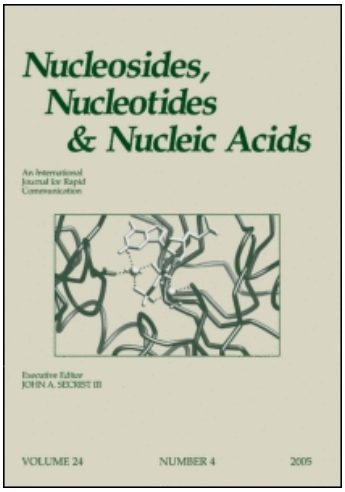

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Lyttle, Matthew H.(1993) 'Chain Cleavage During Deprotection of RNA Synthesized by the 2'-O-Trialkylsilyl Protection Strategy', Nucleosides, Nucleotides and Nucleic Acids, 12: 1, 95 — 106
To link to this Article: DOI: 10.1080/07328319308016197
URL: http://dx.doi.org/10.1080/07328319308016197

NUCLEOSIDES & NUCLEOTIDES, 12(1), 95-106 (1993)

CHAIN CLEAVAGE DURING DEPROTECTION OF RNA SYNTHESIZED BY THE 2'-*O*-TRIALKYLSILYL PROTECTION STRATEGY

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Abstract. The side reaction of inter-ribonucleotide bond cleavage occuring during ammonia treatment for deprotection of support bound 2'-*O*-trialkylsilyl protected synthetic RNA has been assessed by analysis of a series of 15 mers of sequence T_4XT_{10} where X is rA, rC, rG and U. Data derived from HPLC analysis of the products showed, in all cases, cleavage by the anhydrous ammonia to be dramatically reduced compared to that with ethanolic concentrated aqueous ammonia. The anhydrous reagent also gave improved product quality when used in the synthesis of a 50 residue sequence. Complete cleavage of exocyclic amine protection groups with the anhydrous reagent was confirmed by RNAse T2 digests.

INTRODUCTION

Solid phase supported RNA synthesis by the 2'-*O*-trialkylsilyl protection strategy has allowed the synthesis of several long sequences[1]. The extent of removal of the 2'-protection occurring during ammonia treatment resulting in inter-ribonucleotide bond cleavage remains a concern. Addition of ethanol diminishes this problem[2], and the use of anhydrous ethanol is reported by Usman to give dramatic improvement[3]. Our studies[4] show good results with 25 % ethanol in concentrated ammonia, but quantitation is complicated

by the work up which eliminates much of the degraded side products. This paper reports HPLC monitoring of side by side deprotections using the two basic reagents, and other direct comparisons, which demonstrate the better product quality obtained with the Usman procedure.

MATERIALS AND METHODS

Aqueous solutions for RNA de-salting were pre-treated by the addition of 0.1% diethylpyrocarbonate (Sigma Co.). After 24 h at room temperature, the solutions were heated at 115^0 in an autoclave for 45 min - 2 hrs. Sterile Eppendorf tubes were obtained from Bio-Rad, inc. All other equipment for RNA purification was similarly sterilized, and gloves were worn during RNA purification. A Milligen/Biosearch 8750 DNA synthesizer was used for all of the syntheses.

Reagents for Oligonucleotide Synthesis

N^6-Benzoyl-5'-*O*-(4,4'dimethoxytrityl)-2'-*O*-(*tert*-butyldimethylsilyl)adenosine3'-*O*-(2-cyanoethyl*N,N*-diisopropylphosphoramidite),N^4-benzoyl-5'-*O*-(4,4'dimethoxytrityl)-2'-*O*-(*tert*-butyldimethylsilyl)cytidine3'-*O*-(2-cyanoethyl*N,N*-diisopropylphosphoramidite), N^2-benzoyl-5'-*O*-(4,4'dimethoxytrityl)-2'-*O*-(tributylsilyl)guanosine 3'-*O*-(2-cyanoethyl*N,N*-diisopropylphosphoramidite) and 5'-*O*-(4,4'dimethoxytrityl)-2'-*O*-(*tert*-butyldimethylsilyl)uridine 3'-*O*-(2-cyanoethyl*N,N*-diisopropylphosphoramidites)[5,7] were used for the 15 and 20-mer synthesis. 5'-*O*-(4,4'dimethoxytrityl)thymidine (2'-cyanoethyl *N,N*-diisopropylphosphoramidite) was used to assemble the DNA parts of the 15 mers. For the 50 mer, the more reactive N^6-Benzoyl-5'-*O*-(4,4'dimethoxytrityl)-2'-*O*-(*tert*-butyldimethylsilyl) adenosine3'-*O*-(2-cyanoethyl*N,N*-diethylphosphoram idite),N^4-benzoyl 5'-*O*-(4,4'dimethoxytrityl)-2'-*O*-(*tert*-butyldi methylsilyl)-cytidine3'-*O*-(2-cyanoethyl*N,N*-diethylphosphoram idite),N^2-benzoyl- 5'-*O*-(4,4'dimethoxytrityl)-2'-*O*-(tributylsilyl) guanosine 3'-*O*-(2-cyanoethyl*N,N*-diethylphosphoramidite) and 5'-*O*-(4,4'dimethoxytrityl)-2'-*O*-(*tert*-butyldimethylsilyl) uridine 3'-*O*-(2-cyanoethyl-

N,N-diethylphosphoramidites) were used. The supports for RNA sunthesis was the same as those previously described[7] and these and all other synthesis reagents were commercially available[5].

RNA Oligomer Synthesis

20 and 50 mer oligoribonucleotides were made using reaction columns containing 1 micromole of immobilized nucleoside on controlled pore glass (CPG) supports. The synthesis programs were the same as those previously used by us for RNA[4].

RNA Oligomer Deprotection and Purification

The 20 and 50 mer oligoribonucleotides were left with the 5'DMT group on, and dried with helium on the instrument. The CPG was divided into two equal portions by weight, and both of the portions were placed in screw cap Eppendorf tubes. One tube was charged with 1 ml of anhydrous ammonia dissolved in absolute ethanol[6], while the other tube received 1 mL of 25 % ethanol in conc. aqueous ammonia. Both of these were heated at 55^{o} for 18 hrs, after which they were cooled to rt and exposed to the atmosphere. After 1 hr, each solution was decanted and the supernatant was evaporated to dryness on a vacuum centrifuge. The samples were then dried further by good vacuum for 6 to 24 h, and 400 uL of 1 M tetrabutylammonium flouride (TBAF) in tetrahydrofuran (Aldrich Co.), was added. The mixture was agitated briefly and allowed to stand for 18 h. 400 uL of 1.0 M triethylammonium acetate (TEAA, pH 7.0) was added. A reverse phase DNA purification cartridge[5] was prepared by eluting 10 mL of 20 % aqueous ethanol, 10 mL of acetonitrile and 10 mL of 1.0 M TEAA via syringe. The solution containing the RNA then was passed through and repeated twice, maintaining directionality of flow and vertical disposition of the column. The cartridge was then washed with 10 mL of 0.1 M ammonium acetate, pH 7, followed by 10 mL of sterile water. Next, the DMT group was removed by passing 10 mL of 2 % triflouroacetic acid (TFA) through. A pause of 2 min during the TFA treatment ensured complete detritylation. The cartridge was washed

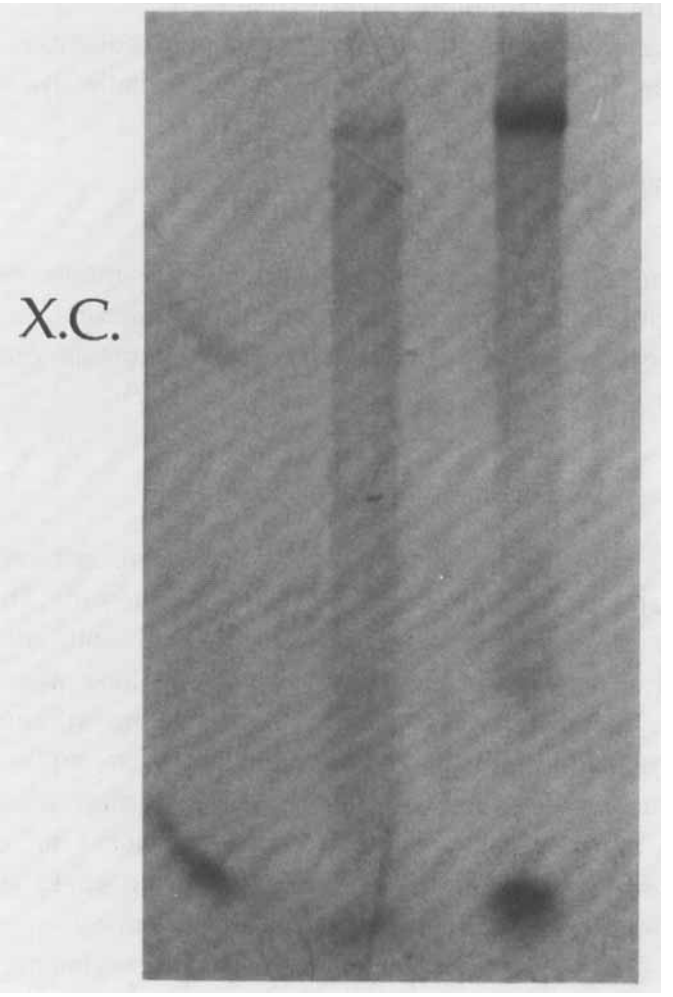


FIGURE 1. PAGE of 50 mers. Left, Hyd. NH_4OH . Right, Anhyd. NH_4OH

with 10 mL of water, and the RNA was eluted with a1 : 1 mixture of acetonitrile and water. 1 mL fractions were collected. 5 o.d. units of RNA were present in the first 1 mL eluted from each of the 50 mer samples, and 25 o.d. of each of the 20 mers were obtained in the first fraction. Very small amounts of RNA were in the second 1 mL fraction of each purification; these were discarded. 2 o.d.s of each 50 mer sample were analyzed on a 2 mm thick 20 % gel run at 75 mA for

Table1. 15 Micromole Synthesis Programs

15 Micromole Mixed DNA/RNA Synthesis Cycle

Step[7]	Duration(sec)	
Wash	20	
Deblock	230	
Wash	294	
Couple[8]	600	(for DNA)
	1937	(for RNA)
Wash	30	
Cap	80	
Wash	20	
Oxidize	50	

3 hrs. The RNA bands were visualized with U.V. light on a fluorescent background. A Polaroid CU - 5 land camera with a 5 " lense using. Polaroid 107c coaterless black and white film was used for photography. See Fig. 1. The 20mers were also analyzed by PAGE, and in this case little difference was seen (data not shown). The two samples generated were used in the RNAse T2 study described below. For the mixed DNA/RNA fragments, a 15 micromole program was used for the incorporation of the T residues. A modified version of this protocol with a double coupling was used to incorporate the ribonucleoside base. See Table 1. The RNA synthesis routine has also been successfully used for 10 micromole RNA synthesis[9]. After each fragment was synthesized, the CPG containing the 15 - mer was washed and dried. From each sample, 10-20 mg portions were weighed into 2 ml glass vials with screw caps.25 % ethanol in

concentrated ammonia was added to 5 of the vials, and ammonia gas dissolved in ethanol was added to the others. The samples were heated at 55° for 1 to 8 days. At the end of the specified time, each sample was cooled, depressurized and removed from the vial. The samples were then evaporated and stored at -20°.

When the last sample was finished, each was dissolved in water and analyzed by a Waters 625 LC system using a Beckmann Ultrasil AX analytical column. The gradient used was from 0 to 50 % B over 20 min. Buffer A was .003 M aqueous potassium phosphate at pH 6.8 (20 % acetonitrile), buffer B was 0.6 M potassium phosphate at pH 6.8 (20 % acetonitrile). See figure 2 for the series of traces obtained with the hydrolysis of TTTTU(2'-*O*-(*tert*-butyldimethylsilyl))-TTTTTTTTTT in anhydrous ethanolic ammonia and 25 % ethanolic aqueous ammonia. HPLC standard samples of TTTTTTTTTT, fragments in the hydrolysis HPLC traces.See Figure 3.

HPLC standard samples of TTTTTTTTTT, TTTTTTTTTT plus PO3TTTTTTTTTT, TTTTU(2'-O-(*tert*-butyl dimethyl silyl))TTTTTTT-TTT and TTTTU(2'-OH)-TTTTTTTTTT were isolated independently and used to confirm the identity of the various fragments in the hydrolysis HPLC traces.See Figure 3.

Enzyme Digest of UCCACGUCAUCCAGGUCAUC

5 o.d.s of 20 mer cleaved with the anhydrous ammonia and 5 o.d.s of the 20 mer cleaved with the aqueous ammonia and ethanol solution were dried and re-dissolved in 800 uL of 0.05 M NH_4OAc (0.002 M EDTA) pH 4.5. 5 units of RNAse T2 (Sigma Co.) were added and the solutions were warmed to 37° overnight. Then 400 uL of 0.1 M TEAA (pH 9) and 30 units of Calf Alkaline Phosphatase (Sigma Co.) were added, and the samples again warmed to 37° overnight. The next day, the solution was filtered through 10000 mw exclusion filters[5] and analyzed by HPLC. A Waters Deltapak C-18 column was used, on the same instrument as that used above for the SAX HPLC, with a gradient of 0 - 15 % B over 15 min, 15 % B for 8 min. A was 0.1 M TEAA pH 7, and B was 50% aqueous acetonitrile. See FIGURE 4.

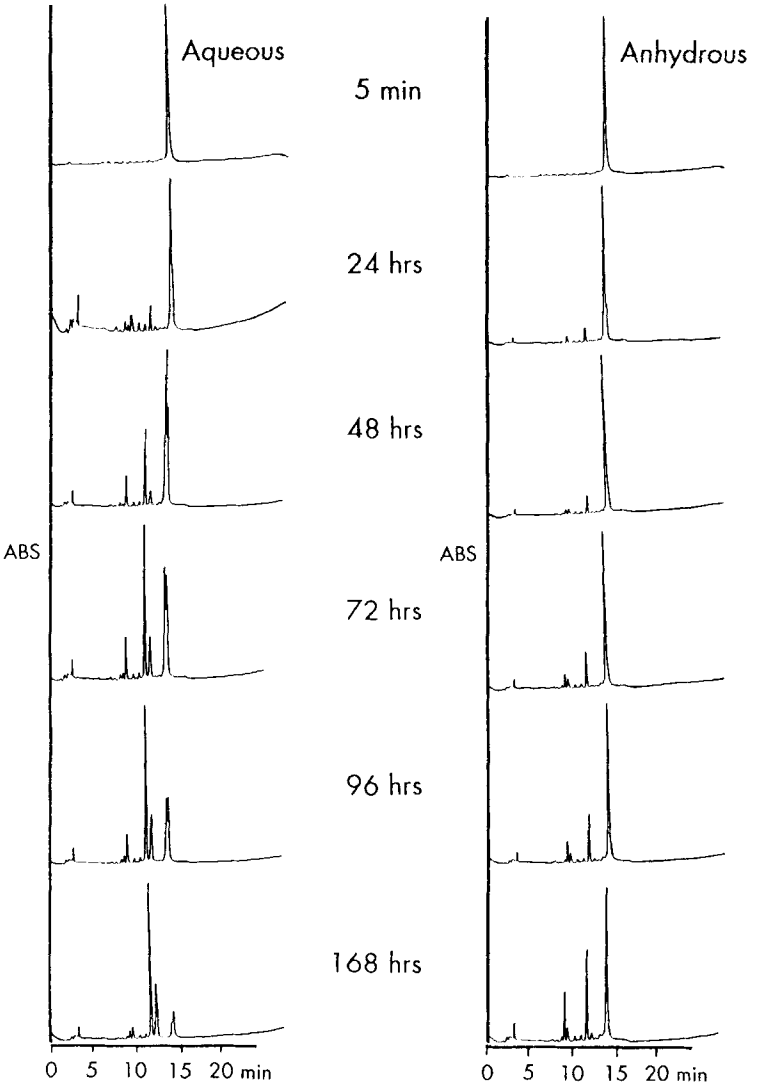


FIGURE 2. SAX HPLC of TTTTU(2'-*O*-*tert*-butyldimethylsilyl)-TTTTTTTTTT Hydrolysis Products

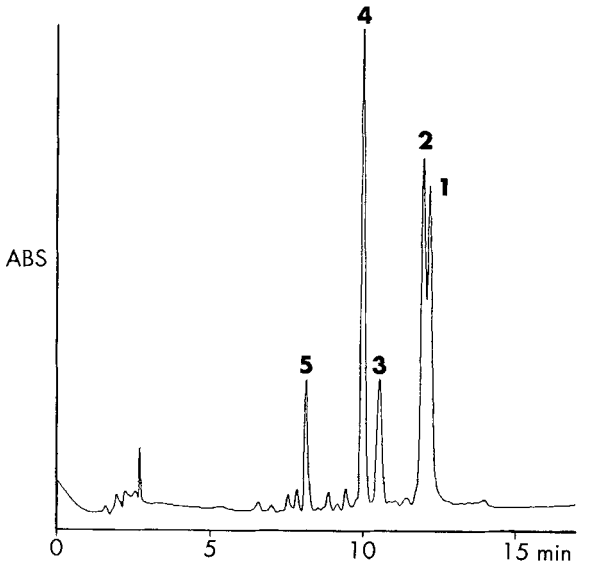


FIGURE 3. Typical Hydrolysis Product HPLC Trace

1 Is TTTTUTTTTTTTTTT Without the Ribonucleoside Silyl Group

2 Is TTTTUTTTTTTTTTT with Silyl Protection Intact

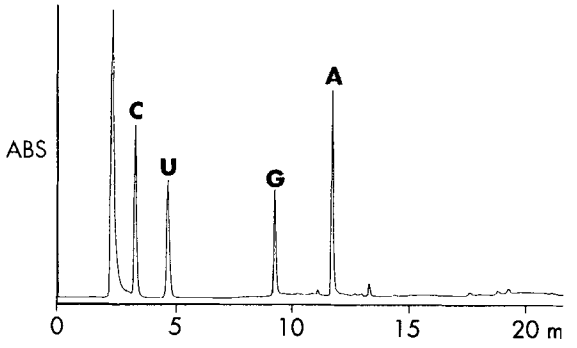
3 Is PO_3TTTTTTTTTT

4 Is TTTTTTTTTT

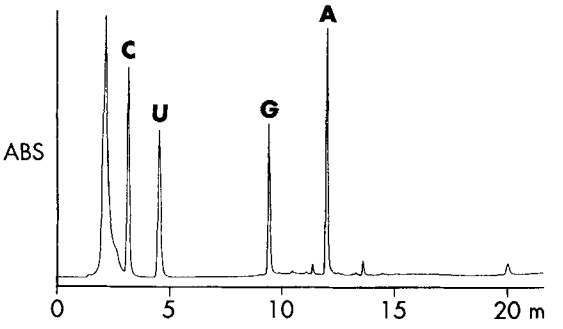
5 is Probably TTTTUPO_3 or TTTTU.

RESULTS AND DISCUSSION

The solid-phase supported synthesis of RNA is more complicated than for DNA, and a few strategies have been proposed. Of these, only the 2'-*O*-trialkylsilyl method has been established as a practical method for routine synthesis[1,3,4,7]. It has been established in simple dimer studies[2, 3] that premature loss of 2'-O-trialkylsilyl groups does occur during ammonia treatment, resulting in subsequent internucleotide bond cleavage. This paper provides an evaluation of this effect. In the first series of experiments, two oligoribonucleotides 20 and 50 base units in length were made, and



Base hydrolysis with 25 % ethanol in Conc. Aq. Ammonia, 18 H at 55°



Base hydrolysis with anhydrous ammonia in absolute ethanol, 18 H at 55°.

FIGURE 4. Reverse Phase HPLC of RNAse T2 and Calf Alkaline Phosphatase Hydrolysis of UCCACGUCAUCCAGGUCAUC.

each of these syntheses were split into two equal portions of CPG. One of each pair was treated with a solution of 25 % ethanol in 28 % aqueous ammonia. The other portion of CPG was treated with a saturated solution of ammonia gas in absolute ethanol. Each sample was heated to 55° for 18 hrs,cooled, and evaporated. the residue was treated with TBAF and this was quenched with buffer after overnight exposure. Each pair was purified with a reverse phase separation cartridge.

Using this procedure, failure sequences which had been generated during the synthesis are eliminated, provided that capping is operating properly during the synthesis[10]. Failure sequences generated by unwanted cleavage during the ammonia treatment will be retained on the cartridge along with the full length product, provided that they contain the 5' end of the sequence which has the DMT group still attached. When the samples were detritylated and analyzed by PAGE, the 50 - mer produced by the use of anhydrous ammonia in ethanol had less shorter sequence material below the product band than the aqueous base treated counterpart. See Figure 1.

With the 20 - mer fragments little improvement was seen in using the anhydrous reagent. This supports our contention that the generation of failures due to unwanted cleavage is a minor problem with 2'-*O*-trialkylsily protection, which will only manifest itself when longer fragments are attempted.

We were concerned that the result with the 50 - mer could be due to the weaker concentration of ammonia in the anhydrous solution, and that the exocyclic amine protecting groups of guanosine in the RNA sequence would still be retained to a varying degree, in contrast to literature claims[3]. This would appear as improved results in this experiment, when in reality products with reduced biological activity were being produced. Indeed, the concentration of ammonia in the anhydrous solution was only 3.3 M when a simple titration was performed, when it should be about 12 M in the aqueous solution, when fresh aqueous ammonia is used.

To allay these fears, RNAse T2 and Calf Alkaline Phosphatase digestion of the 20 mers obtained in the above study was performed, and the results looked nearly identical. A special reverse phase HPLC gradient was used which was designed to show the presence of unhydrolized base protecting groups. Co-injection of $G^{bz}$with one of the samples showed that this material was not present in the enzyme digest products. See Figure 4.

In the second series of experiments, the T_4XT_{10} oligomers were treated with either ethanolic concentrated aqueous ammonia or

anhydrous ethanolic ammonia for time intervals up to 168 hrs. The samples were analyzed by strong anion exchange HPLC without any treatment which could disturb the observed distribution of products. FIGURE 2 shows the the results obtained in the T_4UT_{10} study. The other test samples containing rA, rC and rG gave more complicated traces due to the presence of side chain protected species at the beginning of the hydrolysis. Nevertheless, the same trends were observed. FIGURE 4 shows detail of the 72 hr HPLC profile of T_4UT_{10} hydrolysis obtained with the aqueous reagent, and identifies the components. It is clear (from FIGURE 2) that the anhydrous reagent gives much less degredation. Interestingly, analysis of the data shows that the degredation fits a first order kinetic treatment quite well. The appearance of the desilylated full length product during the hydrolysis (FIGURE 3, compound 1) suggests that hydrolysis of the 2'-hydroxy RNA species, and not loss of the 2'-*O*-silyl group is the rate limiting step.

CONCLUSION

This study confirms that anhydrous ethanolic ammonia is a superior reagent for the deprotection and cleavage of RNA synthesized by the 2'-O-trialkylsilyl protection method. Also, the studies show that chain cleavage with the original reagent, 25 % ethanol in concentrated aqueous ammonia, is sufficiently slow that an RNA strand of 50 residues in length can be synthesized and deblocked with this reagent.

The methods described provide viable tests for evaluating alternative strategies for RNA synthesis.

ACKNOWLEGEMENT

We would like to thank Peter Wright for assistance in the HPLC analysis of the products and Nanda Sinha and Barbara Mueller for their work in adapting the reverse phase cartridge purificaition procedures for use with RNA, and to Derek Hudson and Peter Davis for their help in preparing this manuscript.

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6. Anhydrous ethanol and a lecture bottle of ammonia gas with a valve were purchased from Aldrich Co. and the solution was prepared according to ref. 3. Caution should be observed due to the highly effervescent nature of this solution and the danger of getting it in one's eyes.
7. Wang, Y.; Lyttle, M.; Borer, P.; *Nuc. Acids Res.*,**1990**, 3347-3352.
8. For the DNA base addition, 100 mg of amidite was consumed as a 50 mg / mL solution in acetonitrile. For the RNA base addition, 200 mg of amidite was consumed as a 50 mg / mL solution in acetonitrile.
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10. The reagents for capping give above 95 % capping efficiency, as measured by the decrease in DMT color in two synthetic cycles where the coupling step is eliminated in the first cycle.

Received 6/18/92
Accepted 10/14/92