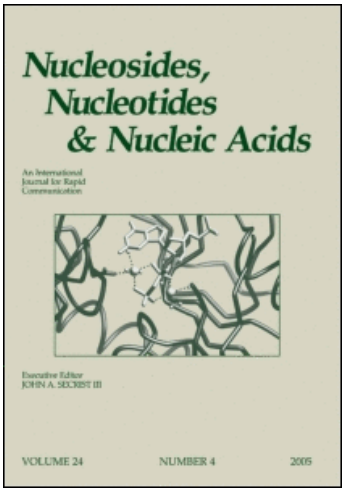

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

SITE SPECIFIC INCORPORATION OF 6-AZAURIDINE INTO THE GENOMIC HDV RIBOZYME ACTIVE SITE

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Online publication date: 31 December 2001

To cite this Article Oyelere, Adegboyega K. and Strobel, Scott A.(2001) 'SITE SPECIFIC INCORPORATION OF 6-AZAURIDINE INTO THE GENOMIC HDV RIBOZYME ACTIVE SITE', Nucleosides, Nucleotides and Nucleic Acids, 20: 10, 1851 — 1858
To link to this Article: DOI: 10.1081/NCN-100107196
URL: http://dx.doi.org/10.1081/NCN-100107196

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(10&11), 1851–1858 (2001)

SITE SPECIFIC INCORPORATION OF 6-AZAURIDINE INTO THE GENOMIC HDV RIBOZYME ACTIVE SITE

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ABSTRACT

The HDV ribozyme is proposed to catalyze its self cleavage reaction by a proton transfer mechanism wherein the N3 of its C75 acts as a general acid. The C75 to U mutation, which raises the N3 pK_a from about 4 to almost 10, abolishes all enzymatic activity. To test if a U analogue with a neutral pK_a can restore ribozyme function we incorporated 6-azauridine (n^6U), a uridine analogue with histidine-like N3 pK_a, into the genomic HDV ribozyme active site by 2′-*O*-ACE oligoribonucleotide protection chemistry. The resulting ribozymes were analyzed for their ability to undergo the HDV ribozyme cis-cleavage reaction. Incorporation of n^6U at nucleotide position 75 did not restore ribozyme function compared to the U75 mutant. This suggests that the HDV ribozyme reaction mechanism involves more than positioning of a neutral nucleobase at the active site and implies that the exocyclic amino group of C75 participates in establishing the proper active site fold.

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INTRODUCTION

Ribozymes were generally thought to be strict metalloenzymes that require accurate positioning of metal ions within their active sites for catalysis; however, recent studies have begun to shed light on the wealth of catalytic mechanisms available to RNA enzymes[1–4]. For example, biochemical and crystallographic analyses on the self cleavage reaction of HDV ribozyme have implicated the N3 of a critical cytidine residue, C75, in a proton transfer process akin to general-acid catalysis[2,4]. To be able to function efficiently in this capacity, the N3 pK_a of C75 is proposed to be raised toward neutrality. However, this pK_a shift has yet to be directly demonstrated, and the functional roles of pK_a perturbation in RNA catalysis remain poorly understood.

The recently solved crystal structure of the self-cleaved genomic HDV ribozyme revealed that the heterocyclic base of C75 is buried in a cleft within the active site where it is in position to act catalytically and the local environment might perturb its N3 pK_a[2]. Additional indirect evidence on the catalytic roles of the N3 of C75 came from a biochemical study where the macroscopic pK_a of the self cleavage reaction of the genomic HDV ribozyme was estimated to be 5.6[4]. Furthermore, mutagenic studies have shown that the replacement of C75 with U resulted in a complete loss of ribozyme activity[4,5]. These results have been used to invoke a direct role for the N3 of C75 as a general acid during the self cleavage reaction[4].

Our goal is to develop biochemical probes to unravel the functional roles of nucleotide pK_a perturbation in RNA function. The approach involves the use of nucleotide analogues with altered heterocyclic ring pK_as coupled with reactivity assays to determine the effects such substitutions have on catalysis when incorporated into ribozymes[6]. We sought to determine if a uridine analog with a near neutral N3 pK_a can rescue the inactivity of the C75U mutant HDV ribozyme. An ideal analogue for this approach is 6-Azauridine **1**, a uridine analogue with enhanced acidity at the N3 position of the heterocyclic ring[7]. Its N3 pK_a is close to neutrality (N3pK_a = 6.7 for **1**,

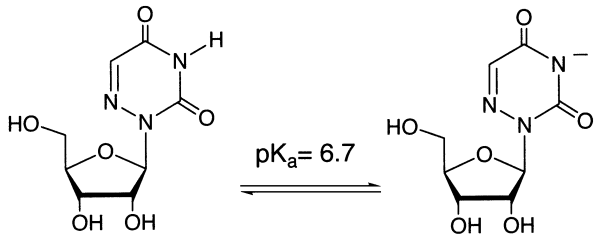

Figure 1. Ionization of 6-azauridine. The N3 pK_a is 6.7,[7] which suggests that a significant proportion of the base exists in a negatively charged form under neutral conditions. Note, only one tautomeric form of the charged species is specified.

compare to N3 $pK_a = 9.7$ for uridine), which is optimum for proton transfer processes. This may make it an ideal nucleoside analogue for probing RNAs where cytidine has been implicated in general acid or general base mechanisms of catalysis[2,4]. Here we report the synthesis of 6-azauridine-5′-*O*-silyl-2′-*O*-orthoester-3′-phosphoramidite **5**, its site specific incorporation into the active site of the full length genomic HDV ribozyme, and the effects this analogue has on HDV ribozyme catalysis.

CHEMISTRY

6-Azauridine **1** was incorporated previously into the hammerhead ribozyme using the 2′-*O*-t-butyldimethylsilyl phosphoramidite approach[8]. As an alternative method, we investigated the behavior of **1** in the recently described approach for RNA synthesis based on the 2′-*O*-bis(2-acetoxyethoxy)-methyl (ACE) orthoester chemistry[9].

Incorporation of 6-azauridine by this approach requires phosphoramidite **5**, which was synthesized from **1** in four steps (Fig. 2). Nucleoside **1** was converted into the 3′,5′-cyclic silyl protected product 2 with an isolated yield of 97% by adapting literature procedures[10]. The 2′-ACE protection

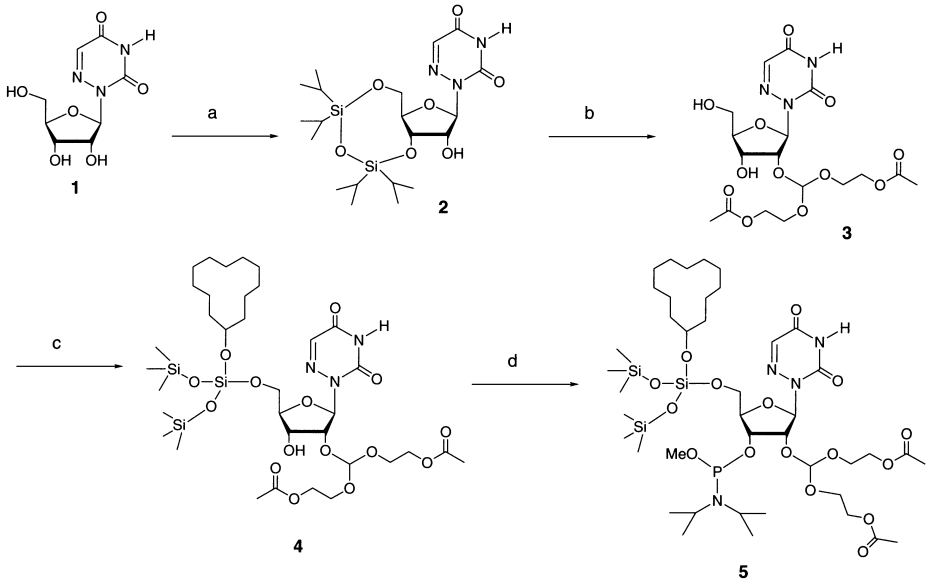

Figure 2. Synthesis of 5′-*O*-silyl-2′-*O*-ACE phosphoramidite **5**: (a) 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane, pyridine, 0 °C to room temperature, 1h; (b) (i) tris(2-acetoxyethoxy) orthoformate, pyridinium p-toluenesulfonate, 55 °C, overnight; (ii) TEMED-HF, CH_3CN, room temperature, 6 h; (c) bis(trimethylsiloxy)cyclododecyloxysilyl chloride, imidazole, THF, 0 °C, 50 min; (d) bis(N,N-diisopropylamine)methoxy phosphine, 1-H tetrazole, CH_2Cl_2, room temperature, 7 h.

and 3′,5′ desilylation of **2** were carried out in a one-pot, two-step reaction to give 2′-ACE nucleoside **3** in 42% yield. The 5′ specific *O*-silylation of **3** with bis(trimethylsiloxy)cyclododecyloxysilyl chloride (DOD-Cl) using published procedures resulted in product **4** with a 58% yield. Phosphitylation of **4** with bis(N,N-diisopropylamine)methoxy phosphine gave the desired 5′-*O*-silyl-2′-*O*-ACE phosphoramidite **5** in 59% yield. The synthesized products were characterized by ^{1}H-NMR, ^{13}C-NMR, HRMS and ^{31}P-NMR (for product **5**)[11].

Phosphoramidite **5** was incorporated by solid phase synthesis into either position 72 or 75 of oligoribonucleotides corresponding to a segment of the genomic HDV ribozyme sequence from positions 65 to 85. Solid phase RNA synthesis was performed at Dharmacon Research Inc., Boulder, Colorado. The coupling efficiency of phosphoramidite **5** was comparable to that of the four standard nucleoside derivatives. The synthesized oligoribonucleotides were 2′-*O*-ACE deprotected as recommended by the manufacturer[9]. The oligoribonucleotides were 5′ end labeled with [γ-^{32}P]ATP using polynucleotide kinase[12] and ligated with T4 RNA ligase onto the 3′ end of a transcribed RNA corresponding to the HDV ribozyme sequence from positions -30 to 53[13]. This construct was engineered to create a nick within a six-nucleotide loop between nucleotide 53 and 65 of the wild-type sequence, which is an ideal substrate for T4 RNA ligase (Fig. 3a). Enzymatic ligation of the oligonucleotide to the transcript resulted in a full-length HDV ribozyme with site specific placement of 6-azauridine at either position 72 or 75 (Fig. 3b).

RIBOZYME ANALYSES

Three ribozyme constructs were made and analyzed for their ability to undergo the HDV cis-cleavage reaction in buffers ranging from pH 5 to 7.5. Ribozyme **A** had C75U and U72n^{6}U substitutions, ribozyme **B** had a U72n^{6}U substitution while ribozyme C contained a C75n^{6}U substitution. These ribozymes were compared to the wild type construct (Fig. 4, panel 1). The cis-cleavage reaction was initiated by addition of a DNA oligonucleotide to activate the ribozyme according to a published procedure[4]. Ribozyme **A**, which replaces the critical C75 with U was inactive, consistent with a report for a similar mutant ribozyme (Fig. 4, panel 2)[4,5]. This is hypothesized to result from the inability of the U75 N3 to participate in the catalytically relevant proton transfer process during the course of the cleavage reaction because the pK_a of U75 is too high. Ribozyme **B** which retains the critical C75 was able to perform the cis-cleavage reaction[14], although it was less active than the unmodified ribozyme within the pH range studied (Fig. 4, panel 3). The reduced catalytic activity of ribozyme **B** may be due to a destabilization of Watson-Crick base pairing between A46 and n^{6}U72 due to ionization of the N3.

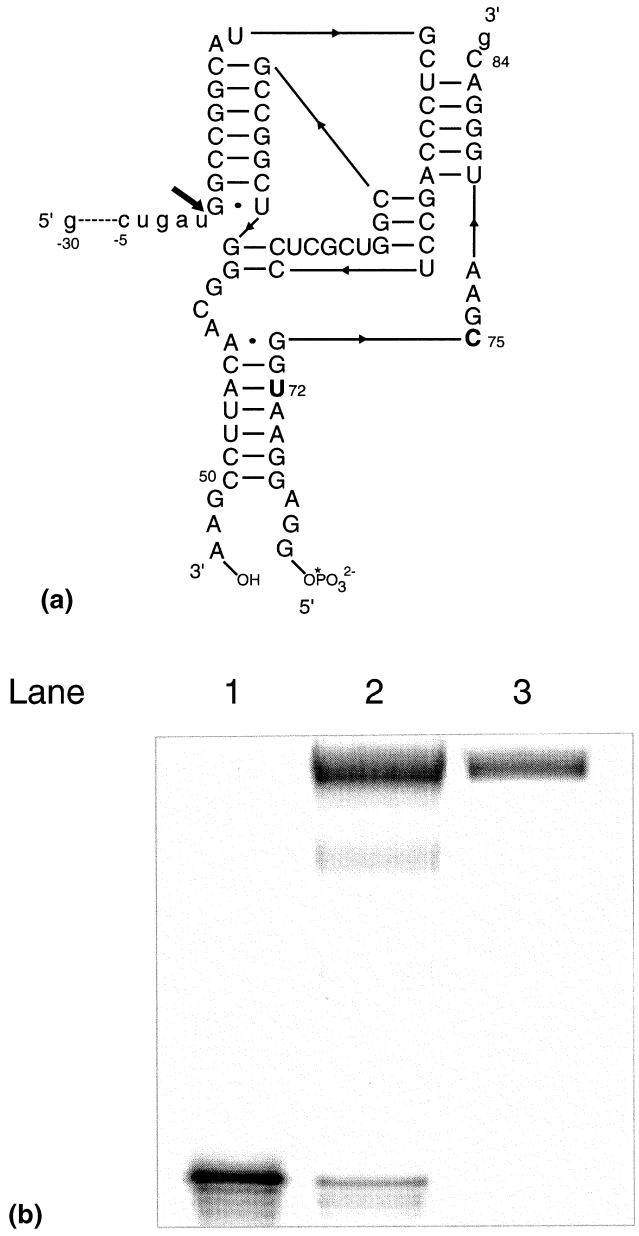

Figure 3. Site specific incorporation of 6-azauridine into the −30 to 85 form of the genomic HDV ribozyme. (a) Schematic depiction of the RNA constructs before ligation. Asterisk indicates ^{32}P label. The final products used in the cleavage experiments are single covalently linked molecules. Site of cleavage by the ligated ribozyme is indicated with an arrow. Numbering is based on the genomic HDV ribozyme wild-type sequence. (b) Autoradiograph of a denaturing polyacylamide gel electrophoresis (PAGE) of the RNA ligation reaction. Lane 1: ^{32}P 5′ end labeled synthesized oligoribonucleotide. Lane 2: ligation reaction mixture consisting of ^{32}P 5′ end labeled synthesized oligoribonucleotide and *in vitro* transcribed HDV ribozyme sequence from −30 to 53 after ligation[13]. Lane 3: 3′ end labeled full length transcribed HDV ribozyme.

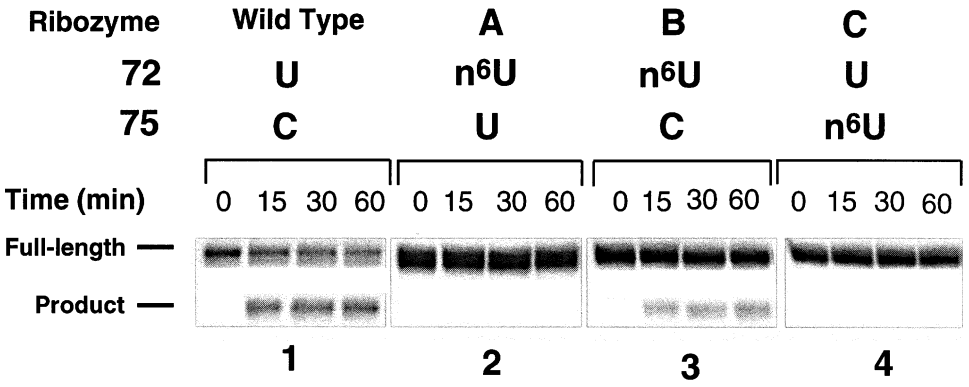

Figure 4. Autoradiograph of a denaturing PAGE of the HDV cis-cleavage reaction performed at pH 6. Ribozyme identity and nucleotide at positions 72 and 75 are specified on top of the gel. Panel numbers are indicated at the bottom of the gel. Bands corresponding to full-length ribozyme and cleaved product are specified to the left of the gel. The incubation times following reaction initiation by oligonucleotide addition are also indicated.

With the enhanced N3 acidity of n^6U, it was anticipated that ribozyme **C** would be able to support the cis-cleavage reaction to a level comparable to C75; however, it was inactive from pH 5 to 7.5 (Fig. 4, panel 4). The failure of n^6U substitution to rescue ribozyme function may be due to several possible factors: (i) Substantial ionization of n^6U may place a negative charge at the active site, which destabilizes the developing negative charge in the transition state of the reaction. (ii) The O4 carbonyl group of n^6U may be unable to participate in an important H-bonding interaction between the C22 phosphate backbone and the C75 NH2 normally present in the wild type ribozyme[2]. This may result in an incorrectly folded active site. (iii) Within the micro-environment of the ribozyme active site, the N3 pK_a of n^6U might be perturbed up by the same mechanism utilized to raise the pK_a of C75. This would render ineffective the n^6U substitution at nucleotide position 75, though this possibility seems the least likely.

CONCLUSIONS

This paper describes the use of 2′-*O*-ACE chemistry for the incorporation of 6-azauridine, a uridine analogue with a neutral N3 pK_a, into two positions within an intact self cleaving genomic HDV ribozyme. The inability of the C75n^6U mutated RNA to restore ribozyme function compared to the U75 mutant suggests that the catalytic mechanism of the genomic HDV ribozyme is more complex than simply a pK_a match at the critical C75. The correct folding of the active site, involving a direct participation of the exocyclic NH2 group of C75, appears to be essential for the creation of a micro-environment suitable for effective catalysis. Analysis of

the ribozyme reaction with cytidine analogues with altered N3 pK_as is now underway to decipher the contributions of the exocyclic amine and the N3 pK_a perturbation to catalysis.

ACKNOWLEDGMENTS

This work was supported in part by NSF grant CHE-9701787. A.K.O. is the recipient of a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund for Medical Research. We thank Dr. Stephen A. Scaringe of Dharmacon Research Inc. for generously providing us with reagents needed for 2′-*O*-ACE chemistry. We also thank Alex Szewczak, Greg Muth, Sean Ryder and Mark Parnell for helpful discussion.

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9. (a) Scaringe, S.A.; Wincott, F.E.; Caruthers, M.H. J. Am. Chem. Soc. **1998**, *120*, 11,820–11,821; (b) 2′-*O*-ACE protection groups were removed from the synthesized oligonucleotides by treatment at 60 °C with 200 mM acetic acid pH 3.0 for 10 min. An equal volume of 300 mM TRIS buffer pH 8.7 was added and the resulting mixture was incubated at 60 °C for 15 min.
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11. Representative spectroscopic data: 2′-*O*-ACE-6-azauridine **3**: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 2.05 (6H, s, CH_3 of ACE), 3.70–3.85 (8H, m, CH_2 of ACE), 4.09–4.21 (3H, m, H- 5′, 5″ and 2′), 4.38 (1H, m, H-4′), 4.70 (1H, m, H 3′), 5.44 (1H, s, CH of ACE), 6.20 (1H, d, H-1′, $J = 4.8$ Hz), 7.49 (1H, s, H5); HRMS (ES, 50% CH_3CN, 0.1% HCOOH) calcd for $[C_{17}H_{25}O_{12}N_3 + Na]^+$ 486.1336, found 486.1339. 5′-*O*-Silyl-2′-*O*-ACE-6-azauridine **4**: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.09 (18H, s, CH_3 of silyl group), 1.21–1.43 (22H, m, CH_2 of cyclododecyl group), 1.63 (1H, m, CH of cyclododecyl group), 2.05 (6H, s, CH_3 of ACE), 3.76–3.84 (8H, m, CH_2 of ACE), 4.00–4.24 (3H, m, H- 5′, 5″ and 2′), 4.35 (1H, m, H-4′), 4.69 (1H, m, H 3′), 5.44 (1H, s, CH of ACE), 6.28 (1H, d, H-1′, $J = 4.2$ Hz), 7.43 (1H, s, H5); ^{13}C-NMR ($CDCl_3$, 120 MHz) δ 170.8, 155.8, 147.8, 135.7, 112.2, 88.0, 83.8, 74.5, 70.4, 62.8,

62.7, 62.4, 60.2, 31.7, 24.1, 23.7, 23.0, 22.9, 20.5, 20.5, 1.3; HRMS (ES, 50% CH_3CN, 0.1% HCOOH) calcd for $[C_{35}H_{65}O_{15}N_3Si_3 + Na]^+$ 874.3621, found 874.3621. 5′-*O*-Silyl-2′-*O*-ACE-6-azauridine phosphoramidite **5**: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.08 (18H, m, CH_3 of silyl group), 1.13–1.45 (34H, m, CH_2 of cyclododecyl group and CH_3 of isopropyl group), 1.63 (1H, m, CH of cyclododecyl group), 2.06 (6H, 2s, CH_3 of ACE), 3.08–4.64 (18H, m, CH_2 of ACE, CH of isopropyl group, H- 5′, 5″, 4′, 3′, 2′ and CH_3 of methoxy group), 5.42 (0.5H, s, CH of ACE), 5.50 (0.5H, s, CH of ACE), 6.31 (0.5H, d, H-1′, $J = 4.8$ Hz), 6.35 (0.5H, d, H-1′, $J = 4.2$ Hz), 7.31 (1H, bs, H5); ^{13}C-NMR ($CDCl_3$, 120 MHz) δ 170.8, 170.8, 160.2, 160.1, 151.1, 151.0, 135.9, 135.8, 111.8, 111.7, 88.4, 87.9, 83.5, 83.3, 83.2, 74.5, 74.1, 74.0, 71.8, 71.4, 71.2, 70.5, 63.2, 63.2, 63.1, 63.1, 63.0, 62.4, 62.4, 61.1, 60.9, 52.7, 50.5, 50.3, 50.2, 44.4, 42.9, 42.8, 42.6, 31.7, 24.6, 24.5, 24.4, 24.0, 23.1, 23.0, 20.7, 20.5, 8.3, 7.8, 1.5; ^{31}P-NMR ($CDCl_3$, 121 MHz, H_3PO_4 external standard) δ − 34.84, − 35.45; HRMS (ES, 50% CH_3CN, 0.1% HCOOH) calcd for $[C_{42}H_{81}O_{16}N_4PSi_3 + Na]^+$ 1035.4591, found 1035.4591. Incorporation of 6-azauridine into the synthetic oligoribonucleotides used to make the ribozymes constructs was confirmed by electrospray mass spectrometry (ESMS, negative ion mode): ribozyme B oligoribonucleotide (U72n^{6}U) GGAGGAAn^{6}UGG-CAAAUGGGACG, 6934.0 (M) and 6955.0 (M + Na); ribozyme C oligoribonucleotide (C75n^{6}U) - GGAGGAAUGGn^{6}UGAAUGGGACG, 6935.0 (M) and 6956.0 (M + Na).

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14. Tris (40 mM, pH 7.5) and MES (25 mM, pHs 5.0 to 6.5) buffers containing 1 mM Mg^{2+} were used. RNAs were refolded at 50 °C for 5 min and cis-cleavage reaction was performed at 37 °C by adding a saturating concentration of DNA oligo, AS1 (TAAGAAAGGATGGAACGCGGACCCCCACAC) as described in ref. 4. AS1 activates the cis-cleavage reaction of the HDV constructs used by base pairing with an inhibitory sequence 5′ to the cleavage site[4].

Received February 7, 2001
Accepted July 2, 2001