# Structure and Cytotoxicity of Dehydrotomatine, a Minor Component of Tomato Glycoalkaloids

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The structure of dehydrotomatine was determined as (3β,22β,25*S*)-spirosol-5-en-3-yl-*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside from the analysis of FAB-MS, UV, and NMR spectra. Dehydrotomatine exhibited cytotoxicity on animal cell lines HepG2, NIH/3T3, and U937 lower than that of tomatine, the major tomato glycoalkaloid.

**Keywords:** *Dehydrotomatine; cytotoxicity; NMR; tomato glycoalkaloid*

## INTRODUCTION

With the advent of transgenic agricultural products due to recent advances in biotechnology, much attention has been focused on food safety. Since the tomato was the first transgenic food to be marketed, the content and toxicities of tomato glycoalkaloids have become of increased interest. Tomatine, a naturally occurring toxicant in all tomatoes, is distributed throughout the tomato plant but is most concentrated in leaves and in opening flowers. The tomatine concentration in tomatoes depends on the degree of ripeness and declines as green fruit ripen to red. From the view of food safety, the principle of "substantial equivalence" has been used to evaluate the acceptable level of tomatine in transgenic tomatoes, that is, whether the levels are "substantially equivalent" to those of ripe tomatoes that have a history of safe use. The maximum reported level of the tomatine concentration in ripe tomatoes is a few milligrams per 100 g of fresh fruit (Bushway et al., 1994; Keukens et al., 1994; Kozukue et al., 1994; Asano et al., 1995).

The analysis of glycoalkaloids in food is a difficult problem to solve because of their complex chemical structure and high molecular weight. As the tomatine has no chromophore, its accurate quantitative analysis employing chromatographic separation requires short-wavelength UV detection or a specific detector such as MS or pulsed amperometric (Friedman et al., 1994). Dehydrotomatine was first reported during the development of tomatine analysis as an impurity in commercial sources of tomatine standard (Friedman et al., 1994; Bushway et al., 1994) and was also found in tomato.

As an alternative for the quantitative analysis of glycoalkaloids in tomatoes, a bioassay method was proposed by Asano et al. (1995), in which tomatine concentration was measured by the cytotoxicity of animal cell cultures. Commercial tomatine was used as a standard to estimate the concentration in tomatoes; however, the effect of dehydrotomatine present was not considered. There are no reports about the cytotoxicity of dehydrotomatine, although chromatographic techniques have allowed detection of tomatine and dehydrotomatine separately (Friedman et al., 1994; Bushway et al., 1994). It is important to evaluate the difference in cytotoxicity between tomatine and dehydrotomatine for quantitative analysis using bioassays as well as for the confirmation of the safety of agricultural products.

The molecular structure of dehydrotomatine (Figure 1) has been depicted on the basis of low-resolution MS data, chromatographic features, and the consideration of the structures of analogous alkaloids (Friedman et al., 1994; Bushway and Perkins, 1995; Bushway et al., 1994), reflecting some ambiguity in its structure. In this paper, we report its structure, unambiguously determined by high-resolution mass spectroscopy (HR-MS) and nuclear magnetic resonance (NMR) analysis prior to determining the cytotoxicity by bioassay.

## MATERIALS AND METHODS

**Reagents.** Tomatine was purchased from Sigma Chemical Co. (St. Louis, MO) containing dehydrotomatine as the main impurity (Bushway et al., 1995). Pure tomatine and dehydrotomatine were then obtained from Wako Chemicals (Tokyo, Japan) by chromatographic separation of the tomatine from Sigma.

**Organisms and Media.** Organisms and media used were selected to be in accord with those of Yamashoji et al. (1995). Phospate-buffered saline (PBS), minimum essential medium (MEM), Dulbecco's modified Eagle's (DME) medium, and RPMI 1640 medium were obtained from Nissui (Tokyo, Japan). Fetal calf serum (FCS) was obtained from Nichirei (Tokyo, Japan). Animal cell lines HepG2 (Aden et al., 1979; Knowles et al., 1980), NIH/3T3 (Jainchill et al., 1969), and U937 (Sundström et al., 1976) were obtained from RIKEN Gene Bank (Tsukuba, Japan).

HepG2 and NIH/3T3 cells were cultured in DME medium containing 10% FCS and 0.4% *l*-glutamine. U937 cells were cultured in RPMI 1640 medium containing 10% FCS and 0.4% *l*-glutamine. These cells were incubated in a $CO_2$ incubator at 37 °C in humidified atmosphere containing 5% $CO_2$ in air.

**Physical and Spectroscopic Data.** UV spectra were recorded on a Hitachi U-3300 spectrophotometer. $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker DRX600 ($^1H$, 600.13 MHz; $^{13}C$, 150.90 MHz) spectrometer as a solution of methanol-$d_4$ at 303 K. All NMR data are reported in ppm ($\delta$) downfield from tetramethylsilane. Mass (MS) spectra were obtained with a JEOL SX-102 spectrometer by fast atom bombardment (FAB) ionization procedure (glycerol, Xe, 70 eV). The purities of pure tomatine and pure dehydrotomatine were shown to be >98% by $^1H$ NMR spectra and/or HPLC analysis.

**Cytotoxicity Test.** The cytotoxicity test using a chemiluminescent assay was performed according to the method of Yamashoji et al. (Yamashoji et al., 1992; Asano et al., 1995). Cell suspensions were prepared from confluent culture dishes

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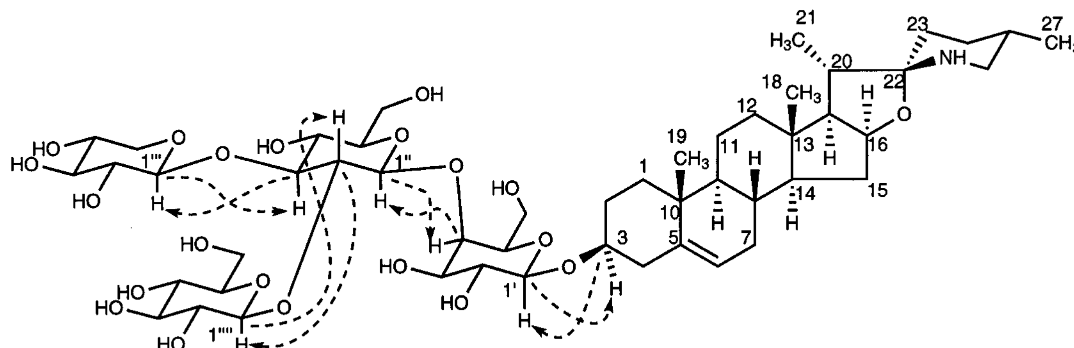


**Figure 1.** Molecular structure of dehydrotomatine. Dashed arrows represent $^1$H-$^{13}$C long-range *J* coupling around oligosaccharide moiety observed by HMBC experiment.

**Table 1. $^1$H and $^{13}$C NMR Spectral Assignments of Dehydrotomatine**

| position | $^1$H δ | | $^1$H *J* coupling (Hz) | $^{13}$C δ | position | $^1$H δ | | $^1$H *J* coupling (Hz) | $^{13}$C δ |
|---|---|---|---|---|---|---|---|---|---|
| 1α | 1.07 | ddd | 13.5, 13.3, 4.3 | 38.51 | 1′ | 4.36 | d | 7.8 | 102.89 |
| 1β | 1.87 | ddd | 13.3, 4.1, 3.6 | | 2′ | 3.62 | dd | 9.7, 7.8 | 73.17 |
| 2α | 1.92 | ddddd | 13.0, 4.3, 4.1, 3.7, 2.6 | 30.67 | 3′ | 3.51 | dd | 9.7, 3.4 | 75.64 |
| 2β | 1.62 | dddd | 13.5, 13.0, 12.1, 3.6 | | 4′ | 4.02 | br d | 3.4 | 79.97 |
| 3 | 3.55 | dddd | 12.1, 11.8, 4.6, 3.7 | 80.14 | 5′ | 3.51 | dd | 7.9, 5.9 | 75.36 |
| 4α | 2.43 | ddd | 13.7, 4.6, 3.5, 2.6 | 39.68 | 6′ | 3.61 | dd | 11.0, 5.9 | 61.07 |
| 4β | 2.27 | dddd | 13.7, 11.8, 3.5, 2.5 | | | 3.90 | dd | 11.0, 7.9 | |
| 5 | | | | 142.02 | 1″ | 4.58 | d | 7.8 | 104.74 |
| 6 | 5.38 | ddd | 4.8, 3.5, 2.0 | 122.54 | 2″ | 3.75 | dd | 9.0, 7.8 | 81.05 |
| 7α | 1.56 | ddd | 18.2, 10.2, 2.0 | 33.21 | 3″ | 3.70 | dd | 9.0, 8.4 | 87.92 |
| 7β | 2.01 | dddd | 18.2, 5.0, 4.8, 2.5 | | 4″ | 3.28 | dd | 10.1, 8.4 | 70.49 |
| 8 | 1.67 | dddd | 10.2, 10.2, 10.2, 5.0 | 32.76 | 5″ | 3.33 | ddd | 10.1, 7.4, 2.2 | 77.52 |
| 9 | 0.98 | ddd | 13.2, 10.2, 5.4 | 51.68 | 6″ | 3.57 | dd | 11.5, 7.4 | 63.15 |
| 10 | | | | 38.03 | | 3.90 | dd | 11.5, 2.2 | |
| 11α,β | 1.49−1.60 | m | | 22.02 | 1‴ | 4.60 | d | 7.7 | 104.97 |
| 12α | 1.21 | qddd | 2.2, 12.4, 12.4, 5.1 | 41.09 | 2‴ | 3.24 | dd | 9.1, 7.7 | 75.28 |
| 12β | 1.78 | dd | 12.4, 6.2 | | 3‴ | 3.31 | dd | 9.1, 8.9 | 78.34 |
| 13 | | | | 41.78 | 4‴ | 3.52 | ddd | 11.2, 8.3, 5.6 | 71.00 |
| 14 | 1.13 | ddd | 14.2, 10.2, 5.8 | 57.23 | 5‴α | 3.26 | dd | 11.2, 11.1 | 67.20 |
| 15α | 1.99 | ddd | 12.3, 7.5, 5.8 | 33.50 | 5‴β | 3.91 | dd | 11.1, 5.6 | |
| 15β | 1.31 | ddd | 14.2, 12.3, 6.0 | | 1⁗ | 4.91 | d | 8.0 | 104.29 |
| 16 | 4.20 | ddd | 9.5, 7.5, 6.0 | 80.37 | 2⁗ | 3.18 | dd | 9.2, 8.0 | 75.90 |
| 17 | 1.70 | dd | 9.5, 6.7 | 63.49 | 3⁗ | 3.36 | m | | 71.60[c] |
| 18 | 0.89 | d | 2.2 | 17.23 | 4⁗ | 3.31−3.38 | m | | 78.02[c] |
| 19 | 1.05 | s | | 19.83 | 5⁗ | 3.33 | ddd | 12.5, 5.4, 2.3 | 78.50 |
| 20 | 1.82 | qd | 6.8, 6.7 | 43.48 | 6⁗ | 3.82 | dd | 12.3, 5.4 | 62.74 |
| 21 | 1.00 | d | 6.8 | 15.85 | | 3.89 | dd | 12.3, 2.3 | |
| 22 | | | | 99.50 | | | | | |
| 23 | 1.5−1.8 | m | | —[a] | | | | | |
| 24 | 1.3−1.4 | m | | 29.04[b] | | | | | |
| 25 | 1.60 | m | | 31.50 | | | | | |
| 26α | 2.68 | br dd | 11, 11 | 50.61 | | | | | |
| 26β | 2.73 | br dd | 11, 4 | | | | | | |
| 27 | 0.87 | d | 6.4 | 19.65 | | | | | |

[a] Not observed, see Discussion. [b] Broad. [c] Assignment uncertain due to signal overlapping.

by washing cells on the dishes with PBS, incubating under normal culture conditions with PBS containing 0.05% w/v trypsin for 5 min until cells began to detach from substrata, drawing off trypsin solution, and flushing cells from the substratum with fresh medium. After the number of cells was counted on a hemocytometer, the suspension was diluted to adjust for the concentration of $5 \times 10^4$ cells/mL for the assay. Solutions of pure glycoalkaloid were prepared at 1000 μg/mL ethanol, and they were diluted with MEM medium (phenol red free) to obtain test solutions of the concentrations 0.02, 0.2, 0.5, 1, 5, 10, and 20 μg/mL. Equivalent volumes of cell suspension (250 μL) and test solution were mixed and incubated at 37 °C for 1 h under 5% $CO_2$-containing atmosphere. Testing conditions were triplicated to perform statistical analysis. The cell viability (percentage) of each compound at various concentrations was calculated by the rapid chemiluminescent assay method (Yamashoji et al., 1989) in which menadione-catalyzed $H_2O_2$ production by viable cells was detected.

## RESULTS AND DISCUSSION

**Structural Determination.** Spectral features of dehydrotomatine, ((3β,22β,25*S*)-spirosol-5-en-3-yl-*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside), were as follows: colorless amorphous solid, UV spectral data shown in Figure 2; FAB-MS, *m*/*z* 1032 ([M + H]$^+$), 1030 ([M − H]$^+$), 576, 442, 414, 396; high-resolution FAB-MS calcd for $C_{50}H_{84}NO_{21}$ ([M + H]$^+$) 1032.5379, found 1032.5385; $^{13}$C and $^1$H NMR assignments shown in Table 1.

The molecular formula of dehydrotomatine was determined as $C_{50}H_{81}NO_{21}$ by high-resolution FAB-MS measurement. Its UV spectrum showed the compound had an absorption band around 190 nm arising from $\pi \rightarrow \pi^*$ transition (Figure 2). Two $^{13}$C NMR signals with olefinic chemical shift (δ 142.02, 122.54) support an

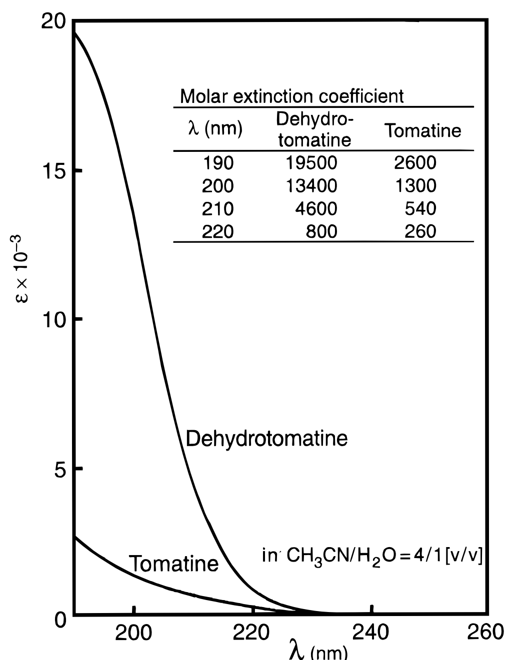


**Figure 2.** UV spectra of tomato glycoalkaloids.

unsaturated structure for the compound. In a 1D $^1$H NMR spectrum, a proton signal appearing at 5.38 ppm was the only signal around the olefinic region. Two-dimensional DQF-COSY and TOCSY experiments enabled the assignment of most of the $^1$H signals (Table 1). The olefinic signal was assigned at H-6 on the aglycon, and the position of C-5 lacked the corresponding proton signal, so that the position of the double bond was determined to be between C-5 and C-6. The H-23 and H-24 did not afford a clear cross-peak on 2D spectrum, while the corresponding $^{13}$C signals were broadened (C-24) or not observed (C-23). These phenomena were also observed in tomatine and were interpreted not only as extreme signal overlapping but also as conformational exchange of the F-ring. A low-temperature experiment at 255 K, under which solubility became a problem for measurement, changed the 1D spectrum around the F-ring, but a much lower temperature was required to observe cross-peaks on the 2D spectra at the resonance frequency of 600 MHz. However, the 2D spectral pattern of dehydrotomatine around the F-ring was identical to that of the corresponding region of tomatine. Hence, dehydrotomatine should have the same stereochemistry of the C-25 position as tomatine. The $^1$H NMR signals of the carbohydrate region were assigned by 2D measurement with cosideration of the *J*-coupling values. The assignment classifed that the oligosaccharide consisted of four $\beta$-pyranose structures, a $\beta$-galactopyranose, a $\beta$-xylopyranose, and two $\beta$-glucopyranose structures. To assign $^{13}$C signals, HMQC and HMBC experiments were made. HMBC experiments revealed that dehydrotomatine had the same oligosaccharide connectivities as tomatine (Figure 1). From the above, we conclude the structure of dehydrotomatine to be (3$\beta$,22$\beta$,25*S*)-spirosol-5-en-3-yl-*O*-$\beta$-D-glucopyranosyl-(1→2)-*O*-[$\beta$-D-xylopyranosyl-(1→3)]-*O*-$\beta$-D-glucopyranosyl-(1→4)-$\beta$-D-galactopyranoside as shown in Figure 1. The structure was identical with that of Friedman et al. (1994), Bushway et al. (1994), and Bushway and Perkins (1995).

Very recently, during the reviewing process of this paper, the same structure was reported by Friedman et al. (1997). They obtained pure dehydrotomatine by separation using chromatography methods of the impure commercial tomatine (Sigma) and adopted chemical degradation, followed by comparison with known compounds using HPLC and MS. While the source of the dehydrotomatine was the same as for the present study, the strategy for the structural elucidation by Friedman et al. (1997) was different. Thus, the structure has been doubly determined and is quite unambiguous.

The ratio of tomatine to dehydrotomatine in the commercial tomatine standard (Sigma) was determined to be 7:1 on the basis of the integral intensity of $^1$H NMR of their assignments. The same ratio value was also obtained from a gated decoupling $^{13}$C NMR experiment in DMSO-$d_6$.

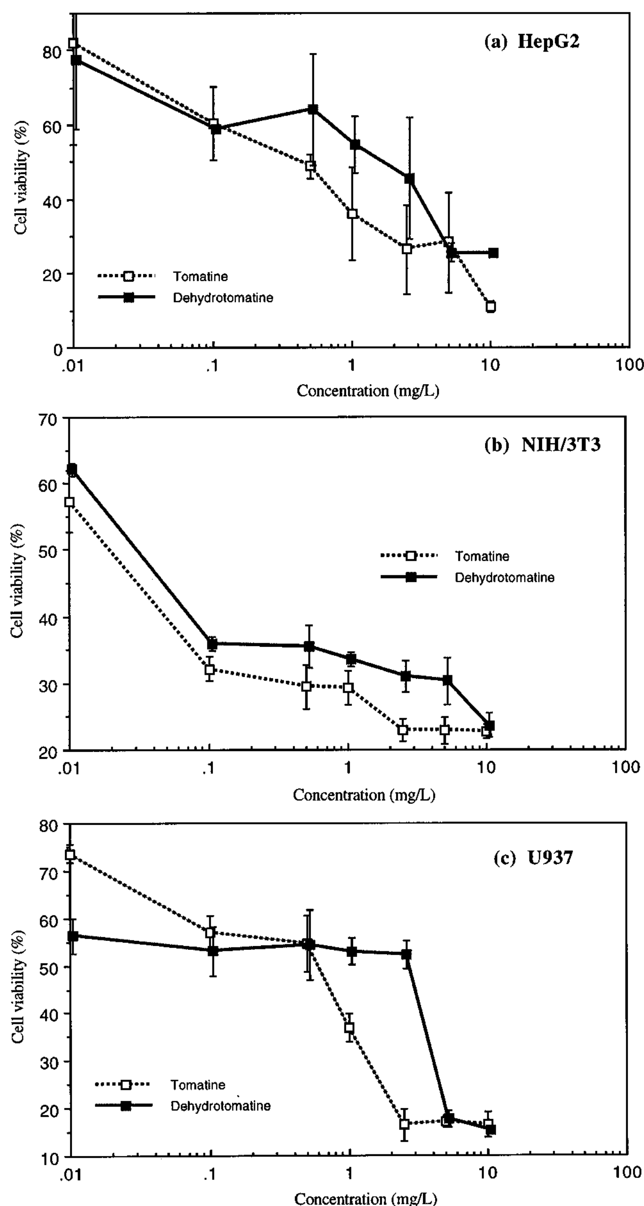


**Figure 3.** Cytotoxic effects of tomato glycoalkaloids. The value of each symbol represents the mean ± SD of triplicate determination.

**Cytotoxicities of Tomato Glycoalkaloids.** The cytotoxicities of tomatine and dehydrotomatine were represented as a relationship between cell viability and concentration (Figure 3). Cell viabilities of tomatine decreased at an earlier stage than those of dehydrotomatine along the concentration increment. The inhibition concentration of cell growth [50% ($IC_{50}$) values] and

**Table 2. $IC_{50}$ Values of Tomato Glycoalkaloids in Animal Cells**

| cell line | tomato glycoalkaloids ($\mu$g/L) | |
|---|---|---|
| | tomatine | dehydrotomatine |
| HepG2 | $302^{+191}_{-247}$ | $872^{+1291}_{-670}$ |
| NIH/3T3 | $13^{+13}_{-7}$ | $34^{+10}_{-7}$ |
| U937 | $200^{+78}_{-56}$ | $186^{+173}_{-100}$ |

their 90% confidence intervals were also calculated (Table 2). Tomatine afforded a 2−3 times smaller (more toxic) value for HepG2 and NIH/3T3 than dehydrotomatine. The equivalent value for U937 could be caused by a few data points around the $IC_{50}$ concentration. After taking into consideration deviation and empirical error, we concluded that tomatine was more toxic than dehydrotomatine.

Many studies for tomatine may have been performed by employing the mixture of the two kinds of tomato glycoalkaloids, because of the dehydrotomatine contamination in the commercial source. Previous studies on toxicity using commercial tomatine should be reevaluated by using pure tomatine and/or dehydrotomatine. However, it is not easy, especially for *in vivo* studies, to use large amounts of pure tomato glycoalkaloids. Taking into account the purity of commercial tomatine (88%), the result of cytotoxicity (less than a half-order difference) implied to us that previous studies on toxicity using commercial tomatine could be relevant to that of pure tomatine. For example, the cytotoxicity of commercial tomatine has been measured with several different kinds of assay from the view of the development of tomatine analysis (Yamashoji et al., 1995). Yamashoji et al. (1995) concluded HepG2 employing the chemiluminescent assay gave the most accurate results in linearity. Our $IC_{50}$ value of HepG2 cell line was similar to the value read from the figure provided in their paper.

However, chromatographic analysis employing UV detection should be interpreted with much care, because tomatine and dehydrotomatine behave in a similar way chromatographically but differ in UV detection. Dehydrotomatine has ca. a 10 times larger molar extinction coefficient (Figure 2) than tomatine at around 200 nm, the wavelength used to detect tomatine.

**Conclusion.** The structure of dehydrotomatine, a minor tomato glycoalkaloid, was determined by NMR and HR-MS analyses. The purity of the commercial tomatine was determined to be 88% on the basis of NMR spectra. The toxicities of both tomatine and dehydrotomatine were evaluated by cytotoxicity on animal cell cultures utilizing a chemiluminescent assay method in which small amounts of glycoalkaloids were required. Tomatine exhibited a 2−3 times smaller $IC_{50}$ value than dehydrotomatine. However, further studies are desired for clarifying their *in vivo* toxicities in detail. For this purpose, it is hoped that in future pure tomato glycoalkaloids will become available from commercial sources.

## ABBREVIATIONS USED

PBS, phosphate-buffered saline; MEM, minimum essential medium; DME, Dulbecco's modified Eagle's; FCS, fetal calf serum; NMR, nuclear magnetic resonance; HR, high resolution; MS, mass spectroscopy; FAB, fast atom bombardment; HPLC, high-performance liquid chromatography; 1D, one dimensional; 2D, two dimensional; DQF-COSY, double quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; HMQC, heteronuclear multiple quantum correlation spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy; $IC_{50}$, inhibition concentration of cell growth 50%; UV, ultraviolet; SD, standard deviation.

## ACKNOWLEDGMENT

We thank Dr. Ann E. Percy for critical reading of the manuscript.

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Received for review March 31, 1997. Accepted August 4, 1997.[⊗]

JF970253K

[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1997.