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Abstract: The preparation of carbon-14 labeled compounds and their use in discovery and development of agrochemicals was discussed.

Keywords: Agrochemicals; Degradates; Insecticide; Spinetoram; carbon-14

USE OF ISOTOPES IN AGROCHEMICAL RESEARCH

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Abstract: Both radioactive and stable isotopes play a significant role in the research, development and registration of agrochemicals. Tritium labels are frequently used in early compound design and in screening of analogs for biological activity and initial fate data. Radioisotopes can be applied in mechanistic studies to determine distribution of agrochemicals in plants and modes of action. Regulatory agencies, such as the United States Environmental Protection Agency, mandate a series of biotic and abiotic degradation studies that are conducted in a wide variety of biological matrices, including animal, plant, water, soil and aquatic systems. Use of radiolabeled test materials, commonly 14C, allows calculation of balance data and tracking of the degradation products. Data on both degradation kinetics and pathways of degradation are required for registration. Studies require maintenance of a 90-110% radiobalance in biologically viable systems for up to a year, with periodic isolation and structure elucidation of degradation products. Radiolabeled test material allows characterization of degradation products have been incorporated into the biological matrices. This multi year data generation process develops a comprehensive picture of the fate of the agrochemicals. Stable isotopes play significant roles as internal analytical standards in residue analysis and in stewardship monitoring.

Keywords: agrochemicals; degradates; risk assessment

Introduction: Both radioactive and stable isotopes play a significant role in the research, development and registration of agrochemicals. Isotopically labeled material, both radioactive and stable, are invaluable in the initial discovery phase, registration data generation studies and in residue analysis. Once a compound is commercially available, isotopes play a significant role in monitoring and stewardship programs.

Discussion: Screening programs may identify a few members of a chemical class which demonstrate a desireable biological activity. In an effort to maximize the desireable activity while decreasing undesireable traits of solubility, stability, phytotoxicty and/or toxicity, a large number of structural analogs are synthesized and tested. As an example, Figure 1 shows members of the sulfonylurea class which have been tested by different companies.

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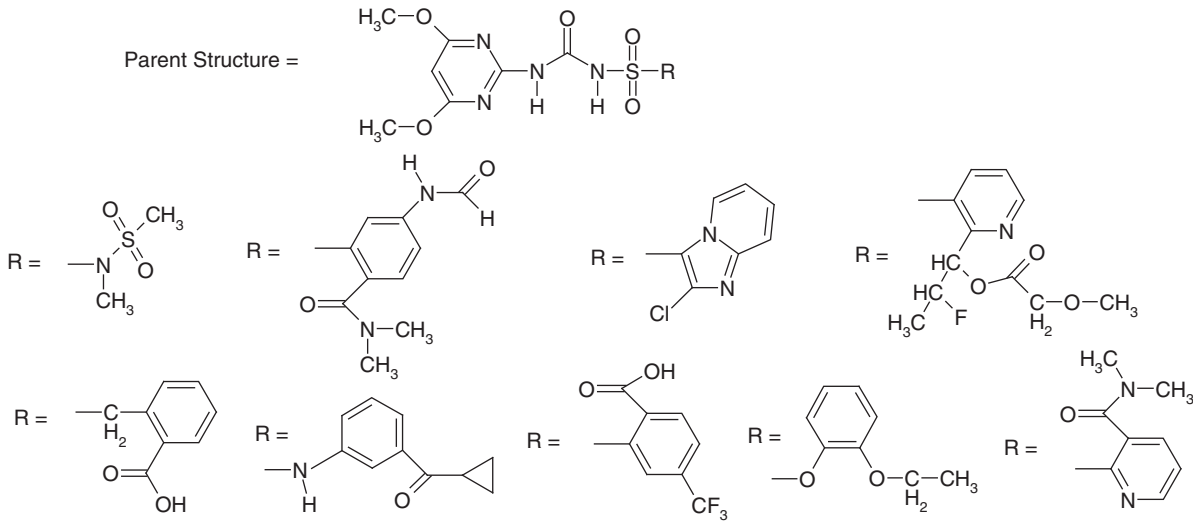

Figure 1. Screening of Members of a Chemical Class for Biological Activity and Initial Environmental Fate.

Once a compound has been identified as having desireable traits for development as a commercial product, a series of studies are initiated to determine the potential risk that use of that chemical might entail. Exposure (chemical plus any degradates of concern)+ Probability (likelihood that nontarget species will be exposed to the chemical or degradates)+Hazard (toxicity of chemical or degradates to nontarget species) all contribute to the determination of risk. Risk characterization will determine whether or not a chemical is a candidate for commercial use. In addition, the risk characterization can dictate the criteria for legal use of the chemical once it is registered and commerically available.

Initial efficacy and toxicity testing can be done with unlabeled test materials. However the focus on both rate and nature of degradation of xenobiotics in biological matrices dictates the use of radiolabeled test material in this tier of the screening process. Because of the relative ease of synthesis, tritium labeled test materials are frequently used to generate initial fate data. The radiolabel allows measurement of the kinetics of degradation and of the mobility of both parent compound and degradates. These characteristics are major determinants whether a compound is a viable candidate for commercial development. Full development and registration of an agrochemical typically costs many tens of millions of dollars and 6–8 years of data generation. Therefore the initial screening process is extremely important.

Radioisotopes and autoradiography can also be applied in mechanistic studies to determine distribution of agrochemicals in biological matrices. This information is useful in determining whether biological activity of the test material is systemic or localized and in predicting areas of potential residue accumulation.

Regulatory agencies, such as the United States Environmental Protection Agency, mandate a series of biotic and abiotic degradation studies that are conducted in a wide variety of biological matrices, including animal, plant, water, soil and aquatic systems. These are both laboratory and field studies, some with biological phases that may run up to a year. Use of radiolabeled test materials, commonly ^{14}C, allows calculation of balance data and tracking of the degradation products. The radiolabel is placed in a relatively stable position to allow monitoring and isolation of the intermediate structures as the chemical degrades. Multiple ring structures often undergo bridge cleavage to yield single ring components. To develop a full understanding of the degradation pathway, either multiple radiolabels are placed in the test substance, or parallel studies are conducted with test material radiolabeled in different locations in the molecule. Samples are pulled at multiple time points during the conduct of the studies. A series of extraction, purification and analytical procedures allows generation of both structure elucidation of the isolated radiolabeled components and calculation of the percent of total dose which each component represents.

Many of the degradation and mobility studies are required to run at proposed agrochemical field use rates. A typical use rate for a herbicide is 200 grams of active ingredient per hectare or 80.94 grams per acre. Assuming the chemical is incorporated into the top 5 cm of the soil and the soil has an average density of 1.5 g/cm^3, the 80.94 grams of chemical would be distributed in 9.25×10^8 grams of soil under normal field use. A laboratory soil study uses 50–100 grams of soil per incubation. Dosing of the laboratory study at the 50 gram sample size and at the field rate would require only 4.35 μg of test material per incubation. The aerobic soil guideline studies require maintenance of a 90–110% radiobalance in a biologically viable system for up to a year, with periodic isolation and structure elucidation of degradation products. The test system is under a constant air flow and volatile components are collected in a variety of trapping materials during the incubation phase. In several of the systems being tested, the degradation radiolabeled products may become incorporated into the biological matrices and are not extractable under normal isolation procedures. Combustion of the extracted residues converts the carbon in the samples to carbon dioxide which is readily trapped as carbonates in traps containing a solution of a strong base. The individual degradation products, both the volatile and nonvolatile, may each account for only a few percent of the initial dosing material. Therefore, the limits of quantitation of the radioassay methods have to show sufficient sensitivity to accurately calculate the quantitative distribution. As shown in Figure 2 use of a variety of commercial fluors allows liquid scintillation counting of the various sample types and the calculation of the limits of quantitation for each assay.

The limit of quantitation will vary depending on the specific activity of the test material used to dose the test system. The 58.9 μCi/mg specific activity used in the example in Figure 2 resulted in limits of quantitation ranging from 0.00032 μg to 0.00063 μg for all assay types. In the example of the aerobic soil metabolism study, these values translate to 0.007% to 0.014% of the 4.35 μg of test material in each incubation flask.

One method for calculating the Limit of Quantitation (Lq).

$$Lq = 50 * \left(1 + \sqrt{[(DPM_{bkg} * Count\_Time)/12.5]}\right)/Count\_Time$$

Example: Compound with Specific Activity = 58.9 μCi/mg

$$\text{Specific Activity} \times (2.2210^{6\ \text{dpm}}/\mu\text{Ci}) \times \text{mg}/1000\ \mu\text{g} = \text{dpm}/\mu\text{g} = 130,758\ \text{dpm}/\mu\text{g}$$

Example: Radioassay*
61 dpm/130,758 dpm/μg = 0.00053 μg

Type of Assay	Type of Fluor	Amount of Fluor	Approximate Limit of Quantitation (dpm)	Limit of Quantitation (μg)
Radioassays*	Hionic	10 mL	69	0.00053
Radioassays**	Scintisafe 30%	6-12 mL	61	0.00047
HPLC Quantitation	Ultima Flo M	4 mL	42	0.00032
Radioassays***	Oxosol	15 mL	82	0.00063

* Radioassays of potassium hydroxide volatile fractions
**Radioassays include assays of the foam plugs, aqueous samples, TLC quantitations of components
*** Radioassays of nonextractable fractions
Limit of Detection (μg) was based on the specific activity and LSC background

Figure 2. Calculation of Limit of Quantitation of Radiolabeled Components of Metabolism Samples.

Therefore, the limit of quantitation ranged from 0.00032 μg to 0.00063 μg for all assay types.

Mobility studies are the other major group of mandated studies for which radiolabeled compounds are vital to generation of accurate environmental fate profiles. In one type of mobility study, carbon-14 labeled test material is applied at rates equivalent to proposed field use rates to the top of a 36 cm column of soil. The equivalent of 20 inches of rainfall is applied to the column either immediately if the primary interest is the parent test material, or after a period of aging if parent and degradate mobility are the primary focus of the study. Table 1 shows the significant difference in distribution of the radiolabel in different soil types. Use of the radiolabel allows isolation/identification and quantitation of the components in each of the 6 cm segments and in the leachate.

Quantitative and qualitative assays of multiple time points during the conduct of the studies allow calculation of the degradation kinetics of the applied test material, and of the formation and degradation kinetics of the degradates. Degradates may have potential biological or toxicological activity, both in the target species and in nontarget species, and therefore are an integral component of the risk assessment. The kinetics of both parent and degradates help determine the potential exposure component of the risk assessment for a chemical. Figure 3 shows an example of the type of kinetic relationship and prediction of metabolite persistence that can be developed from the study data.

Table 1. Example of an Aged Column Leaching Study Using Six Soil Types

	Sandy Clay Loam	Loam	Sandy Loam	Sand	Niagrara Loam	Silty Clay Loam
0–6 cm	60.4	43.4	41.7	54.5	53.4	36.8
6–12 cm	11.4	8.9	26.0	9.0	4.7	9.2
12–18 cm	9.0	9.9	4.5	0.9	5.0	6.3
18–24 cm	6.7	9.1	4.1	0.8	4.9	7.9
24–30 cm	3.2	9.6	4.5	0.8	5.2	8.8
30–36 cm	1.4	7.1	3.9	0.8	6.1	8.9
Leachate	0.2	7.6	11.0	25.8	21.3	18.4

Test Material was aged in aerobic soil 30 days before initiation of leaching 1030 ml 0.01 M aqueous calcium chloride (equivalent to 20 inches of rain) used for leaching

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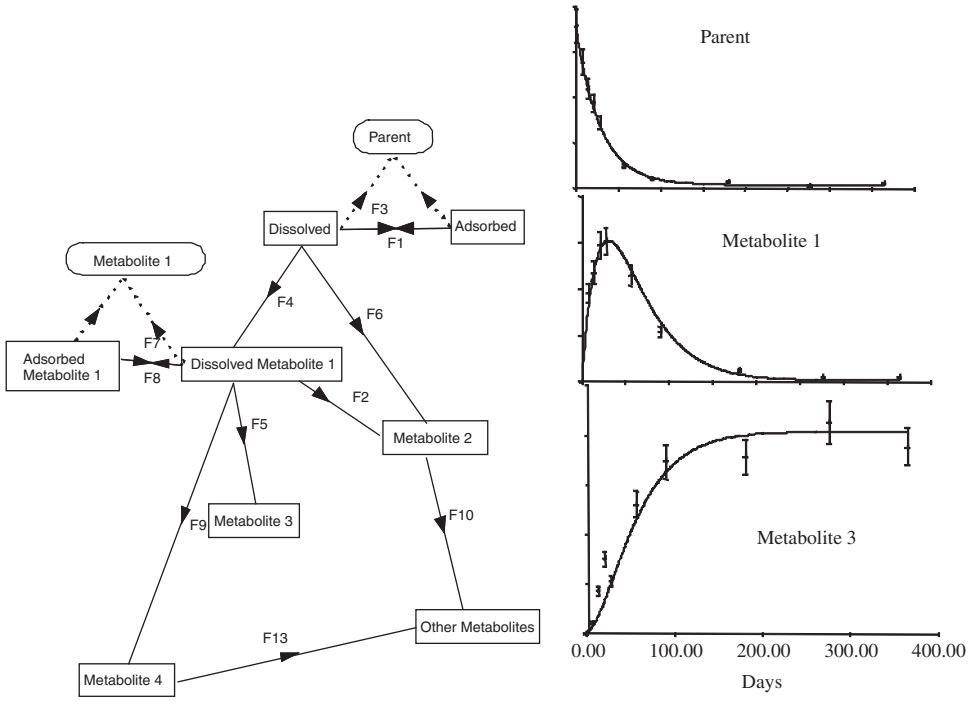

Figure 3. Example of the Formation and Degradation Kinetics of a Xenobiotic in an Aerobic Soil Study.

The environmental mobility studies and the accumulated degradation studies in environmental matrices, plants and animals are combined with toxicological effect studies conducted on both target and nontarget species to generate a database for submission to regulatory bodies in support of registration of a chemical for commercial use. The regulatory agencies will utilize this database to determine if the calculated risks for registration and subsequent use of the chemical are acceptable. Even if the registration criteria are addressed successfully, restrictions on the labeled uses and use patterns may be mandated. Figure 4 shows a generalized representation of this process for the U.S. EPA, but similar processes are used for regulatory bodies in other regions/countries.

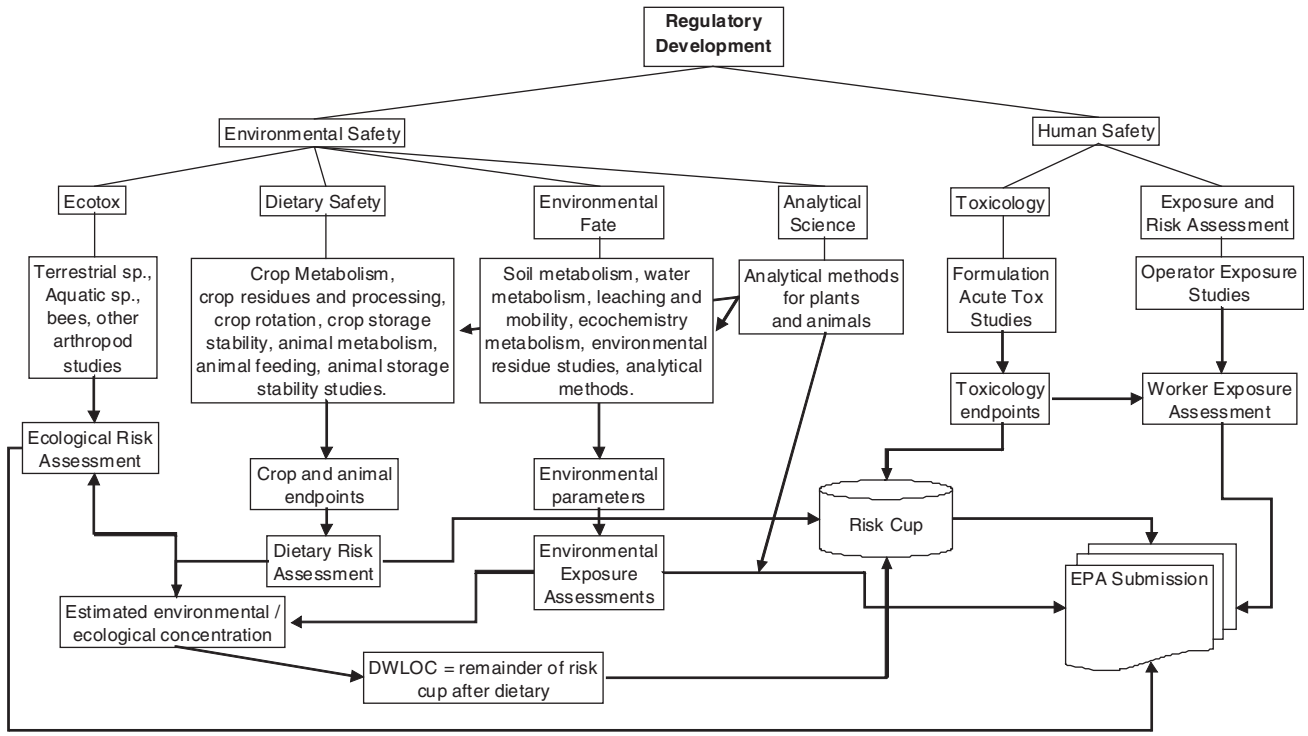

Figure 4. Generation of Database for Registration Submissions to Regulatory Agencies.

Stable isotopes also play a significant role in the generation of data in agrichemical studies. One common usage is as internal analytical standards in residue analysis in field studies and in stewardship monitoring programs. The use of stable isotope and analyte ratios, particularly in MS/MS methodology, allows the accurate determination of very low concentrations of multiple analytes in complex biological samples that have high levels of matrix contamination.

Summary: Radioactive and stable isotopes play an integral role in agrochemicals, from the initial research/development/registration phases to stewardship activities once the agrochemicals are in commercial use.

PREPARATION OF ISOTOPICALLY LABELED STANDARDS OF SPINETORAM

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Spinetoram is a semi-synthetic natural product composed primarily of two major factors, spinetoram J and L. Isotopically labeled standards (^{13}C, ^{14}C and ^{2}H) of both spinetoram J and L, and their major metabolites, were prepared. These standards were used to support registration studies and metabolite identification.

Keywords: spinosyn; spinetoram; carbon-14; carbon-13; deuterium; stable isotope; radioactive; insecticide; synthesis; fermentation; mass spectrometry

Introduction: Spinosyns are a family of bacterial natural products produced by fermentation of actinomycete *Saccharopolyspora spinosa*.[1] *S. spinosa* was discovered in the early 1980s from a soil sample taken from an abandoned rum distillery in the Caribbean. Of the many factors produced by the fermentation, the two predominate factors are spinosyn A and D (Figure 1). Fermentation extracts containing this mixture, known as spinosad, show broad insecticidal activity. The broad spectrum activity combined with a novel mode of action and favorable environmental and toxicological profile has lead to the commercialization of several products containing spinosad as the active ingredient, including Tracer®, Success®, Spintor® and Conserve®.

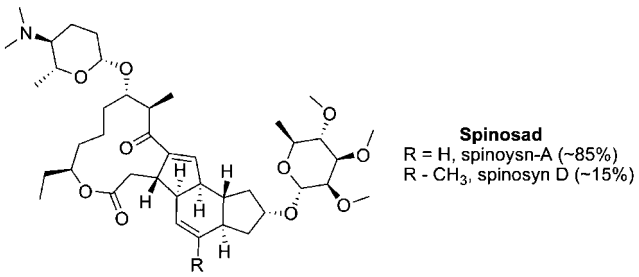
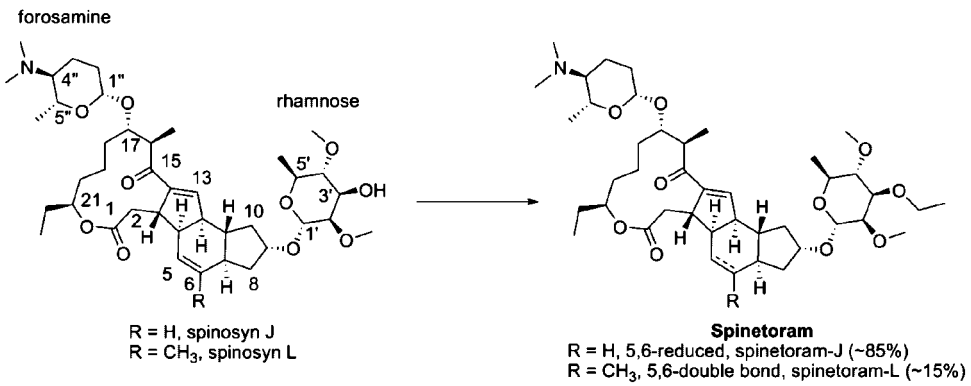


Figure 1. Structure of spinosad.

Efforts to improve the efficacy and spectrum of activity of the spinosyns lead to the discovery of a semi-synthetic product known as spinetoram.[2] A fermentation strain of *S. spinosa* was discovered that produces spinosyn J and L. Ethylation of the free hydroxyl group on the rhamnose sugar and selective reduction of the isolated double bond of spinosyn J yields the semi-synthetic product with better activity while maintaining the favorable mode of action, environmental and toxicological profile (Scheme 1).



Scheme 1. Conversion of spinosyn J/L to spinetoram J/L.

Through the development and registration phases of the project there was a need to prepare stable and radioactive isotopes of the mixture as well as the individual components, spinetoram-J and spinetoram-L. The stable isotopes (^{2}H and ^{13}C) would be used as

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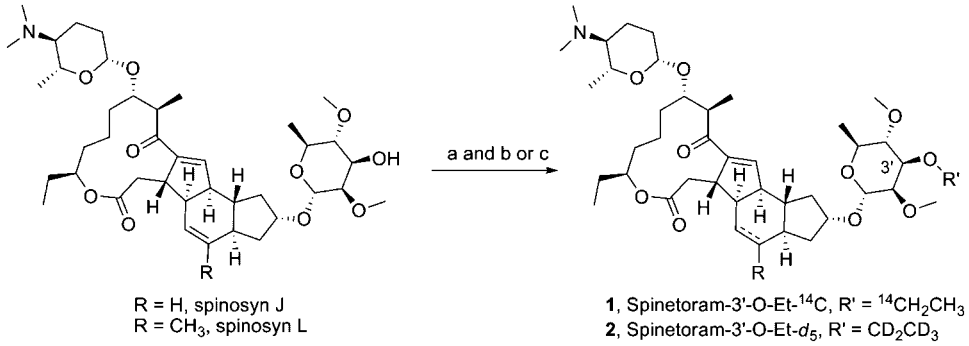
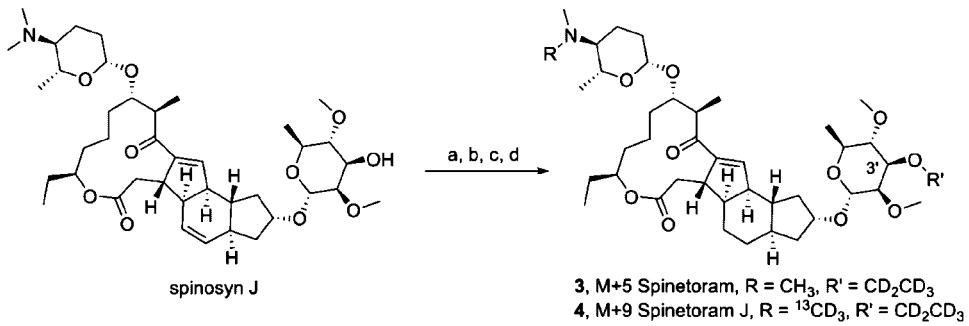
internal standards for method development as well as aiding in metabolite identification. The radioactive isotope (^{14}C) would be used for environmental fate and plant and animal metabolism studies. Mixed isotopically labeled standards that contained ^{2}H and/or ^{13}C in combination with ^{14}C were also prepared yielding a unique isotope pattern that was used to aid in the rapid identification of metabolites utilizing LC/MS positive electrospray ionization technology.

Results and Discussion: One of the easiest ways to incorporate a label into spinetoram is to start with a mixture of spinosyn J and L and selectively reduce the 5,6-double bond of spinosyn J with Wilkinson's catalyst (Scheme 2). The free hydroxyl group in this mixture of 5,6-dihydrospinosyn J and spinosyn L can then be alkylated with either iodoethane-1-^{14}C to give a radioactive tracer **1** or iodoethane-d_5 to produce a stable isotope **2**. The poor yield observed with the alkylation using iodoethane-1-^{14}C was the result of the labeled iodoethane being contaminated with unlabeled iodomethane.



Scheme 2. (a) $ClRh(PPh_3)_3$, H_2, toluene, 86%; (b) I-$^{14}CH_2CH_3$, KOH, DMSO, 7%; (c) I-CD_2CD_3, KOH, DMSO, 51%.

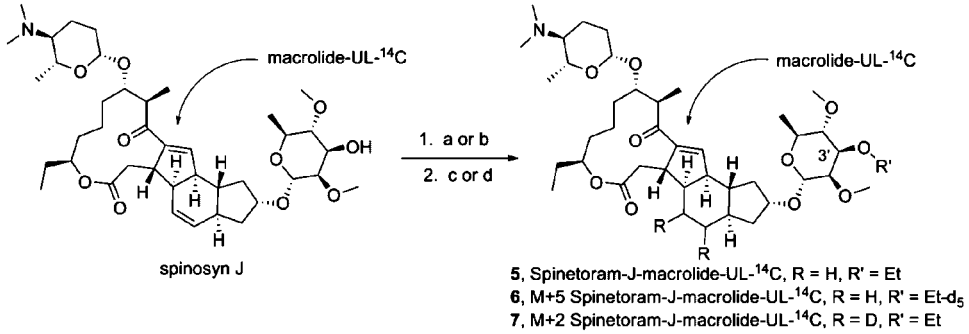
The individual components, spinetoram J and L, were also selectively labeled by starting with spinosyn J or L respectively (Scheme 3). Reduction of the 5,6-double bond of spinosyn J with rhodium on alumina gave near quantitative yield of 5,6-dihydrospinosyn J which was then alkylated with bromoethane-d_5 under phase transfer conditions to give a good yield of an M+5 stable isotope of spinetoram-J (**3**). The forosamine nitrogen could then be demethylated with Selectfluor[3] followed by re-alkylation with iodomethane-^{13}C-d_3 to give a decent yield of an M+9 stable isotope of spinetoram-J (**4**).



Scheme 3. (a) 5% Rh/Al, H_2, IPA, 100%; (b) Br-CD_2CD_3, KOH, H_2O, $Bu_4N^+Br^-$, 93%; (c) Selectfluor, pyridine, CH_3CN, 0°C 70%; (d) $^{13}CD_3$-I, $(i\text{-}Pr)_2NEt$, THF, 74%.

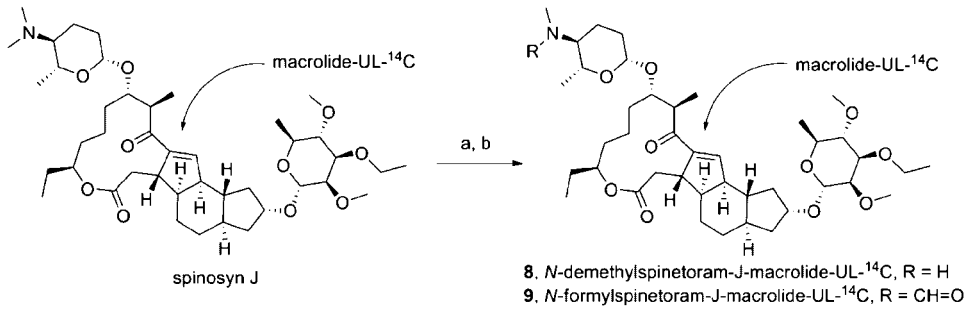
Carbon-14 was incorporated into the macrolide of spinosyn J and L by fermentation with carbon-14 labeled acetate. The fermentation was performed by an external vendor who also separated the two factors by preparative reverse phase HPLC. The final chemical steps were then performed in-house to prepare macrolide-^{14}C labeled standards of spinetoram-J and L.

Reduction of the 5,6-double bond of spinosyn J-macorlide-UL-^{14}C followed by phase transfer catalyzed ethylation of the 3′-hydroxy group provided a carbon-14 macrolide label of spinetoram-J, **5**, in high yield (Scheme 4). The alkylation could also be run with bromoethane-d_5 to provide a tracer that contained carbon-14 in the macrolide ring as well as a stable isotope in the rhamnose sugar **6**. Alternatively, the reduction of the 5,6-double bond could be run in the presence of deuterium gas and the intermediate alkylated with bromoethane to provide a standard which now had both radioactive and stable isotopes in the macrolide portion of spinetoram-J (**7**).



Scheme 4. (a) 5% Rh/Al, H_2, IPA, 3 h, 100%; (b) 5% Rh/Al, D2, DME, 3 h, 90%; (c) Br-CH_2CH_3, KOH, H_2O, $Bu_4N^+Br^-$, 81%; (d) Br-CD_2CD_3, KOH, H_2O, $Bu_4N^+Br^-$, 83%.

Two of the major metabolites of spinetoram that are observed in the environment are the *N*-demethyl and *N*-formyl derivatives. Tracers of both of these metabolites were prepared from spinetoram-J-macrolide-UL-^{14}C (Scheme 5). Treatment of spinetoram-J-macrolide-UL-^{14}C with Selectflour in the presence of pyridine produced the *N*-demethyl derivative **8** in good yield. Refluxing *N*-demethylspinetoram-J-macrolide-UL-^{14}C with ethyl formate gave a good of the *N*-formyl metabolite **9**.



Scheme 5. (a) Selectfluor, pyridine, CH_3CN, 0°C, 78%; (b) ethyl formate, reflux, 8 h 90%.

A number of isotopically labeled standards of spinetoram-L were also prepared as shown in Figure 2. These standards were prepared from spinosyn L using the same chemical transformation as those described for the corresponding analog of spinetoram-J.

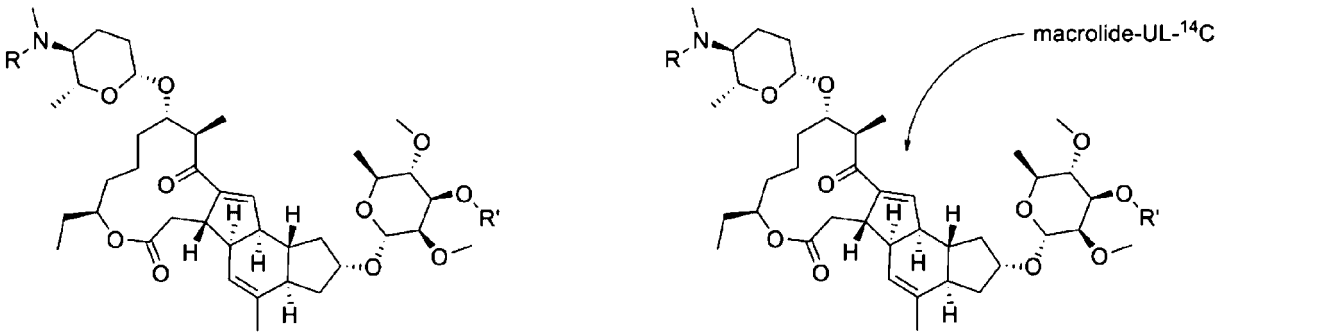


Figure 2. Isotopically labeled standards of spinetoram-L.

Registration of a new insecticide requires a number of studies for which isotopically labeled standards are needed. These studies include plant and animal metabolism studies as well as environmental fate studies which include both aerobic and anaerobic soil studies and aqueous photolysis and hydrolysis studies. The challenge associated with these studies is identifying metabolites from a complex molecule and extracting from complex matrices when combined with low dose levels.

To aid in the identification of trace level spinetoram metabolites a unique labeling scheme was developed. By combining C-14 labeled and stable isotope labeled forms of spinetoram a unique isotopic 'fingerprint' was produced which could readily be distinguished using mass spectrometry. An example of the unique isotope pattern observed for the parent ion when spinetoram-J is mixed with an M+5 stable isotope of spinetoram-J is shown in Figure 3. Assuming that the isotopically labeled portion of the molecule remains intact, the mass spectrum of all spinetoram metabolites generated in subsequent environmental studies must contain this unusual isotopic pattern. Spinetoram is readily ionized by electrospray ionization (ESI), and under MS/MS conditions

produces fragment ions resulting from loss of the sugar moieties. These MS/MS fragments ion provide key information for the identification of spinetoram metabolites. In subsequent studies this unique isotope pattern was used to confirm that the observed masses were related to the applied material. A combination of applying a mixture of C-14 labeled and stable isotope standards of spinetoram J and L was found to greatly aid in the rapid identification of a number of metabolites from these complex systems.

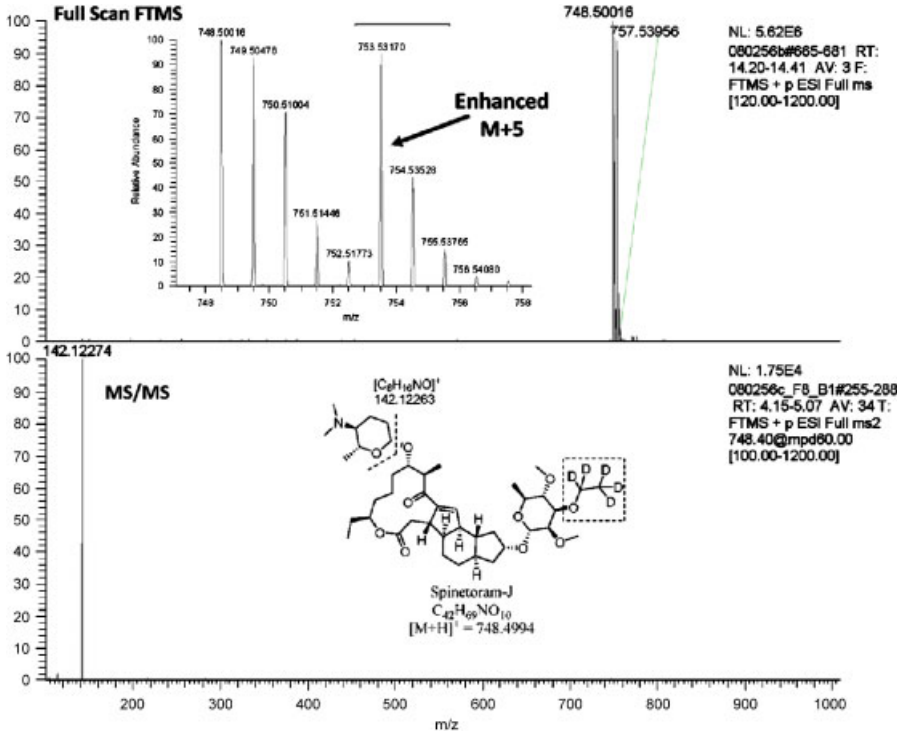


Figure 3. Unique isotope pattern produced from spinetoram J and M+5 spinetoram J.

Conclusion: Several isotopically labeled standards of spinetoram and it's individual components, spinetoram-J and spinetoram-L, were prepared synthetically by incorporation of ^{2}H, ^{13}C and ^{14}C into molecules starting from either spinosyn J or L. Fermentation was used to label the macrolide ring of spinosyn J and L with carbon-14. The ^{14}C-ring labeled materials were then synthetically transformed to spinetoram-J and L. These labeled materials were utilized as internal standards and for registration studies. Combinations of $^{2}H-$, $^{13}C-$, ^{14}C-labeled and unlabeled parent material were used to produce a unique isotope pattern which allowed low level metabolites in environmental fate studies to be rapidly identified by the use of mass spectrometry.

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