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REGULATION OF STEROL BIOSYNTHESIS IN SUNFLOWER BY 24(R,S),25-EPIMINOLANOSTEROL, A NOVEL C-24 METHYL TRANSFERASE INHIBITOR

W. David Nes*, Giselle G. Janssen, Robert A.Norton, Malgorzata Kalinowska$, Farrist G. Crumley, Beni Tal, Annika Bergenstrahle+, and Lisbeth Jonsson+

Plant and Fungal Lipid Research , Microbial Products Research Unit
Richard B. Russell Research Center, 950 College Station Rd. Athens, Ga. 30613

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SUMMARY- Whereas sitosterol and 24(28)-methylene cycloartanol were competitive inhibitors (with K_i=26 μM and 14 μM, respectively), 24(R,S)-25-epiminolanosterol was found to be a potent non-competitive inhibitor (K_i=3.0 nM) of the S-adenosyl-L-methionine-C-24 methyl tranferase from sunflower embryos. Because the ground state analog, 24(R,S)-oxidolanosterol, failed to inhibit the catalysis and 25-azalanosterol inhibited the catalysis with a K_i of 30 nM we conclude that the aziridine functions in a manner similar to the azasteriod (Rahier, A., et al., *J. Biol. Chem.* (1984) **259**, 15215) as a transition state analog mimicking the carbonium intermediate found in the normal transmethylation reaction. Additionally, we observed that the aziridine inhibited cycloartenol metabolism (the preferred substrate for transmethylation) in cultured sunflower cells and cell growth.

In sunflower, as in other crop plants, the synthesis of 24-ethyl sterol membrane inserts proceeds stereoselectively from cycloartenol (the product of squalene oxide cyclization) to 24-α ethyl cholesterol (sitosterol) via 24(28)-methylene cycloartanol (1). Previously, we hypothesized that the enzyme that catalyzes the first committed step in the C-24 alkylation pathway should be inhibited by the ultimate end product, which in sunflower is sitosterol (1-3). It was thought that HMG CoA-reductase may catalyze the overall rate-determining step in phytosterol genesis thereby limiting cycloartenol availability (1-3). However, as we now demonstrate, in this and our companion paper (2), (S)-adenosyl-L methionine C-24 methyl transferase (SMT), and its putative

*To whom correspondence should be addressed.

$Visiting Scientist -- permanent address: Department of Biochemistry Warsaw University, Warsaw, Poland.

+Visiting Scientist -- permanent address: Department of Plant Physiology, Swedish University of Agricultural Sciences, Uppsala, Sweden.

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isoenzyme cogeners (2, 3) may be considered the key regulatory enzyme in the conversion of cycloartenol to sitosterol, for several reasons. First, recent substrate specificity studies performed with enzymes that act on the tetracyclic moiety indicate that these enzymes exhibit a high degree of specificity, in contrast to earlier assertions (2, 3 and ref. cited therein). Additionally, the kinetic findings coupled with distribution data on cycloartenol transformation products show the degree of C-4 demethylation may be the initial molecular feature which acts as the pivotal structural determinant for binding and transformation of cycloartenol by enzymes which may use it as a substrate ie., the C-4 demethylase (3), cyclopropyl to $\Delta^{8,9}$-isomerase (4), the SMT (2), $\Delta^{24,25}$-reductase (3), and $\Delta^{22,23}$-desaturase (3): of the five enzyme systems, only one-the SMT catalyzing the first C_1-transfer, prefers a substrate with the geminal C-4 methyl group left intact on the substrate. Second, for the initial transmethylation to proceed as the regulatory step the concentrations of substrates and products involved in the catalysis should be far from thermodynamic equilibrium, which they are (2). Third, the maximal velocity of the regulatory enzyme, as measured in cell extracts under optimal conditions, should be one of the slowest of all enzymes in the pathway and the SMT reaction appears to be slower than the reactions catalyzed by other plant sterol enzymes studied (4, 5). Fourth, as we now demonstrate, the concentration of the substrate of the enzyme should increase without undergoing significant transformation eg., undergo C-4 demethylation, by cells treated with a sterol biosynthesis inhibitor (SBI) designed to selectively interfere with the rate-limiting enzyme of the pathway. The SBI which we prepared to examine the regulatory phenomena was 24(R,S)-25-epiminolanosterol (IL). Previously, we found that IL completely inhibited in the micromolar range (1-50 μM) the growth of cancer cells (6), pathogenic fungi (7) and pathogenic algae (8). As expected for mechanistic reasons (9), each of the IL-treated cells accumulated sterols with the 24,25-double bond intact. For an explanation of the lack of metabolism of the 24,25-double bond in the IL-treated cells, we postulated that the carbonium ion formed during C-24 alkylation or 24,25-double bond reduction leads to the formation of a high energy intermediate where the carbonium ion appears at C-25 and attack at C-24 is achieved by addition of CH_3^+ (alkylation)

or H^+ (reduction). Thus, IL, by acting as a transition state analog of the reaction, should interfere with the metabolism of the 24,25-double bond. The results presented herein provide additional support for the mechanism of action of IL and that C-24 alkylation may be the rate-limiting step in the production of phytosterols.

MATERIALS AND METHODS

Plant material -- Sunflower (*Helianthus annus*) seeds were germinated for 3-5 days and the sprouts homogenized in the manner described to provide a microsomal preparation (2). The kinetic properties and substrate specificity of the microsome bound C-24 methyl transferase of sunflower embryos has also been characterized by us (2) and the assay is described as follows: A microsomal suspension (0.5 ml) from the embryo tissue preparation was incubated for 45 min at 30°C in the presence of 50 µl [methyl-^{3}H] S-adenosyl-L-methionine (SAM) (0.5-1 µCi/ml, Amersham), 50 µl substrate dissolved in 50 µl of a Tris-HCl buffer and Tween 80 (final concentration of 0.1% w/v) to give a total volume of 0.6 ml and final concentration of substrate of 50 µM. The reaction was terminated by the addition of 0.5 ml of 6% KOH in ethanol. The resultant neutral lipids were extracted into hexane and the pooled extracts dried, then analyzed.

Tissue cultures -- Cultured sunflower cells derived from hypocotyl tissue were prepared and maintained on MS medium supplemented with hormones as described (10).

Sterol analysis -- The nonsaponifiable lipid fractions of enzyme extracts and culture cell pellets were chromatographed by TLC and HPLC to separate the sterols from other lipids and from one another and structurally defined by GC-MS and ^{1}NMR (11-13). Radioactivity was assayed as described (14).

Source of compounds -- 24(R,S)-25-Epiminolanosterol and 25-azalanosterol were synthesized as described (7, 15). The azasteroid was purified by HPLC from a minor contaminant, 25-azadihydroagnosterol. 24(R,S)-25-Oxidolanosterol was a gift of Dr. Edward Parish, Auburn University. The Parish sample was purified by HPLC and the structure of the HPLC pure material was confirmed to be the oxysterol of interest by GC-MS and NMR. The details of the purification protocols will be presented elsewhere. Cycloartenol and 24(28)-methylene cycloartanol were isolated from cactus pollen (16). [Methyl-^{3}H]SAM (15 Ci/mmol) was purchased from Amersham.

RESULTS AND DISCUSSION

The sterol composition of 4-day sprouts, microsomes derived from sprouts (embryo tissue), and cultured cells, also derived from sprouts, is given in Table 1. When IL was fed as an ethanolic solution to mid-log phase cultured cells at 10 ppm (Figure 1), cell growth became arrested. The major sterol which accumulated in the IL-treated cells after five days of incubation was cycloartenol (Figure 2). When IL was added to cultures then inoculated with the same amount of cells supplied as inoculum to control flasks and incubated for 24 days the I_{50} for IL was determined to be ca. 200 nM. Cell growth was not restored by supplementation with either 10 ppm sitosterol or the natural mixture of 4-desmethyl sunflower cell sterols when added together

TABLE 1. STEROL COMPOSITION OF SUNFLOWER[1]

STEROL	Sterols of sprouts[2]	Sterols of microsomes[3]	Sterols of cultured cells[4]		Sterols of 1 ppm IL-treated cells[5]	Sterols of seeds	Sterols of calli[6]
			A	B			
cholesterol	tr.	2	1	tr.	tr.	tr.	tr.
campesterol	5	{9}	6	{10}	{7}	{8}	{13}
24-epicampesterol	3		4				
sitosterol	44	59	65	45	10	51	58
stigmasterol	18	16	13	36	31	9	23
stigmast-7-enol	11	11	5	2	tr.	10	tr.
cycloartenol	tr.	1	tr.	tr.	48	15	tr.
24(28)-methylene cycloartanol	1	tr.	3	1	tr	7	tr.
lanosterol	N.D.	tr.	tr.	1	tr.	N.D.	N.D.
24(28)-methylene lanosterol	N.D.	tr.	1	3	tr.	N.D.	N.D.
24,25-dihydro lanosterol	N.D.	N.D.	N.D.	tr.	tr.	N.D.	N.D.
others	18	2	2	2	4	tr.	N.D.
Total sterol µg/g fr. wt.	(15 µg/sprout) 2480	N.D.	120	115	48	(83 µg/seed) 1314	230

[1]As percent total sterol: N.D. -- not detected, tr. -- trace.
[2]Sunflower seed germinated in moist vermiculite for 4 days in the dark. Sprouts measure ca. 2.7 cm. (N = 74 ± .3 cm).
[3]Microsomes prepared from 4-day sprouts.
[4]Light-grown cultured cells derived from sprouts (hypocotyl tissue): A) 20-day cultures; B) 24-day cultures.
[5]Cells cultures for 24-days on 1 ppm IL. In this experiment the control yielded 8.5 g dry wt. cells and the IL-treated cells yielded 1.5 g dry wt. cells.
[6]Calli derived from hypocotyl tissue were cultured in the dark on agar plates supplemented with MS medium and hormones.

with 10 ppm IL. Similar results were obtained by supplementing the cultures with 25-azalanosterol (data not shown). The sterol composition of the IL-treated cultures harvested at 24-days is shown in Table 1. The sterol profile of the cells contained, as expected, an accumulation of only one sterol intermediate cycloartenol. The lower sterol content observed in the inhibited cultures compared with the control should not be confused with the possibility that sterol production is diminished by IL-treatment. We observed that sterol production was coordinately regulated so that the increase in cell mass was proportional to the increase in sterol mass (data not shown). Thus the lower sterol content in the IL-treatments relates to the lower cell mass. Because the IL-treated cells possessed a total sterol mass equal to control (ca. 30,000 fg/cell) sufficient sterol was available to perform the bulk membrane role. Thus, the diminished growth may be related to the lack of biosynthetic renewal of sufficient 24-alkyl sterols to mediate one or more of the multiple sterol-controlled functions (1). When 25 ppm IL or 25-azalanosterol were incubated with sunflower seeds during the initial period of germination i.e., during water

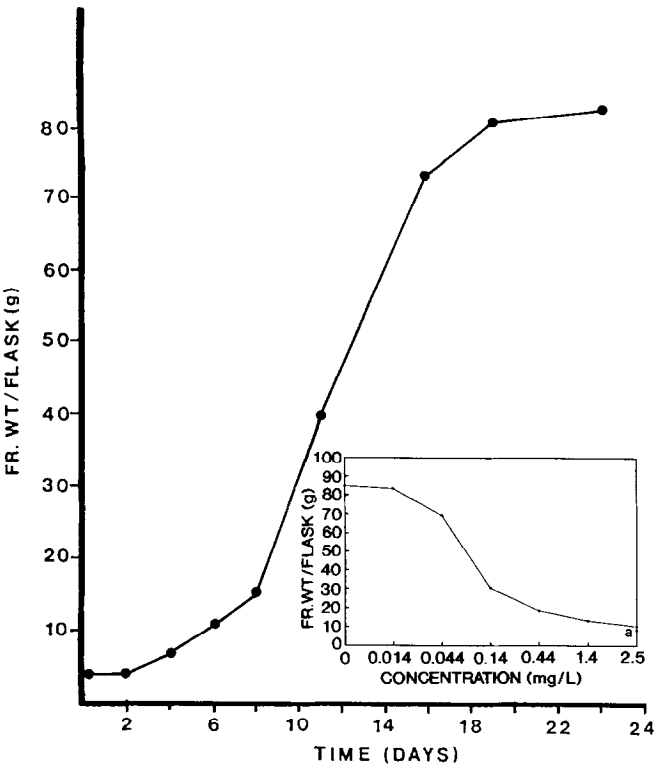


Figure 1. Growth of cultured sunflower cells. The tissue cultures were grown in 1 liter Erlenmeyer flasks supplemented with 350 ml MS medium and shaken at 125 rpm on a rotary shaker under normal laboratory light conditions. Insert is the growth response of cultured cells to increasing concentrations of 24(R,S)25-epiminolanosterol.

absorption and sprout formation, no inhibition of the germination events were apparent. Therefore, it appears that the N-steroids were not accumulated by the seeds of sprouts.

To assess the kinetic parameters of inhibition, a microsomal preparation obtained from sprouts was incubated with SAM, cycloartenol, and one of several putative C-24 methyl transferase inhibitors. A control which lacked the inhibitors was incubated in parallel. The reactions were stopped by the addition of 6% methanolic KOH and the neutral lipids examined as described (2). Previously, we determined that the K_m values for the natural substrates of the methyl transfer reaction viz., cycloartenol and S-adenosyl-L-methionine, were the same at ca. 30 μM (2). We observed similar values for the two substrates in this study which were near their previously determined physiological concentrations (2). From the Lineweaver-Burk representations of the inhibition of SMT activity given in Figure 3, it may be concluded that IL

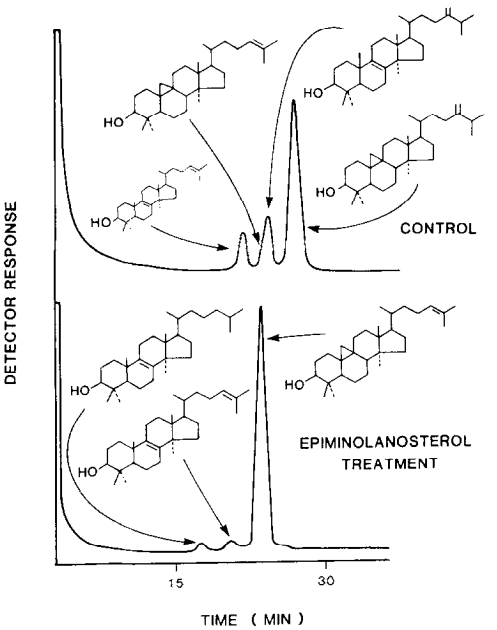
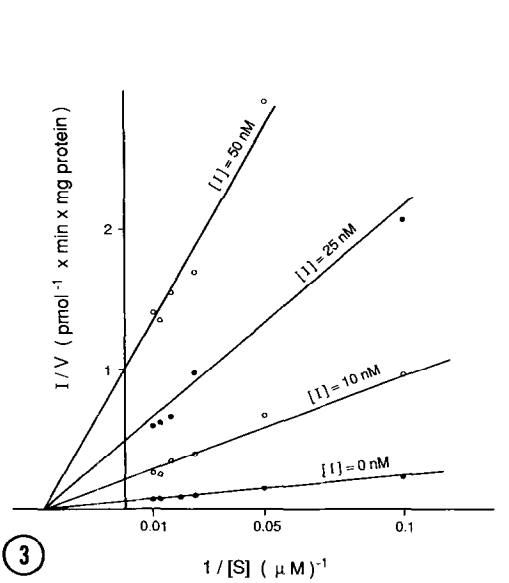


Figure 2. GLC chromatogram (3% OV-17 packed column operated at 245°) of the 4,4-dimethyl TLC band of control and IL-treated cultured sunflower cells.

is a non-competitive inhibitor with respect to cycloartenol. The calculated K_i (3 nM) was four orders of magnitude lower than the K_m (30 μM) for cycloartenol. For an assessment of the inhibition specificity five additional compounds were assayed; 25-azalanosterol, 24(R,S), 25-oxidolanosterol, 24(28)-methylene cycloartanol, sitosterol and cholesterol. At the highest concentration tested (200 μM) neither cholesterol or 24(R,S)-25-oxidolanosterol prevented methylation of cycloartenol whereas the other three compounds were effective inhibitors of the transmethylation reaction. The apparent K_i for 25-azalanosterol was found to be 18 nM, 14 μM for 24(28)-methylene cycloartanol and 26 μM for sitosterol. From the Lineweaver-Burk representations of the inhibition of SMT activity given in Figure 4, it may be concluded that sitosterol is a competitive inhibitor with respect to cycloartenol. Whereas the nitrogen in IL and 25-azalanosterol can mimic either an electrophilic or nucleophilic center, depending on its state of protonation (Fig. 5), the nitronium ion most likely bears a positive charge at physiological pH. Otherwise oxidolanosterol should have interfered with transmethylation through its electron pair on the oxygen binding to an electrophilic site on the enzyme rather than to a nucleophilic site as



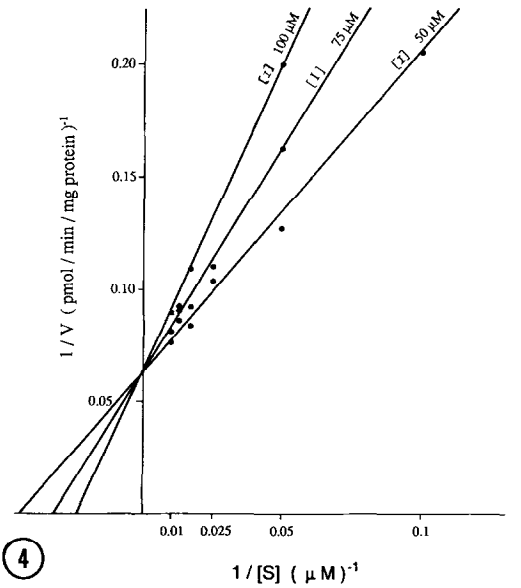


Figure 3. A Lineweaver-Burk plot of the inhibition of the sunflower C-24 methyl transferase by 24(R,S)-25-epiminolanosterol. The conditions of the experiments were as follows: a fixed concentration of [methyl-^{3}H]SAM (50 nM) was added together with varying concentrations of cycloartenol (10, 20, 40, 60, 80 and 100 μM) in Tween 80 (0.1%, w/v) and IL (5, 10, 25 and 50 μM) to a microsomal preparation of enzyme (pH 7.5). Each incubation was performed at 30°C for 45 min. The protein concentration in each microsomal suspension was ca. 2.2 mg/ml.

Figure 4. A Lineweaver-Burk plot of the inhibition of the sunflower C-24 methyl transferase by sitosterol. The conditions of the experiment were the same reported in the IL assay except a fixed concentration of [methyl-^{3}H]SAM (50 μM) was added together with varying concentrations of cycloartenol (10, 20, 40, 60, 80 and 100 μM) in Tween 80 (0.1% w/v) and sitosterol (25 (not shown), 50, 75 and 100 μM).

would be the mode of action for N-steroids, such as IL and 25-azalanosterol. Compounds containing aziridine rings are often powerful alkylating agents (17). However, these biologically active molecules possess, unlike IL, additional functionalization and other heteratoms which are necessary for the alkylation. The oxirane ring could also mimic an alkyl group by providing steric bulk which then might prevent transmethylation as was observed by incubation with 24(28)-methylene cycloartenol. However, the side chain oxysterol lacked inhibitory potency. It follows that conversion of 2,3,22,23-dioxidosqualene cyclization in plants to 24,25-oxido cycloartenol (16) may not regulate sitosterol genesis via the C-24 alkylation pathway (Figure 6).

From the data presented herein we conclude that neither cholesterol nor oxidolanosterol interfered with the transmethylation reaction whereas the other compounds were inhibitory

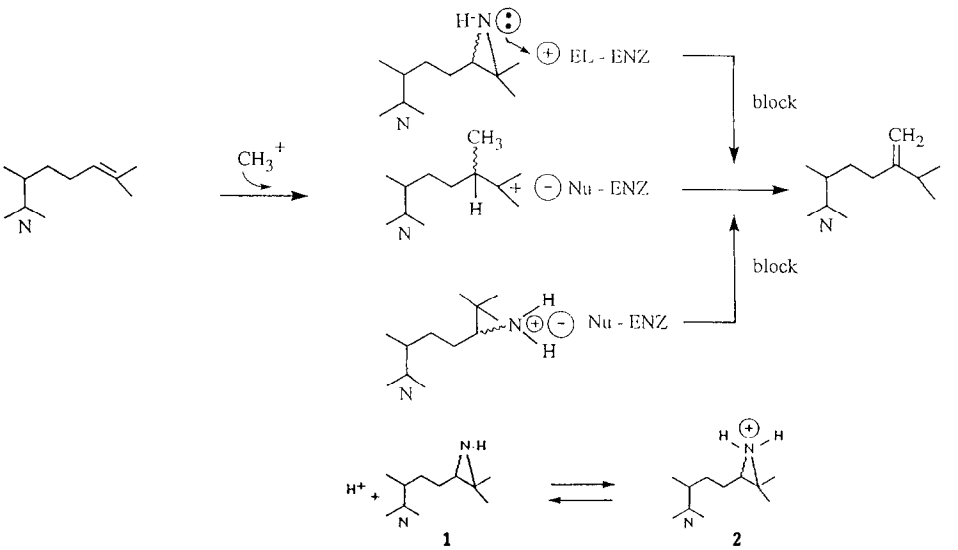


Figure 5. Proposed mechanism of C-24 methylation of cycloartenol and interference by 24(R,S)-25-epiminolanosterol: EL, electrophille; NU, nucleophille.

because: 1) the SMT likely possesses at least two critical domains: one which binds 24-alkyl sterols, another which binds N-steroids; and 2) feedback regulation on 24-alkyl sterol biosynthesis by growth arrested cells (diminished membrane turnover) may result from sitosterol as the membrane level of sitosterol obtains steady-state. In support of this view we note that ontogenetic regulation of sterol biosynthesis in sunflower is coordinated with side chain

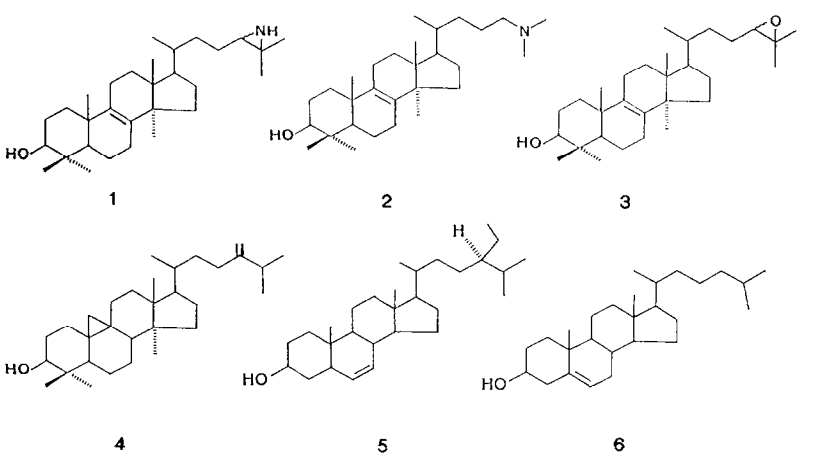


Figure 6. Structures of possible C-24 methylation inhibitors assayed in the present study: 1, 24(R,S)-25-epiminolanosterol; 2, 25-azalanosterol; 3, 24(R,S)-25-oxidolanosterol; 4, 24(28)-methylenecycloartanol; 5, sitosterol; 6, cholesterol.

modifications (1). Alternatively, that cycloartenol continued to be synthesized in SMT inhibited IL-treatments indicates that the C-24 alkylation pathway is not coordinated with the early enzymes of the isopentenoid pathway.

REFERENCES

1. Nes, W. D., (1990) *Rec. Adv. Phytochem.* **24**, 283-327.
2. Nes, W. D., Janssen, G. G., and Bergenstrahle, A., (1991) *J. Biol. Chem.* in press.
3. Janssen, G. G, Kalinowska, M., Norton, R. A., and Nes, W. D. (1991) (S)-Adenosyl-L-methionine: Δ^{24}-sterol methyl transferase: mechanism, enzymology, inhibitors and physiological importance. In *Physiology and Biochemistry of Sterols* (G. W. Patterson and W. D. Nes, eds.), AOCS press, Champaign IL. in press.
4. Rahier, A., Taton, M. and Benveniste, P. (1989) *Eur. J. Biochem.* **181**, 615-626.
5. Taton, M., Benveniste, P. and Rahier, A. (1989) *Eur. J. Biochem.* **185**, 605-614.
6. Popjak, G., Meenan, A., Parish, E. J., and Nes, W. D., (1989) *J. Biol. Chem.* **264**, 6230-6238.
7. Nes, W. D., Xu, S., and Parish, E. J. (1989) *Arch. Biochem.Biophys.* **272**, 323-331.
8. Norton, R. A., Janssen, G. G and Nes, W. D. (1990) *Fed. Proc.* **4**, A2162
9. Rahier, A., Genot, J., Schuber, F., Benveniste, P., and Narula, A. S., (1984) *J. Biol. Chem.* **259**, 15215-15223.
10. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473-497.
11. Kalinowska, M., Nes, W. R., Crumley, F. G., and Nes, W. D. (1990) *Phytochemistry* **29**, 3427-3434.
12. Xu, S., Norton, R. A., Crumley, F. G. and Nes, W. D. (1988) *J. Chromatgr.* **452**, 377-398.
13. Nes, W. D., Benson, M., Lundin, R. E., and Le, P. H. (1988) *Proc. Natl. Acad. Sci.* **85**, 5759-5763.
14. Nes, W. D. and Bach, T. J. (1985) *Proc. R. Soc. Lond. B*, **225**, 425-444.
15. Oehlschlager, A .C., Angus, R. H. Pierce, A. M., Pierce, H. D., Jr., and Srinivasan, R. (1984) *Biochemistry* **23**, 3582-3589.
16. Nes, W. D. and Schmidt, J. O. (1988) *Phytochemistry* **27**, 1705-1708.
17. Dermer, O. C.and Ham, G. E. (1969) Ethylenimine and other aziridenes. Academic Press New York. pp.394-450.