FEBS 16945
FEBS Letters 385 (1996) 7–10

Site-directed mutagenesis of *Saccharomyces cerevisiae* β-tubulin: interaction between residue 167 and benzimidazole compounds

Jing Li, Santosh K. Katiyar, Thomas D. Edlind*

Department of Microbiology and Immunology, Medical College of Pennsylvania and Hahnemann University, 2900 Queen Lane, Philadelphia, PA 19129, USA

Received 7 March 1996

Abstract **Benzimidazoles are widely used as anthelmintic agents and systemic fungicides. In susceptible organisms, benzimidazoles bind to β-tubulin and block microtubule polymerization. To further characterize this interaction, site-directed mutagenesis followed by gene replacement was used to change *Saccharomyces cerevisiae* β-tubulin residue Phe-167 to Tyr. Consistent with previous studies, this mutation resulted in at least 3–4-fold decreased sensitivity to the benzimidazole derivatives carbendazim and nocodazole. The Tyr-167 mutant was cold sensitive, implying a direct effect on benzimidazole binding rather than a nonspecific increase in microtubule stability. Surprisingly, the mutant had 8-fold increased sensitivity to the derivative benomyl, which is structurally identical to carbendazim except at position 1. This suggests that residue 167 interacts with benzimidazoles in the vicinity of the 1-position.**

Key words: Benzimidazole; Microtubule; β-Tubulin; *Saccharomyces cerevisiae*

1. Introduction

The benzimidazole derivative thiabendazole was introduced in the early 1960s as an anthelmintic agent [1], and soon shown to possess antifungal activity as well [2]. Subsequently, a series of benzimidazole derivatives with varying substitutions at the 1, 2 and 5 positions (Fig. 1) were commercially developed as human and veterinary anthelmintics (e.g. mebendazole and albendazole) and as agricultural fungicides (benomyl and carbendazim) [3-5]. With one exception (nocodazole), benzimidazoles are selectively toxic to lower eukaryotes, including certain protozoa [6]. However, there is wide variation in the activity of different derivatives against susceptible organisms. For example, the protozoan *Trichomonas vaginalis* is about 100-fold more sensitive to mebendazole than to benomyl [6,7], while the reverse is true for the yeast *Saccharomyces cerevisiae* (Li, unpublished data).

Biochemical and genetic analyses have identified microtubules as the primary benzimidazole target, specifically the β-tubulin subunit [4,5]. Studying the amino acid changes in fungal mutants with altered benzimidazole sensitivity provides potentially useful information about benzimidazole-tubulin interaction. Mutations in β-tubulin residues 241 (Arg to His) and 167 (Phe to Tyr) conferring benomyl resistance were identified in, respectively, *S. cerevisiae* [8] and *Neurospora crassa* [9]. A more extensive analysis of benomyl-resistant *Aspergillus nidulans* identified mutations in residues 6, 198 and 200 [10]. A mutation in *A. nidulans* β-tubulin residue 165 (Ala to Val) conferred resistance to thiabendazole [11]. However, this mutant was supersensitive to benomyl, carbendazim and nocodazole. In aqueous solution, benomyl breaks down to carbendazim with a half-life of approx. 1 h [12]. Since thiabendazole and carbendazim differ only at their 2 positions (Fig. 1), Jung and Oakley [11] deduced that this position interacts with residue 165. In the work reported here, site-directed mutagenesis and gene replacement were employed to change the β-tubulin residue 167 from Phe to Tyr in *S. cerevisiae*. Analogous to the *N. crassa* mutant [9], the *S. cerevisiae* mutant was resistant to carbendazim and nocodazole, but surprisingly was supersensitive to benomyl. A specific interaction between residue 167 and benzimidazole compounds is suggested by these results.

2. Materials and methods

S. cerevisiae strain W303-1A was obtained from Dr. Alan Hudson (Medical College of Pennsylvania and Hahnemann University). The plasmid pCT-01L, a pUC18 derivative containing a complete *S. cerevisiae* β-tubulin gene with a *LEU2* gene inserted downstream [13], was a gift from Dr. Seiji Matsumoto (Tokyo Metropolitan Institute of Medical Science, Japan). A full length *S. cerevisiae* β-tubulin gene was amplified by PCR and cloned into M13. Single stranded DNA was prepared following growth in *dut*−, *ung*− *E. coli* strain CJ236 [14]. A phosphorylated mutagenic oligonucleotide substituting TAC (Tyr) for TTC (Phe) in codon 167 was annealed to this DNA and extended by incubation with T4 DNA polymerase plus dNTPs. Following ligation with T4 DNA ligase, the mutagenized DNA was transformed into *E. coli* XL1-Blue. The presence of the expected mutation and the absence of any additional mutations were confirmed by DNA sequencing. An *Xba*I-*Hin*dIII fragment (encoding β-tubulin residues 68 to 393) was subcloned from M13 RF DNA into *Xba*I-*Hin*dIII cleaved pCT-01L, generating pCT-01L167Y. This plasmid was linearized by digestion with *Sca*I and *Bam*HI, and used to transform *S. cerevisiae* W303-1A by the lithium acetate method [15]. Transformants were selected on leu− plates at 30°C, then restreaked on fresh leu− plates to obtain pure colonies.

To prepare genomic DNA, cells were grown in 2 ml YPD (1% yeast extract, 1% peptone, 2% dextrose) overnight, and collected by centrifugation. Following 1 min vortexing with glass beads, the cells were treated with proteinase K plus sodium dodecyl sulfate at 37°C for 2 h. After phenol extraction, DNA was precipitated twice with ethanol. Southern blot analysis of *Nco*I-digested genomic DNA was performed by standard procedures [16]. The blots were probed under stringent conditions with a ^{32}P-labelled *S. cerevisiae* β-tubulin gene fragment. For selected transformants, β-tubulin gene fragments were amplified by PCR, gel purified, and cloned into M13. Transformants possessing the desired mutation at residue 167, as well as transformants with wild-type sequence, were identified by sequencing.

The sensitivities of selected transformants towards different benzimidazoles (dissolved in dimethyl sulfoxide) were determined by serial dilution in YPD medium. Cultures (1×10^4 cells/ml) were incubated at 30°C for 18 h, the cell numbers were determined in a hemocytometer, and drug concentrations inhibiting growth 50% (IC_{50}) were estimated. Sensitivities were also determined towards freshly diluted benomyl, and benomyl that was preincubated in YPD at 30°C for 12 h. Growth rates at 14, 30, and 37°C were determined by counting the cells every 2 h, starting at 1×10^4 cells/ml. Average cell division times during log phase were then estimated.

*Corresponding author. Fax: (1) (215) 848 2271.
E-mail: edlind@medcolpa.edu

PII S0014-5793(96)00334-1

3. Results

3.1. Site-directed mutagenesis of S. cerevisiae β-tubulin

Orbach et al. [9] isolated a spontaneous benomyl resistant mutant of the filamentous fungus *Neurospora crassa* and demonstrated that resistance was due to a change in residue 167 from Phe to Tyr. To examine the generality of this result and to study the phenotype of this mutation more closely, the identical change was introduced into a cloned copy of the β-tubulin gene from the yeast *S. cerevisiae*. Following transformation, genomic DNA was prepared from leu+ colonies. As shown in Fig. 2, Southern blot analysis identified several transformants with the expected change in β-tubulin band size, indicating gene replacement through homologous recombination. The β-tubulin genes from four of these transformants were amplified, cloned, and sequenced. Two of the transformants (designated WL167Y) included the desired mutation of residue 167, and two (designated WL167F) were wild type due to recombination occurring downstream of codon 167. Sequencing of the entire WL167Y β-tubulin gene confirmed that only codon 167 was mutated.

3.2. Benzimidazole sensitivities

The sensitivities of the *S. cerevisiae* parent (W303-1A) and recombinant strains were determined for the benzimidazole derivatives carbendazim, nocodazole, thiabendazole, and benomyl (Table 1). Structurally, nocodazole differs from carbendazim only at the 5-position, thiabendazole differs from carbendazim only at the 2-position, and benomyl differs from carbendazim only at the 1-position (Fig. 1). The mutant strain WL167Y was at least 4-fold less sensitive to nocodazole and at least 3-fold less sensitive to carbendazim compared to both the parent strain W303-1A and the transformant with the wild-type sequence WL167F (the actual reductions in sensitivity could not be determined due to limitations in the aqueous solubility of nocodazole and carbendazim). There was negligible difference in the sensitivity of the three strains to thiabendazole. Surprisingly, with respect to benomyl, WL167Y was 8-fold more sensitive than W303-1A and WL167F. Similar results were obtained for an additional pair of WL167Y and WL167F isolates.

3.3. Effects of temperature on growth rate

The growth rates of strains W303-1A, WL167Y, and WL167F were determined at 14, 30 and 37°C in drug-free medium. All three strains divided approximately every 2 h at 30 and 37°C. At 14°C, W303-1A and WL167F division time increased to 6–8 h, but the effect on WL167Y growth was more pronounced, with a division time of more than 48 h. Thus, WL167Y is cold-sensitive.

3.4. Effects of preincubation on benomyl activity

Benomyl breaks down spontaneously to carbendazim with a half-life of approx. 1 h [12]. Indeed, the antimicrotubule activity of benomyl is widely attributed to carbendazim alone; i.e. benomyl is considered a prodrug [5]. In light of this, it was important to confirm that benomyl was responsible for WL167Y supersensitivity. The sensitivities of strains W303-1A, WL167Y and WL167F were tested in parallel using benomyl and carbendazim that was either freshly diluted (from dimethyl sulfoxide) or preincubated in YPD for 12 h at 30°C. For carbendazim, there were no apparent differences. An IC_{50} of 6.5 μg/ml was obtained in this experiment for strains W303-1A and WL167F using both fresh and preincubated carbendazim, while the IC_{50} for WL167Y remained over 10 μg/ml in both cases. For benomyl, however, a clear difference was observed. With freshly diluted benomyl the IC_{50} for WL167Y

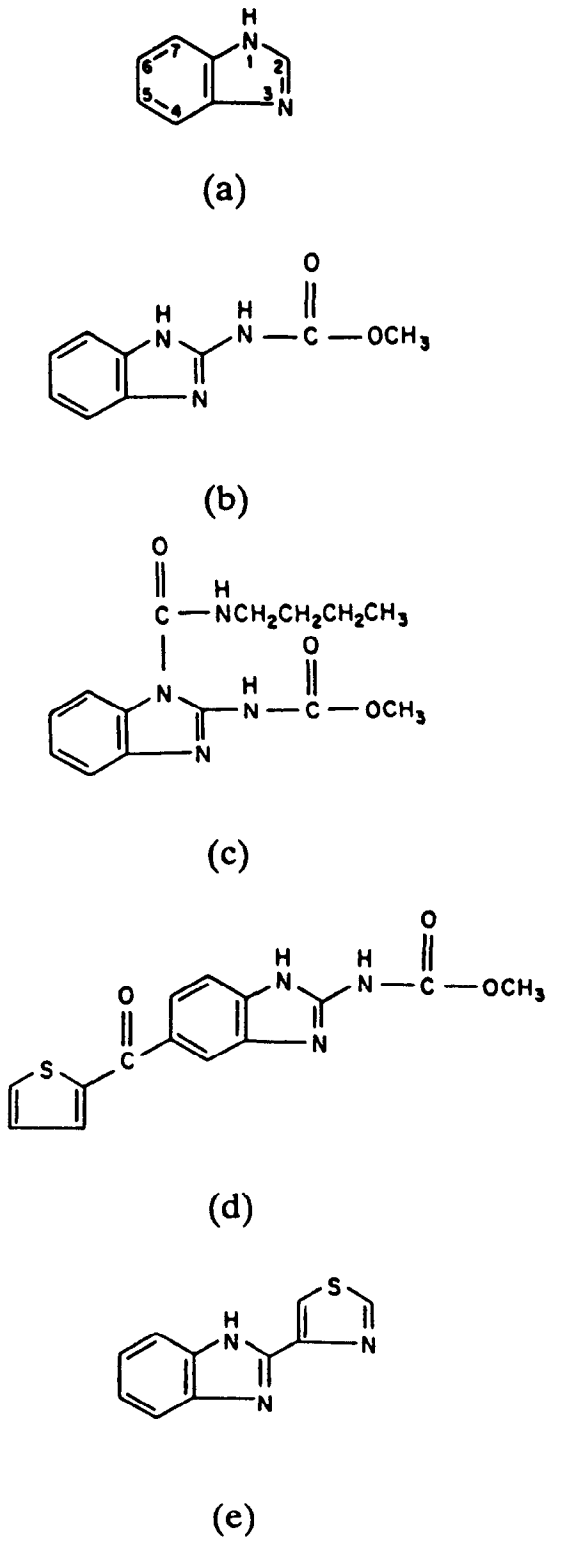

Fig. 1. Structures of (a) benzimidazole, and benzimidazole derivatives (b) carbendazim, (c) benomyl, (d) nocodazole, and (e) thiabendazole.

was 0.15 μg/ml, while with preincubated benomyl the IC_{50} increased to 6.4 μg/ml. For W303-1A and WL167F, the benomyl IC_{50} increased from 1.0 to 6.6 μg/ml with preincubation.

4. Discussion

There were several reasons for selecting β-tubulin residue Phe-167 for analysis by site-directed mutagenesis. First, Orbach et al. [9] previously identified the mutation Phe-167 to Tyr in the filamentous fungus *N. crassa* following selection for resistance to the benzimidazole derivative benomyl. However, Phe-167 is conserved in nearly all β-tubulins, from both benzimidazole sensitive and resistant organisms. Furthermore, the protozoan *Trichomonas vaginalis* is highly sensitive to certain benzimidazole derivatives [6], and yet its β-tubulin has Tyr-167 [7]. Thus, one goal was to resolve these apparent inconsistencies regarding the role of residue 167 in benzimidazole sensitivity by studying the phenotype of Tyr-167-substituted β-tubulin in the well defined organism *S. cerevisiae*. A second reason for studying this mutation was its proximity to residue 165, which has been implicated in the binding of the 2 position side chain of benzimidazoles. Specifically, the mutation Ala-165 to Val in the filamentous fungus *Aspergillus nidulans* resulted in thiabendazole resistance but carbendazim supersensitivity [11]. Since these two derivatives differ only in their 2 position, it was deduced that this position interacts with residue 165. A third reason is that residues 155 to 174 represent one of three peptides implicated in GTP binding by UV-crosslinking studies [17-19]. Two GTP molecules per tubulin dimer are required for microtubule assembly. One GTP binds to α-tubulin, while the other binds to an 'exchangeable' site on β-tubulin and, unlike the α-tubulin GTP, undergoes hydrolysis following microtubule assembly [20]. However, in binding studies with mammalian tubulin, benzimidazoles (i.e. nocodazole) and GTP did not compete with each other [5]. Finally, β-tubulin residue 167 was of interest because it borders a hydrophilic region which is reactive with antibody [21]; thus, it is on or near the microtubule surface. This would facilitate its interaction not only with GTP or benzimidazoles, but also potentially with other cellular proteins. For example, residue Thr-166 represents a potential phosphorylation site [22], and hence a potential target for regulators of microtubule stability or function.

The site-directed mutagenesis of *S. cerevisiae* β-tubulin residue Phe-167 to Tyr resulted in at least 3–4-fold decreased sensitivity to the benzimidazole derivatives carbendazim and nocodazole (Table 1). This result is consistent with the *N. crassa* study, in which this mutation was identified following selection for benomyl resistance on agar medium [9]. Decreased sensitivity or resistance to microtubule-destabilizing drugs, such as the benzimidazoles, can result from two types of mutations: those that directly affect drug binding, and those that increase microtubule stability and hence indirectly confer resistance. The cold sensitivity of the Tyr-167 mutant implies a less stable microtubule (e.g. low temperatures are routinely used to induce depolymerization in microtubule pur-

Table 1. *S. cerevisiae* transformants' benzimidazole sensitivities

	IC_{50} (μg/ml)		
	W303-1A	WL167F[a]	WL167Y[b]
Nocodazole	1.3	2.4	> 10[c]
Carbendazim	3.7	3.9	> 10[c]
Benomyl	1.3	0.83	0.11
Thiabendazole	18	24	17

[a]WL167F is a W303-1A leu+ transformant which encodes wild-type Phe at residue 167.
[b]WL167Y is a W303-1A leu+ transformant which encodes Tyr at residue 167.
[c]Limit of carbendazim and nocodazole solubility.

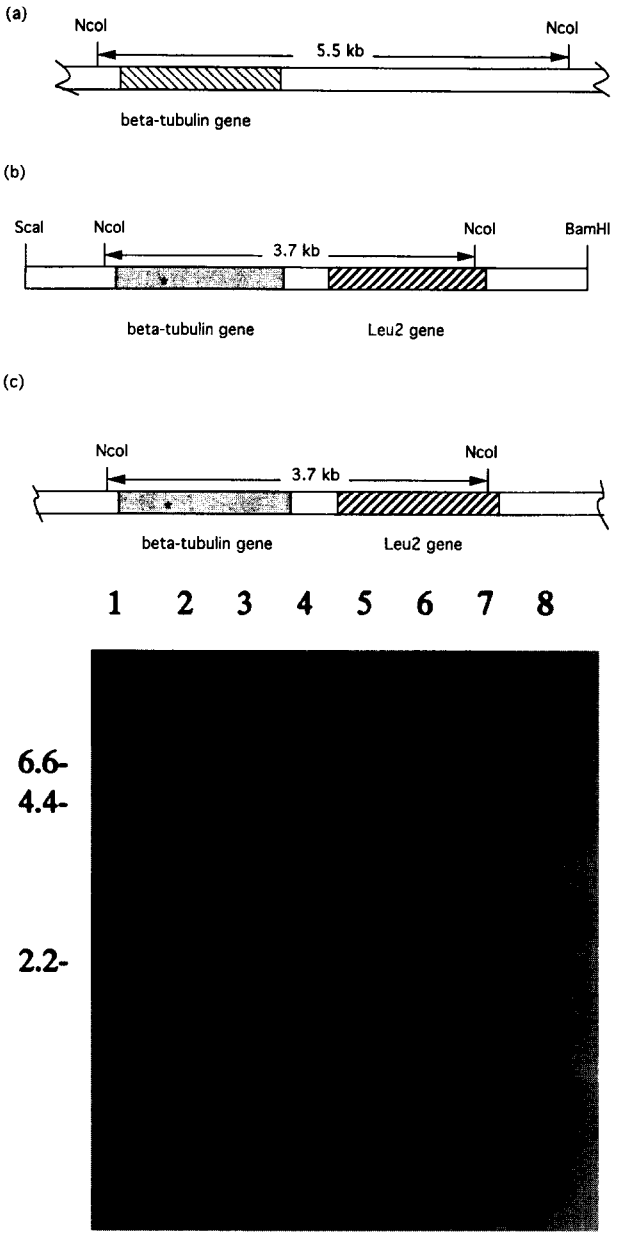

Fig. 2. Replacement of wild type with mutated β-tubulin gene. (a) Chromosomal β-tubulin locus of parent strain W303-1A. (b) *Sca*I-*Bam*HI fragment from pCT-01L167Y which was used for transformation. (c) Expected gene arrangement in chromosomal β-tubulin locus following pCT-01L167Y transformation and homologous recombination. The expected lengths of *Nco*I restriction fragments are indicated (diagrams are not drawn to scale). Mutated residue 167 is shown (*). (d) Southern blot of genomic DNAs digested with *Nco*I and probed with a ^{32}P-labelled β-tubulin gene fragment. Lane 1 is parent strain W303-1A; lanes 2–8 are pCT-01L167Y transformants. Lanes 2, 3, and 6–8 represent the expected replacement; lanes 4 and 5 represent insertion events that were not characterized further.

ification procedures). Thus, the Tyr-167 mutation most likely affects benzimidazole binding directly.

In contrast to the results reported for *N. crassa*, the *S. cerevisiae* Tyr-167 mutant was supersensitive to the derivative benomyl. This phenotype was only observed in liquid medium with benomyl freshly diluted from a dimethylsulfoxide stock. This difference can be understood by considering benomyl structure and stability. Benomyl differs from carbendazim only at the 1 position, in which benomyl is substituted with butylcarbamyl (Fig. 1). This side chain is lost by spontaneous intramolecular catalysis in aqueous solutions, yielding carbendazim [23]. Since the half-life of benomyl is about 1 h [12] it is generally considered a carbendazim prodrug without activity of its own. However, the division time for *S. cerevisiae* in our assays was about 2 h; thus, cells were exposed for 1 to 2 cell divisions to significant benomyl concentrations. Given the potentially irreversible (lethal) activity of benzimidazoles [6], this length of exposure is sufficient for activity. The observed supersensitivity was lost after a 12 h preincubation of the benomyl in medium; i.e. with conversion to carbendazim. Consequently, we conclude that benomyl supersensitivity resulted from the interaction of the 1 position butylcarbamyl with Tyr-167. In the *N. crassa* study [9], benomyl was added to agar medium prior to pouring, leading to its conversion to carbendazim well before the addition of cells.

An interaction between β-tubulin residue 167 and the benzimidazole 1 position (either the butylcarbamyl side chain, or the imidazole ring itself) coincides well with the earlier conclusion that residue 165 interacts with the 2 position side chain [11]. Our results suggest a plausible although still speculative model for benzimidazole interaction with residue 167. In this model, the phenyl side chain of Phe-167 forms a hydrophobic bond with the benzimidazole core. When mutated to Tyr, this hydrophobic bond is weakened, decreasing benzimidazole binding and sensitivity. On the other hand, the Tyr-167 side chain interacts favorably with butylcarbamoyl at the benomyl 1-position, perhaps through the formation of a hydrogen bond. This enhanced binding generates supersensitivity.

Acknowledgements: This work was supported by Grant AI32433 from the National Institute of Allergy and Infectious Diseases.

References

[1] Brown, H.D., Matzuk, A.R., Ilves, I.R., Peterson, J.H., Harris, S.A., Sarett, L.H., Egerton, J.R., Yakstis, J.J., Campbell, W.C. and Cuckler, A.C. (1961) J. Am. Chem. Soc. 83, 1764–1765.
[2] Robinson, H.J., Silber, R.H. and Graessle, O.E. (1969) Texas Rep. Biol. Med. 27, 553–560.
[3] Van den Bossche, H., Rochette, R. and Hörig, C. (1982) Adv. Pharmacol. Chemother. 19, 67–120.
[4] Davidse, L.C. (1986) Annu. Rev. Phytopathol. 24, 43–65.
[5] Lacey, E. (1988) Int. J. Parasitol. 18, 885–936.
[6] Katiyar, S.K., Gordon, V.R., McLaughlin, G.L. and Edlind, T.D. (1994) Antimicrob. Agents Chemother. 38, 2086–2090.
[7] Katiyar, S.K. and Edlind, T.D. (1994) Mol. Biochem. Parasitol. 64, 33–42.
[8] Thomas, J.H., Neff, N.F. and Botstein, D. (1985) Genetics 112, 715–734.
[9] Ohrbach, M.J., Porro, E.B. and Yanofsky, C. (1986) Mol. Cell. Biol. 6, 2452–2461.
[10] Jung, M.K., Wilder, I.B. and Oakley, B.R. (1992) Cell Motil. Cytoskel. 22, 170–174.
[11] Jung, M.K. and Oakley, B.R. (1990) Cell Motil. Cytoskel. 17, 87–94.
[12] Clemons, G.P. and Sisler, H.D. (1969) Phytopathol. Notes 59, 705–706.
[13] Takahashi, M., Matsumoto, S., Iwasaki, S. and Yahara, I. (1990) Mol. Gen. Genet. 222, 169–175.
[14] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[15] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[16] Davis, L.G., Dibner, M.D. and Battey, J.F. (1986) Basic Methods in Molecular Biology, Elsevier, New York.
[17] Hesse, J., Thierauf, M. and Ponstingl, H. (1987) J. Biol. Chem. 262, 15472–15475.
[18] Linse, L. and Mandelkow, E.M. (1988) J. Biol. Chem. 263, 15205–15210.
[19] Shivanna, B.D., Mejillano, M.R., Williams, T.D. and Himes, R.H. (1993) J. Biol. Chem. 268, 127–132.
[20] Erickson, H.P. and O'Brien, E.T. (1992) Annu. Rev. Biophys. Biomol. Struct. 21, 145–166.
[21] Arévalo, M.A., Nieto, J.M., Andreu, D. and Andreu, J.M. (1990) J. Mol. Biol. 214, 105–120.
[22] Barahona, I., Soares, H., Cyrne, L., Penque, D., Denoulet, P. and Rodrigues-Pousada, C. (1988) J. Mol. Biol. 202, 365–382.
[23] Calmon, J.P. and Sayag, D.R. (1976) J. Agric. Chem. 24, 311–314.