Tritritionikkomycin Z, [*uracil*-5-^{3}H,*pyridyl*-2,4-$^3H_2$]: Radiolabeling of a Potent Inhibitor of Fungal and Insect Chitin Synthetase

Tetsu Ando,‡ Berhane Tecle,§ Robert F. Toia, and John E. Casida*

Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, California 94720

Nikkomycin Z (NZ) (a potent fungicide, insecticide, miticide, and inhibitor of fungal and insect chitin synthetase) was converted to a mixture of specific mono-, di-, and tribromo derivatives (BrNZ, Br_2NZ, and Br_3NZ, respectively) on reaction with *N*-bromosuccinimide in *N*,*N*-dimethylformamide. Substitution by bromine occurred first at the 2-position of the 3-hydroxypyridyl moiety, second at the 5-position of the uracil moiety, and finally at the 4-position of the 3-hydroxypyridyl moiety as observed both for NZ and for mixtures of uridine and 3-hydroxy-6-methylpyridine as model compounds representative of the moieties of NZ. Following fractionation of the various bromonikkomycin derivatives by HPLC, their structures were assigned by NMR, MS, and UV analyses. Catalytic reductive debromination of Br_3NZ with tritium gas over palladium on carbon gave [*uracil*-5-^{3}H,*pyridyl*-2,4-$^3H_2$]NZ. This material has sufficiently high specific activity (~60 Ci/mmol) and suitable positions of labeling to study its uptake, distribution, metabolism, and possible target site interactions in fungal and insect systems.

INTRODUCTION

Chitin synthetase (CS) is a preferred target in the rational design of selective fungicides and insecticides (Marks et al., 1982; Hajjar, 1985; Kramer and Koga, 1986). Nikkomycin Z (NZ) (Figure 1), a fermentation product of *Streptomyces tendae* (Dähn et al., 1976), and some closely related nucleoside peptide antibiotics (Hori et al., 1971; Kobinata et al., 1980) are the most potent known inhibitors of CS of fungi (Gow and Selitrennikoff, 1984; Furter and Rast, 1985) and insects (Cohen and Casida, 1980). NZ also has high fungicidal, insecticidal, and miticidal activity (Zoebelein and Kniehase, 1985). The possible use of NZ or related compounds in medicine and agriculture is limited by species differences in CS sensitivity (Gow and Selitrennikoff, 1984; Furter and Rast, 1985; Zoebelein and Kniehase, 1985) and presumably also by differences related to transport phenomena to the active site (Emmer et al., 1985). Variations in metabolic detoxification processes as noted for various polyoxins and neopolyoxins (e.g., peptidase cleavage) may also play a role (Uramoto et al., 1980; Shenbagamurthi et al., 1986).

The effectiveness of NZ and related compounds against target pest species might be enhanced by structural modifications that improve the affinity for CS, facilitate transport, and reduce metabolic detoxification. These developments require further knowledge of NZ mode of action and metabolism through receptor binding and other assays and therefore the availability of radiolabeled NZ of sufficiently high specific activity. It is advantageous to label both the pyridyl and uracil portions since the parent compound is potentially labile to peptide hydrolysis. The stereochemistry of NZ remains to be fully determined, and, to date, only a partial synthesis has been achieved (Hahn et al., 1987). From a practical point of view, therefore, the multiple introduction of ^{3}H into the molecule, as opposed to ^{14}C, represents the only feasible experimental approach at this time. Monotritio-NZ, prepared earlier at 4 Ci/mmol (Yadan et al., 1984), does not satisfy the criteria noted above.

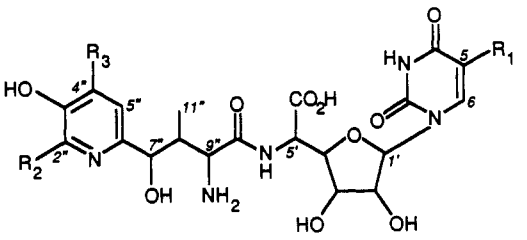

Figure 1. Structures of nikkomycin Z and derivatives showing the numbering system used in this paper: nikkomycin Z (NZ), $R_1 = R_2 = R_3 = H$; tritritionikkomycin Z ($^3H_3$NZ), $R_1 = R_2 = R_3 = {}^3H$; bromonikkomycin Z (BrNZ), $R_1 = R_3 = H$, $R_2 = Br$; dibromonikkomycin Z (Br_2NZ), $R_3 = H$, $R_1 = R_2 = Br$; tribromonikkomycin Z (Br_3NZ), $R_1 = R_2 = R_3 = Br$.

This paper describes the preparation of high specific activity tritritio-NZ ($^3H_3$NZ) labeled at specific centers in both the uracil and pyridyl moieties by reductive debromination of tribromo-NZ (Br_3NZ).

MATERIALS AND METHODS

Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75.5 MHz, respectively, for solutions in 5-mm glass NMR tubes with a Bruker WH-300 spectrometer. The ratios of the products were established by integration of the relevant peaks in the ^{1}H NMR spectra. ^{1}H and ^{3}H NMR spectra of $^3H_3$NZ were measured at 300 and 320 MHz, respectively, for solutions in NMR tubes fitted with Teflon inserts with an IBM AF-300 spectrometer (National Tritium Labeling Facility, University of California, Berkeley). Fast atom bombardment (FAB) MS was accomplished with a Kratos MS-50 magnetic sector instrument (Department of Chemistry, University of California, Berkeley) in the positive ion mode with xenon as the reagent gas at 7 kV, from a thioglycerol matrix. UV spectra were recorded with a Perkin-Elmer 576 ST spectrophotometer.

* To whom correspondence should be addressed.

‡ Permanent address: Department of Plant Protection, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan.

§ Present address: Agricultural Research Division, American Cyanamid Co., P.O. Box 400, Princeton, NJ 08540.

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Chromatography. TLC utilized silica gel 60 F_{254} chromatoplates (0.25-mm layers) (Merck, Darmstadt, Germany). HPLC utilized a Beckman 344 gradient liquid chromatograph equipped with a UV detector (280 nm) and fitted with either an Ultrasphere C_{18} 5-μm column (4.6 mm i.d. × 25 cm) (Beckman, Fullerton, CA) for analysis of a Partisil M20 ODS-3 column (20 mm i.d. × 25 cm) (Whatman, Clifton, NJ) for preparative purposes. For the purification of ^3H_3NZ the above analytical column was fitted to a Hewlett-Packard 1040A diode array detector HPLC system coupled, in series, to a flow-through scintillation counter such that UV spectra (200–400 nm) and the levels of radioactivity of the eluted materials could be monitored continuously.

NZ. NZ was supplied 95% pure by Bayer AG (Leverkusen, Germany). NMR analysis of this sample indicated the absence of nikkomycin X. The numbering system used in this paper for NZ is as indicated in Figure 1.

Syntheses. Bromination of Uridine. 5-Bromouridine was prepared by *N*-bromosuccinimide (NBS) bromination of uridine as a solution in *N*,*N*-dimethylformamide (DMF), as described by Srivastava and Nagpal (1970). For the sake of comparison, the previously unreported NMR data are presented here. ^{1}H NMR (δ, in D_2O) (6)H 8.38, (1′)H 5.87, (2′)H 4.33, (3′)H 4.25, (4′)H 4.14, (5′)2H 3.84 and 3.97, $J_{1',2'}$ = 3.5 Hz, $J_{2',3'}$ = 5 Hz, $J_{3',4'}$ = 5.5 Hz, $J_{4',5'}$ = 3.5 and 2.5 Hz, $J_{5',5'}$ = 13 Hz; ^{13}C NMR (δ, in D_2O) (2) 153.7, (4) 164.5, (5) 99.2, (1′) 92.5, (2′) 76.9, (3′) 71.7, (4′) 86.9, (5′) 63.0.

Bromination of 3-Hydroxy-6-methylpyridine. NBS (2 mmol, 356 mg) was added to a stirred solution of 3-hydroxy-6-methylpyridine (1 mmol, 109 mg) in DMF (1 mL) at 0 °C. After 1 h at this temperature, the mixture was further stirred for 16 h at room temperature. Removal of the DMF in vacuo gave the crude product as a brown oil which was purified by preparative TLC (*n*-hexane:acetone 3:2). In addition to the recovery of traces of starting material (R_f = 0.29), the 2-bromo derivative (R_f = 0.53, 16 mg, yield 9%) and the 2,4-dibromo derivative (R_f = 0.59, 182 mg, yield 68%) were obtained. 2-Bromo-3-hydroxy-6-methylpyridine: ^{1}H NMR (δ, in acetone-d_6) (4)H 7.25, (5)H 7.08, CH_3 2.37, $J_{4,5}$ = 8 Hz; ^{13}C NMR (δ, in acetone-d_6) 150.3, 149.5, 130.0, 124.7, 123.9, 22.8. 2,4-Dibromo-3-hydroxy-6-methylpyridine: ^{1}H NMR (δ, in acetone-d_6) (5)H 7.43, CH_3 2.39; ^{13}C NMR (δ, in acetone-d_6) 151.4, 147.0, 130.8, 127.8, 121.0, 22.5.

Bromination of a Mixture of Uridine and 3-Hydroxy-6-methylpyridine. A mixture of uridine (0.25 mmol, 61 mg) and 3-hydroxy-6-methylpyridine (0.25 mmol, 27 mg) in DMF (1 mL) was treated with NBS (0.4 mmol, 71 mg) as described above. After removal of the solvent, the residue was analyzed by ^{1}H NMR. The process was repeated, as above, varying only the quantity of NBS to 0.7 mmol.

Bromination of NZ. A solution of NZ (0.1 mmol, 50 mg) in DMF (3 mL) was brominated with NBS (0.3 mmol, 53 mg) as described above. After removal of the DMF in vacuo, the crude product mixture was fractionated by preparative HPLC [solvent; water:methanol:trifluoroacetic acid (TFA) (72:28:0.25); 7 mL/min] to give monobromo-NZ (BrNZ) (Rt = 13.4 min, 13 mg) and dibromo-NZ (Br_2NZ) (Rt = 19.1 min, 6 mg). When the product mixture obtained on repeating the reaction with double the ratio of NBS to NZ was fractionated, the preparative HPLC separation used water:methanol:TFA (64:36:0.25) as solvent to yield Br_2NZ (Rt = 12.3 min, 9 mg) and Br_3NZ (Rt = 17.7 min, 8 mg). The relevant spectroscopic data are presented in Tables I and II.

Reductive Debromination of the Bromo Derivatives. A mixture of BrNZ (14 μmol, 8 mg), palladium on carbon (10%, 5 mg), ethanol (250 μL), and water (250 μL) was vigorously stirred under hydrogen gas (1 atm) at room temperature. The reaction was monitored by TLC and HPLC (see Table I) every 30 min. BrNZ was completely consumed within 2 h, and a single product with the same R_f and Rt values as NZ was detected. After the catalyst was filtered and the solvent was removed in vacuo, the residue was purified by preparative HPLC to give NZ (5 mg, yield 70%). The ^{1}H NMR spectrum was coincident with that of the authentic sample. Br_2NZ and Br_3NZ were also reduced smoothly to NZ under the same reaction conditions.

Table I. Chromatographic Behavior, FAB–MS, and UV Absorption of Nikkomycin Z (NZ) and Its 2″-Monobromo, 5,2″-Dibromo, and 5,2″,4″-Tribromo Derivatives (BrNZ, Br_2NZ, and Br_3NZ, Respectively)

compd	TLC R_f[a]	HPLC Rt,[b] min	FAB–MS MH^+ (rel intensity) base ion	UV λ_{max},[c] nm
NZ	0.21	3.6	496 (64%) 185	264, 290 (sh)
BrNZ	0.51	6.4	574, 576 (15%) 245	264, 290 (sh)
Br_2NZ	0.63	12.6	652, 654 (15%), 656 245	284
Br_3NZ	0.83	32.8	730, 732 (7%), 734, 736 231	284

[a] Chromatography on a normal-phase TLC plate with *n*-butanol:water:acetic acid (4:2:1). [b] Chromatography on a reversed-phase column (Ultrasphere C_{18}) with water:methanol:TFA (80:20:0.25) at a flow rate of 1.0 mL/min. [c] Absorption in the above HPLC solvent. Approximate ϵ values of 10 400, 7600, 7700, and 9700 for NZ, BrNZ, Br_2NZ, and Br_3NZ, respectively.

Tritiation of Br_3NZ. Catalytic reductive debromination with tritium gas (59.8 Ci/mmol) was accomplished at the National Tritium Labeling Facility. Thus, palladium on carbon (10%, 10 mg), the surface of which was preactivated with tritium gas, was dropped into a mixture of ethanol (200 μL) and water (50 μL) solution of Br_3NZ (7 μmol, 5 mg). This suspension was stirred under tritium gas (1 atm) at room temperature for 4 h. After the catalyst was removed by filtration, the solvent was lyophilized and the residue dissolved in water (1 mL). The crude product was purified by HPLC (to remove tritiated water, other byproducts, and residual starting compound), yielding 106 mCi of ^3H_3NZ with a specific activity of ca. 60 Ci/mmol. The yield for the conversion was ca. 25%.

RESULTS AND DISCUSSION

Model Reactions. Uridine is converted to 5-bromouridine on treatment with NBS in DMF at room temperature (Srivastava and Nagpal, 1970). This indicates that NZ may also undergo bromination at the 5-position of the uracil moiety. To examine bromination reactions of the pyridyl moiety, 3-hydroxy-6-methylpyridine was treated with NBS in DMF. The major product was the 2-bromo derivative with 1 equiv of NBS and the 2,4-dibromo derivative with 2 equiv. To establish the relative reactivity of the uracil and pyridyl moieties in NZ bromination, an equimolar mixture of uridine and the hydroxypyridine in DMF was treated with 0.8 molar equiv, relative to total reactants, of NBS. Uridine and 5-bromouridine were obtained in a 3:2 ratio and the 2-bromo and 2,4-dibromo derivatives of the hydroxypyridine in a 5:2 ratio. Only a trace of the starting hydroxypyridine remained. When the amount of NBS was increased to 1.4 molar equiv, uridine was completely converted to its 5-bromo derivative, but both the 2-bromo and 2,4-dibromo derivatives of the hydroxypyridine were still observed. These results show that NBS is an appropriate reagent for bromination of both moieties of NZ and suggest that three bromine atoms will be introduced, sequentially, at the 2″-, 5-, and 4″-positions.

Bromination of NZ. NZ is converted in moderate yields to a mixture of BrNZ, Br_2NZ, and Br_3NZ and some unidentified byproducts on reaction with NBS in DMF. This mixture is readily fractionated by TLC or HPLC, or combinations thereof, allowing each bromo derivative to be obtained pure. The molecular formulas of these compounds, established by FAB–MS, and their R_f and Rt values are given in Table I. As evidenced by comparison of the relative mobilities on normal-phase TLC and reversed-phase HPLC, the lipophilicity of the compounds increases as the number of bromine atoms increases.

Table II. ^1H and ^{13}C NMR Data for Nikkomycin Z (NZ) and Its 2″-Monobromo, 5,2″-Dibromo, and 5,2″,4″-Tribromo Derivatives (BrNZ, Br_2NZ, and Br_3NZ, Respectively)

	^1H NMR chemical shifts[a]				^{13}C NMR chemical shifts[b]			
position	NZ[c]	BrNZ	Br_2NZ	Br_3NZ	NZ[d]	BrNZ	Br_2NZ	Br_3NZ
2					152.4	152.5	151.9	151.7
4					165.8	165.8	161.3	161.2
5	5.88 (8)[e]	5.88			103.3	103.3	97.9	97.8
6	7.56 (8)	7.58	7.97	7.97	142.7	142.7	142.1	142.0
1′	5.80 (4.5)	5.80	5.82	5.83	91.7	91.7	91.8	91.6
2′	4.45 (6, 4.5)	4.45	4.38	4.38	74.1	74.2	74.4	74.4
3′	4.57 (6.5, 6)	4.55	4.52	4.50	71.4	71.5	71.5	71.5
4′	4.33 (6.5, 4)	4.34	4.36	4.32	84.9	85.1	85.2	85.2
5′	4.88 (4)	4.87	4.85	4.81	55.3	55.3	55.2	55.1
6′					171.6	171.6	171.6	171.6
2″	8.26 (2.5)				130.0	130.7	130.7	131.7
3″					157.7[f]	153.7[g]	153.7[h]	155.9[i]
4″	8.03 (9, 2.5)	7.70	7.65		133.9	124.8	124.9	122.4
5″	7.88 (9)	7.64	7.60	7.76	126.3	122.2	122.2	126.0
6″					148.7[f]	151.9[g]	151.8[h]	149.2[i]
7″	5.57 (7, 2.5)	5.34	5.29	5.12	72.6	76.4	76.4	75.9
8″	2.78 (3.5, 2.5)	2.75	2.72	2.70	41.4	40.3	40.4	40.2
9″	4.55 (3.5)	4.55	4.55	4.52	57.9	58.3	58.3	58.4
10″					169.4	169.7	169.7	169.6
11″	0.78 (7)	0.81	0.81	0.80	6.8	6.6	6.6	6.5

[a] Relative values (parts per million) from internal 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt, in an acidic D_2O solution (0.2 N DBr). [b] Relative values (parts per million) from the center (49.0 ppm) in the heptet methyl signal in methanol-d_4 solution. [c] See also König et al. (1986). [d] Recrystallized from HPLC solvent used for purification of the bromo derivatives. [e] Coupling constants (hertz). [f–i] These assignments may be reversed.

In the assignment of structures to these bromo derivatives, the UV data (Table I) suggest that Br_2NZ and Br_3NZ both have bromine at the 5-position since uridine and 5-bromouridine in acidic solutions show widely differing λ_{max}, at 263 and 281 nm, respectively. Detailed structural assignment to each of the bromo derivatives was subsequently achieved by NMR spectroscopy (Table II). ^1H NMR spectra of NZ and BrNZ are almost the same except for the aromatic signals of the pyridyl residue. In BrNZ the (2″)H signal and the long-range coupling between (2″)H and (4″)H are absent. Introduction of bromine at the 2″-position as a first point of functionalization is anticipated from the previously reported iodination of NZ with chloramine T (Yadan et al., 1984). Br_2NZ and Br_3NZ both lack the signal for the (5)H otherwise present from the uracil portion of the molecule. In addition, Br_3NZ does not show the (4″)H signal. Assignment of the ^{13}C resonances of NZ is readily carried out by H–C COSY NMR methods. In general, the ^{13}C chemical shifts of NZ and the bromo derivatives show excellent coincidence, with the exception of those carbon centers attached directly to or approached by a bromine atom. The NMR spectra also reveal that each of the bromo derivatives is discrete and not contaminated with other positional isomers. This, in turn, indicates that the bromination is regioselective. When the amount of NBS is increased, the relative proportions of Br_2NZ and Br_3NZ are increased, sequentially. These results establish that the ease of bromination of NZ is 2″ > 5 > 4″, and this order is in agreement with that predicted by the model experiments.

Conversion of Br_3NZ to NZ and ^3H_3NZ. As a model reaction for the desired tritiation experiment, Br_3NZ was reduced with hydrogen gas over a palladium on carbon catalyst in a water and ethanol mixture. The protic solvent mixture was preferred, even though in the subsequent experiment some exchange was expected to occur between hydrogen atoms present in the solvent and the tritium gas, since it gave a cleaner product mixture. Reduction proceeded smoothly, and NZ was recovered in excellent yield. However, given the nature of this exchange process, it was considered critical to establish that the recovered NZ retains all the stereochemical features of the starting material. While ^1H NMR spectroscopy should readily indicate epimerization at any of the chiral centers, caution was exercised with this approach since the ionizable residues in NZ lead to pH-dependent shift values for some resonances. Accordingly, authentic NZ and material recovered from the reductive debromination were compared at various pH values. Identical behavior was observed for both samples, with all pH-dependent shifts being reversible.

The reductive debromination was repeated by using tritium gas. The tritiated product mixture, separated by HPLC and monitored by UV and radioactivity, included ^3H_3NZ (peak I), ditritiomonobromo derivatives (peaks II and III), monotritiodibromo derivatives (peaks IV and V), and unreacted Br_3NZ (peak VI) (Figure 2). The products appearing as peaks II and IV showed UV λ_{max} at 282 and 284 nm, respectively, indicating formation of the 5-bromouridine analogues, and the λ_{max} values of peaks III and V of 264 and 262 nm, respectively, suggested uridine-unmodified derivatives. The structures of these byproducts indicate that the substitution of the bromine atoms in Br_3NZ by the tritium atoms occurred in random order, a result in contrast to the sequential introduction of the bromine substituents into NZ.

^3H_3NZ was purified by HPLC and analyzed by NMR. The ^1H-decoupled ^3H NMR spectrum (Figure 3) showed three resonances corresponding to tritium in the 5-, 2″-, and 4″-positions of NZ, thereby establishing the successful preparation of ^3H_3NZ. In the ^1H-coupled spectrum these ^3H signals appeared as a doublet, a doublet, and a double doublet, respectively. On ^1H NMR analysis, ^3H_3NZ also showed ^1H resonances for the 5-, 2″-, and 4″-positions but with ca. 30% of the intensities relative to the unlabeled material (Figure 3). The presence of these signals indicates that some scrambling has occurred between the tritium gas and the solvent mixture. Since a specific activity of 90 Ci/mmol can be achieved if the three bromine substituents are replaced solely by tritium atoms, the NMR result indicates a specific activity for the ^3H_3NZ of ca. 60 Ci/mmol. Except for these differences in signal intensities, the ^1H NMR spectrum of ^3H_3NZ coincides well with that of NZ, further supporting the argument that no isomer-

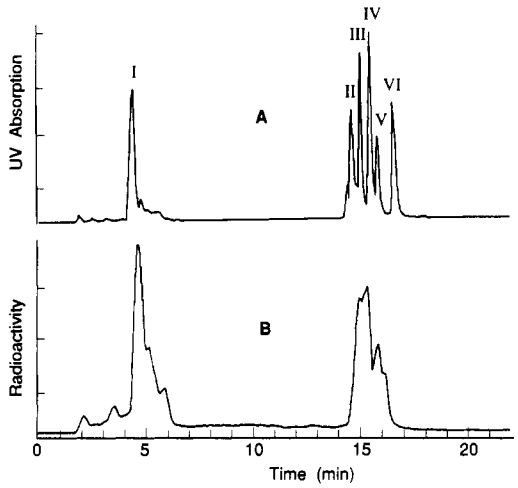

Figure 2. HPLC traces of the tritiated products from catalytic reduction of Br_3NZ with palladium on carbon under tritium gas monitored by UV absorption (A) and radioactivity (B). The Ultrasphere C_{18} column was eluted with water:methanol:TFA (95:5:0.25) for 7 min followed by a change in the solvent ratio to 60:40:0.25, both at a flow rate of 1.5 mL/min. The UV and radioactivity detectors were connected in series, resulting in a small offset of the latter graph to longer retention times. Peak identities are based on radioactive content and on Rt and UV λ_{max} values. Peak I, ^3H_3NZ; peaks II and III, ditritiomonobromo derivatives; peaks IV and V, monotritiodibromo derivatives; peak VI, Br_3NZ.

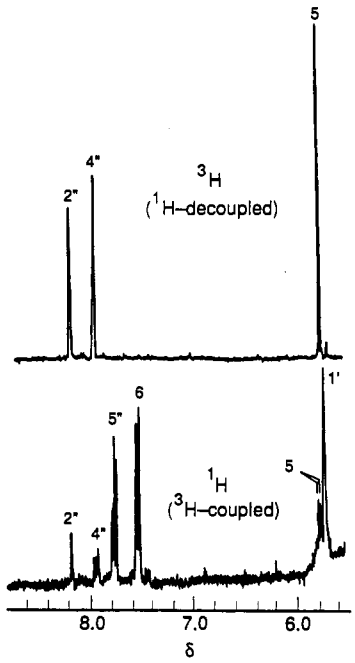

Figure 3. ^3H (^1H-decoupled) and ^1H (^3H-coupled) NMR spectra for the uracil and pyridyl regions of ^3H_3NZ.

ization occurs during either the bromination or the tritiation process.

Preliminary attempts to use ^3H_3NZ as a radioligand for insect CS proved disappointing. No specific binding was achieved by filtration assay with active CS preparations from *Tribolium castaneum* larvae (Cohen and Casida, 1980) and integumental membranes from *Musca domestica* and *Manduca sexta* larvae. Further studies are warranted on optimization of the enzyme source and assay conditions. ^3H_3NZ is an ideal probe to examine the distribution and metabolic factors limiting its insecticidal, miticidal, and fungicidal activity.

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