

The effectiveness of any cleaning procedure used should be demonstrated by preparation and analysis of reagent blanks. In general, all reusable glass- and plastic ware should be washed in hot tap water with laboratory grade, non-phosphate detergent, rinsed several times with tap water, rinsed several times with deionized water, rinsed once with acetone, and allowed to fully dry before use. Care should be taken to avoid working with high levels of the analyte being monitored in the same laboratory where samples are being extracted and analyzed.

4.2.2 Preparation and Stability of Reagent Solutions

The following procedures may be adjusted to prepare different volumes.

1 M Formic Acid

Add 4.5 mL of concentrated formic acid to approximately 80 mL of de-ionized water, and dilute to 100 mL with de-ionized water. The solution may be stored at room temperature and should be stable for 6 months.

1 mM Formic Acid in Water

Add 1.0 mL of 1 M formic acid solution to approximately 800 mL of de-ionized water, and dilute to 1 L with de-ionized water. The solution may be stored at room temperature and should be stable for 6 months.

1 M Ammonium Hydroxide

Add 7 mL of concentrated ammonium hydroxide to 93 mL of de-ionized water. The solution may be stored at room temperature and should be stable for 6 months.

0.02 M Ammonium Hydroxide in Acetonitrile

Add 20 mL of 1 M ammonium hydroxide solution to 980 mL of acetonitrile. The solution may be stored at room temperature and should be stable for 6 months.

0.02 M Formic Acid

Add 20 mL of 1 M formic acid solution to approximately 800 mL of de-ionized water, and dilute to 1 L with de-ionized water. The solution may be stored at room temperature and should be stable for 3 months.

50:50 Methanol:0.02 M Formic Acid (Dilution Solution)

Add 100 mL methanol to 100 mL of 0.02 M formic acid solution. The solution may be stored at room temperature and should be stable for up to 1 year.

0.1% Formic Acid in Water (Mobile Phase A)

Add 2 mL concentrated formic acid (99%) to approximately 1.5 L of de-ionized water, and dilute to 2 L with de-ionized water. The solution may be stored at room temperature and should be stable for 3 months.

0.1% Formic Acid in Methanol (Mobile Phase B)

Add 2 mL concentrated formic acid (99%) to approximately 1.5 L of methanol, and dilute to 2 L with methanol. The solution may be stored at room temperature and should be stable for up to 1 year.

1:1:1 Acetonitrile:Methanol:Water (Needle Rinse Solution)

Add equal parts of acetonitrile, methanol, and water. Mix well and sonicate. The solution may be stored at room temperature and should be stable for 3 months.

Note: The expiration dates of the above listed solvents and reagents may be extended if their suitability has been verified with acceptable control and fortification recovery data.

4.2.3 *Stock Standard Preparation and Stability*

Individual stock solutions are required for each analyte. Use an analytical balance that provides a weight precision to at least three significant figures. To prepare a stock solution of 100 µg/mL, weigh approximately 10.0 mg (adjusted for purity) of the analyte in a tarred 100-mL volumetric flask. Add approximately 100 mL of acetonitrile and sonicate to dissolve. **IN-JCZ38, IN-K5A78, IN-K5A79, and IN-PLT97** may require dimethyl sulfoxide (DMSO) to dissolve. If analyte does not go into solution with acetonitrile, add DMSO in 10 mL increments and sonicate after each addition. Once analyte dissolves, dilute to a total volume of 100 mL with acetonitrile. These solutions are stored in a refrigerator at approximately 4°C and are stable for at least six months. Stock standards use may be extended if supported by stability test data.

4.2.4 *Standard Preparation and Stability*

The following standard preparation procedures are examples and may be adjusted to prepare different volumes. The individual stock standards are combined in the fortification standards.

Prepare fortification solutions from dilutions of the individual stock solutions. Prepare a 1.0-µg/mL fortification intermediate in order to prepare 0.10-µg/mL and 0.010-µg/mL fortification solutions for sample fortification at the 10 × LOQ and LOQ, respectively. Alternative concentrations may be prepared as needed for other fortification levels. Store fortification solutions at or below 4°C and replace monthly.

1.0 µg/mL Solution (Fortification intermediate)

Dilute 1.0 mL of the stock solution for each analyte into a common 100-mL volumetric flask and fill to line with acetonitrile, cap and mix well.

0.10 µg/mL Solution

Dilute 10.0 mL of the 1.0-µg/mL fortification intermediate into a 100-mL volumetric flask and fill to line with acetonitrile, cap and mix well.

0.010 µg/mL Solution

Dilute 10.0 mL of the 0.10-µg/mL fortification standard into a 100-mL volumetric flask and fill to line with acetonitrile, cap and mix well.

4.2.5 *Calibration Standard Preparation and Stability*

LC Calibration standards are prepared from dilutions of fortification standards with methanol and 0.02 M formic acid. Five or more calibration standards are recommended. The concentration of sample fortified at the LOQ and carried through the extraction is equivalent to a final concentration of 1.0 ng/mL for the each analyte. Keep calibration standards refrigerated and they should be stable for up to a week.

Calibration standards can be prepared according to the following table (alternative or additional standards may be prepared as needed). Prepare weekly.

Initial Standard (µg/mL)	Volume of Initial Standard (mL)	Volume of Methanol (mL)	Volume of 0.02 M Formic Acid (mL)	Final Concentration (ng/mL)
0.10	0.25	2.25	2.50	5.0
0.10	0.15	2.35	2.50	3.0
0.10	0.10	2.40	2.50	2.0
0.010	0.50	2.00	2.50	1.0
0.010	0.40	2.10	2.50	0.8
0.010	0.30	2.20	2.50	0.6
0.010	0.25	2.25	2.50	0.5

4.2.6 *Source and Characterization of Samples*

Samples were characterized under GLP by AGVISE Laboratories of Northwood, ND. Results of the characterization are listed below:

Water Type	pH	Calcium (ppm)	Sodium (ppm)	Hardness (mg equiv $CaCO_3$/L)	Conductivity (mmhos/cm)	Sodium Adsorption Ratio	Total Dissolved Solids (ppm)	Turbidity (NTU)
Pond	8.2	166	66	720	1.44	1.07	1178	5.53
Stream	8.2	126	100	616	1.33	1.76	1022	2.08
Well	8.1	156	18	670	1.10	0.30	860	1.09
Tap	7.9	25	6.4	89	0.21	0.30	102	0.18

4.2.7 *Storage of Samples*

Samples should be stored refrigerated at approximately 4°C and analyzed within 30 days of receipt.

4.2.8 *Sample Fortification Procedure*

All fortifications were made directly to the water sample in the centrifuge tubes after measuring the sample. Twenty milliliter samples were fortified with the 0.10-µg/mL and 0.010-µg/mL multi-analyte fortification standards.

Sample Identification	Amount (mL)	Fortification Solution		Fortification (ppb)
		µg/mL	mL	
LOQ Fort	20 ± 0.1	0.010	0.20	0.10
10×LOQ Fort	20 ± 0.1	0.10	0.20	1.0

4.2.9 *Analyte Extraction Procedure for Water Samples*

1. Measure 20 mL of water sample into a 50-mL centrifuge tube.
2. Fortify sample, if necessary, and mix sample rigorously using a vortex mixer.
3. Condition 6 cc/500 mg ENV SPE cartridges with 1 column volume methanol followed by 1 column volume of 1 mM formic acid (aq) allowing the eluate to go to waste.
4. Attach a 25-mL reservoir to the ENV cartridge and add an additional 5 mL of 1 mM formic acid (aq) allowing the eluate to go to waste.
5. Add the samples from Step 2 to the reservoirs and allow them to flow through the cartridges using gravity flow at approximately 1-5 mL/min.
6. When the samples are through the cartridges, remove the reservoirs and wash the ENV cartridges with 5 mL of HPLC-grade water.
7. Apply vacuum for approximately 20-30 seconds to remove any remaining solution. Analytes are retained on the ENV cartridge.
8. Elute the analytes with 3 x 5 mL of 0.02 M ammonium hydroxide in acetonitrile into a glass centrifuge tube using gravity flow.
9. Evaporate to dryness on an N-Evap using a heated water bath. (Recommended temperature is around 40°C.)
10. Reconstitute the extracts in 1.0 mL of methanol. Mix the extracts using a vortex mixer for approximately 20 seconds and sonicate for approximately 2 minutes.
11. Add 1.0 mL of 0.02 M formic acid (aq), mix using a vortex mixer for approximately 20 seconds, and sonicate for approximately 2 minutes.
12. Filter a portion of the sample through a 13 mm 0.20 µm PTFE filter into an autosampler vial and submit for LC/MS/MS analysis.

4.3 Instrumentation

4.3.1 *Analysis of DPX-HGW86 and Metabolites*

This method uses gradient-elution reversed-phase UPLC on a polar-RP column. The column choice reflects experimental results indicating optimum chromatographic separation from co-extractants. Alternative chromatographic conditions can be used provided the analytical method is validated and acceptable recoveries are obtained. Comparative analysis of DPX-HGW86 and metabolites using HPLC along with HPLC instrument parameters are located in Appendix 5.

4.3.2 UPLC Operating Conditions

System:	Waters Acquity
Column:	50 × 3.00 mm, Synergi Polar-RP analytical column with 2.5-μm diameter packing.
Column Temperature:	30°C
Injection Volume:	5.0 μL
Autosampler Temperature:	10°C
Flow Rate:	0.500 mL/min
Conditions:	Time %A %B Flow Rate (mL/min) 0.00 50.0 50.0 0.500 3.00 30.0 70.0 0.500 6.00 30.0 70.0 0.500 7.00 5.0 95.0 0.500 8.00 5.0 95.0 0.500 8.10 50.0 50.0 0.500 10.00 50.0 50.0 0.500 A: 0.1% Formic acid in Water B: 0.1% Formic acid in Methanol
Approximate Retention Times	(minutes)
DPX-HGW86	2.8
IN-J9Z38	6.2
IN-JCZ38	1.8
IN-JSE76	2.4
IN-K5A77	3.6
IN-K5A78	4.4
IN-K5A79	2.1
IN-PLT97	3.7
Total Run Time:	10.0 minutes

Triple Quadrupole MS Operating Conditions

Splitter: None (all flow from column goes to source)

Interface: Turbospray

Mode: MRM

Resolution: Unit

TIS Source: Positive

ANALYTE	Q1 (*M/Z*)	Q3 (*M/Z*)	DWELL (MIN)	CUR (PSI)	GS1 (PSI)	GS2 (PSI)	TEM (°C)	IHE	IS (V)	CAD (PSI)	DP (V)	EP (V)	CE (V)	CXP (V)
DPX-HGW86	475.0	286.0	2.80	15	60	40	600	on	5500	10	85	10	28	19
	473.0	284.0												
IN-J9Z38	457.0	188.0	6.10	15	60	40	600	on	5500	10	150	10	44	11
	455.0	186.0												
IN-JCZ38	493.1	286.0	1.80	15	60	40	600	on	5500	10	90	10	22	18
	491.0	284.0												
IN-JSE76	494.2	463.2	2.30	15	60	40	600	on	5500	10	90	10	26	31
	492.2	461.2												
IN-K5A77	475.2	299.1	3.60	15	60	40	600	on	5500	10	200	10	54	19
	473.0	297.0												
IN-K5A78	474.0	186.0	4.40	15	60	40	600	on	5500	10	200	10	46	12
	476.0	188.0												
IN-K5A79	480.1	463.1	2.00	15	60	40	600	on	5500	10	90	10	24	34
	478.0	461.1												
IN-PLT97	460.1	424.1	3.70	15	60	40	600	on	5500	10	150	10	36	15
	462.1	426.1												

4.3.3 *Calibration Procedure and Sample Analysis*

A chromatographic standard should be analyzed prior to the start of analyses to establish that the instrument is working properly. Operating parameters must be tailored to the particular instrument used, especially if it is to be an alternate vendor's instrument, and should be checked daily. Note that it may be necessary to add some ion channels other than those used for development of this method when utilizing this method on other instrumentation. Each ion channel used for sample analysis/ quantitation must be checked to insure it is free of interference. A control will be used to demonstrate that baseline interference is less than signal-to-noise 3:1. Begin each sample set by injecting a minimum of 2 calibration standards. The first injection of a calibration standard should always be disregarded.

4.4 Calculations

4.4.1 *Methods*

The response factor, RF, for each analytical standard is the ratio of the analyte concentration to the analyte peak area.

$$\text{RFstd} = \frac{\text{Concentration of analyte (ng/mL)}}{\text{Analyte peak area}}$$

The average response factor, RF_{ave}, calculated from each standard level analyzed in an analytical set containing control, fortified, or treated samples was used to calculate the concentration of DPX-HGW86 and its metabolites in these samples.

$$RF_{ave} = \frac{(RFstd1 + RFstd2 + RFstd3 + ...RFstdn)}{\text{Total Number of Standards Injected}}$$

The concentration (ppb) of analyte found in each sample was calculated as follows:

ppb analyte Found =

$$\frac{[\text{Peak Area x } RF_{ave}] \text{ x } [\text{Aliquot Factor x Final Vol.(mL) x Dilution Factor}]}{\text{Sample Volume (mL)}}$$

Where:

Total Extract Volume = 20 mL

Aliquot Taken = 20 mL

Aliquot Factor = Total Extract Volume / Aliquot Taken = 1

Final Sample Volume (Final Vol.) = 2 mL

Sample Volume = 20 mL

The percent recovery found was calculated as follows:

$$\% \text{ Recovery} = \frac{(\text{ppb found})}{(\text{Fortification level, ppb})} \text{ x } 100\%$$

4.4.2 *Example*

The calculation below shows the concentration of DPX-HGW86 from a pond water sample fortified at 0.10 ppb, (LOQ 2) in the validation set. See chromatogram, Appendix 4 and Residue Summary Sheet for values to substitute into calculations:

RF_{ave} =2.7128E-5 ng/mL / peak area

Peak Area HGW86 Fortified Sample: = 32910

Total Extract Volume: = 20 mL

Aliquot Taken: = 20 mL

Final Sample Volume: = 2 mL

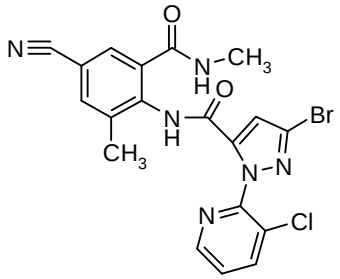
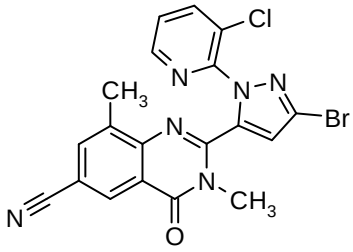
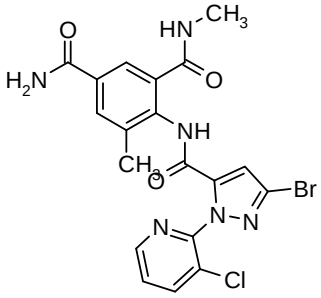
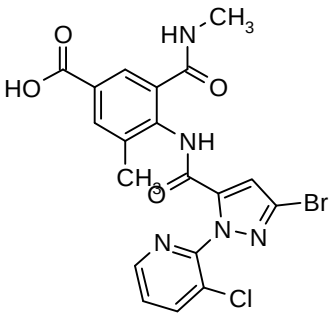
LC Dilution Factor: = 1

Sample Volume: = 20 mL

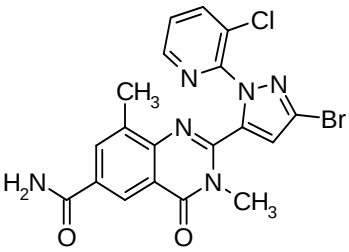
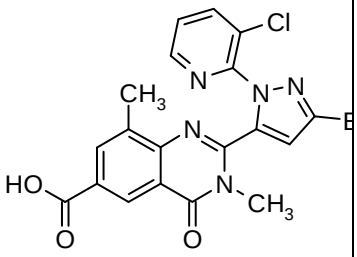
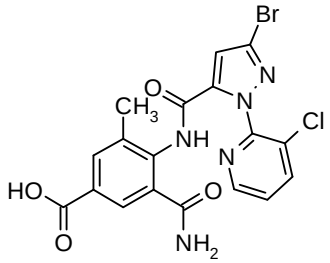
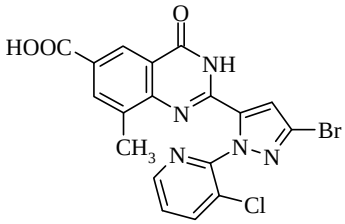
$$\text{ppb HGW86 Found} = \frac{(32910 \text{ x } 2.7128E-5) \text{ x } (1 \text{ x } 2\,\text{mL} \text{ x } 1)}{20\,\text{mL}} = 0.089280 \text{ ppb}$$

$$\% \text{ Recovery} = \frac{0.089280\,\text{ppb}}{0.10\,\text{ppb}} \text{ x } 100\% = 89\%$$

APPENDIX 2 STRUCTURES OF DPX-HGW86 AND METABOLITES

IDENTIFIER	DESCRIPTION	STRUCTURE	IUPAC
DPX-HGW86	Parent M.W. 473.72		3-Bromo-N-[4-cyano-2-methyl-6-(methylcarbamoyl)phenyl]-1-(3-chloropyridin-2-yl)-1*H*-pyrazole-5-carboxamide
IN-J9Z38	Condensed or Cyclized Metabolite M.W. 455.70		2-[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]-3,8-dimethyl-4-oxo-3,4-dihydroquinazoline-6-carbonitrile
IN-JCZ38	A mido anthranilamide M.W. 491.73		4-({[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]carbonyl}amino)-N3,5-dimethylisophthalamide
IN-JSE76	M.W. 492.72		4-({[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]carbonyl}amino)-3-methyl-5-(methylcarbamoyl)benzoic acid

APPENDIX 2 STRUCTURES OF DPX-HGW86 AND METABOLITES (CONTINUED)

IDENTIFIER	DESCRIPTION	STRUCTURE	IUPAC
IN-K5A77	M.W. 473.72		2-[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]-3,8-dimethyl-4-oxo-3,4-dihydroquinazoline-6-carboxamide
IN-K5A78	M.W. 474.70		2-[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]-3,8-dimethyl-4-oxo-3,4-dihydroquinazoline-6-carboxylic acid
IN-K5A79	M.W. 478.69		4-({[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]carbonyl}amino)-3-carbamoyl-5-methylbenzoic acid
IN-PLT97	M.W. 460.68		2-[3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl]-8-methyl-4-oxo-3,4-dihydroquinazoline-6-carboxylic acid