*DEVELOPMENT OF CYTOKININ ACTIVITY BY REARRANGEMENT OF 1-SUBSTITUTED ADENINES TO 6-SUBSTITUTED AMINOPURINES: INACTIVATION BY N^6, 1-CYCLIZATION**

BY NELSON J. LEONARD, SELIM ACHMATOWICZ, RICHARD N. LOEPPKY,† KERMIT L. CARRAWAY,‡ WOLFGANG A. H. GRIMM, ALICJA SZWEYKOWSKA,§ HAMZI Q. HAMZI,** AND FOLKE SKOOG

DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING, UNIVERSITY OF ILLINOIS, URBANA, AND DEPARTMENT OF BOTANY, UNIVERSITY OF WISCONSIN, MADISON

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Since the occurrence of cytokinin[1] activity has been limited mainly to 6-substituted purine derivatives,[2-4] it was of special interest to determine the origin of the kinetin-like growth-promoting activity of the 1-substituted adenines, 1-(γ,γ-dimethylallyl)adenine (I, tautomeric forms shown), and 1-benzyladenine, which were made[5] and tested[6] in these laboratories. While consideration has been given to the possibility that the 1-substituted adenines might be active as such, our interpretation of the results previously reported has been that the development of activity was the consequence of chemical change to the N^6-isomeric or other active N^6-substituted compounds.[1, 6] Accordingly, our experiments have been directed toward determining the conditions under which 1- to N^6-isomer conversion will occur.

First, it was necessary to ascertain that we could obtain *pure* 1-(γ,γ-dimethylallyl)adenine (I), free from any of the isomeric 6-(γ,γ-dimethylallylamino)purine ("N^6-2-isopentenyladenine") (II), the highly active cytokinin[4, 7-9] which was originally obtained synthetically[5, 10] and has been found more recently to be naturally occurring.[11-14] The 1-(γ,γ-dimethylallyl)adenine was prepared by the procedure previously reported:[5] alkylation of adenosine with γ,γ-dimethylallyl bromide in dimethylformamide at room temperature followed sequentially by hydrochloric acid hydrolysis in ethanol, cautious neutralization in ethanol with potassium carbonate to pH 6.8, and isolation of the initial product by an evaporative procedure during which the solution was kept below 35°. The product was recrystallized 10–12 times from ethanol. Cellulose column chromatography of both the crystals and the mother liquor from the last recrystallizations did not show the presence of any impurity. Thus, we could conclude that the level of impurity was less than 0.1 per cent since the chromatography of an artificially prepared mixture of 500 mg of 1-(γ,γ-dimethylallyl)adenine and 0.5 mg of 6-(γ,γ-dimethylallylamino)-purine permitted easy detection of the latter by ultraviolet spectra of the eluted fractions. The pure compound I was characterized by the following properties: mp 237–238°,[5] R_f 0.55 (butanol, water, acetic acid —75:20:5); λ_{max}^{EtOH} 273 mμ (ϵ 12,300), λ_{min} 246 (3900); λ_{max} (acidic) 260 (13,400), λ_{min} 233 (3,900); λ_{max} (basic) 274 (15,200), 268 (15,000, dual peak), 300 (sh) (4,100), λ_{min} 242 (4,500).

Next, it was necessary to minimize chemical change of the 1-(γ,γ-dimethylallyl)adenine in the test for cytokinin activity. The procedures and media employed were essentially those of Murashige and Skoog[15] using tobacco callus tissue (*Nicotiniana tabacum* var. *Wis. #38*) as described earlier.[6] Solutions of *pure* 1-(γ,γ-dimethylallyl)adenine were sterilized by filtration and added to the medium

TABLE 1

CYTOKININ ACTIVITY OF 1-(γ,γ-DIMETHYLALLYL)ADENINE IN THE TOBACCO BIOASSAY (GROWTH PERIOD: JULY 15–AUGUST 19, 1965)

Treatment	Conc. (μM/l)	Yield/Callus Piece	
		Fresh wt(gm)	Dry wt(mg)
Autoclaved in the medium	0.01	0.72	42
	0.05	2.3	74
	0.1	2.9	85
Autoclaved apart from medium (at 5 μM/l)	0.01	2.4	74
	0.05	3.0	100
	0.1	2.8	88
Filter sterilized	0.01	0.08	5
	0.05	0.14	8
	0.1	0.12	7
Kinetin control (KC)	0.14	6.1	250
Basal control (BC)	0.0	0.17	14

after it had been autoclaved and allowed to cool nearly to the gelation point (50–40°). Using these precautions, the compound was inactive in concentrations of 0.01, 0.05, and 0.1 μM/l (Table 1). By contrast, cytokinin activity was shown at the same concentrations when the compound was added to the medium and then autoclaved, or when a solution of the compound (at 5 μM/l) was autoclaved apart from the medium and added in appropriate proportion (Table 1, Fig. 1). The development of activity in the autoclaving process is significant, especially since the rearrangement of 1-substituted adenines to N^6-substituted aminopurines has been observed under alkaline conditions;[16–20] whereas the growth medium was buffered at pH 5.8 and the separately autoclaved aqueous solution of 1-(γ,γ-dimethylallylamino)purine (I) contained no added base. The behavior of I was therefore tested in a buffer of pH 5.8 and in aqueous solution.

The total crude product recovered from the autoclaving of I in pH 5.8 buffer (at 5 μM/l) for 2 hr at 120° and 14–16 psi confirmed the development of cytokinin activity (Table 2). This sample was subjected to paper chromatography, and the paper section corresponding to $R_f > 0.6$, that is, greater than that of 1-(γ,γ-dimethylallylamino)purine, was cut up, extracted with small volumes of water, and the aqueous solution was incorporated into the bioassay medium. The high activity shown by this chromatographic fraction indicated that partial conversion of I had taken place and was suggestive of the N^6-substituted isomer.[5] The autoclaving was then carried out on a preparative scale (2.5 gm of I in 500 ml of 1 mM KH_2PO_4, brought to pH 5.8 with dilute hydrochloric acid, in a pressure cooker at 120° and 14–16 psi for 48 hr). The solution was evaporated in vacuum and the remaining solid was extracted thoroughly with boiling methanol. Concentration of the methanol solution afforded adenine by crystallization and recrystallization, and the combined mother liquors were chromatographed on a cellulose column, eluting with ether, ether-methanol (3:1), and finally methanol. Fractions having similar UV spectra were combined, evaporated, purified, and identified: 6-(γ,γ-dimethylallylamino)purine (II) (0.5%), starting material (I) (10%), adenine (89%, total). Identification of II was based upon the UV spectra,[14] single spot on

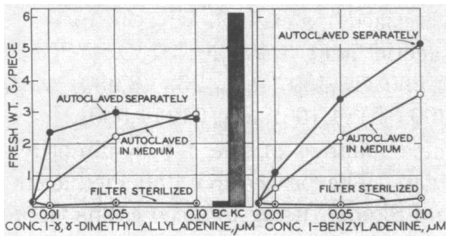
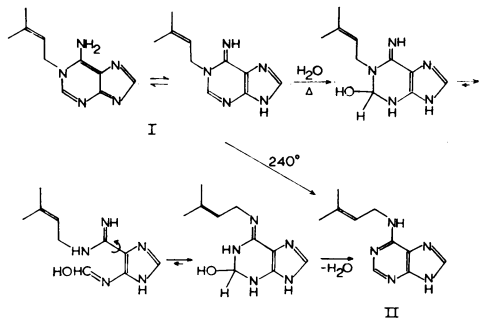


FIG. 1.

paper chromatography, R_f 0.93 (butanol, water, acetic acid −75:20:5), mp 210–212°, mixture mp 209–212°, and mass spectral comparison with an authentic sample.[5] The UV spectra and the fast movement on paper chromatography were not sufficient to differentiate readily between 6-(γ,γ-dimethylallylamino)purine (II) and the isomeric 6-(α,α-dimethylallylamino)purine (III), which might have resulted from a conversion similar to a Claisen rearrangement; however, direct comparison with a sample of the latter, mp 197°, synthesized from 6-chloro-9-(2-tetrahydropyranyl)purine and α,α-dimethylallylamine, removed this possibility.[21]

The instability of 1(γ,γ-dimethylallyl)adenine in aqueous solution alone was shown by refluxing 100 mg of I in 20 ml of water (very concentrated compared with the solution used for the biological testing). In less than 2 hr, crystals started to separate, and after 2.5 hr, 99 mg of pure 6-(γ,γ-dimethylallylamino)purine (II) was obtained, identified by mp 210–212°, mixture mp 210–212°, UV spectra: λ_{max}^{EtOH} 268 mμ, (acidic) 278, (basic) 275 with a shoulder, TLC and paper chromatography, and NMR and mass spectrometry. The mass spectra of the 6-γ,γ- and 6-α,α-isomers are especially useful for differentiation, since that of II has a base peak of m/e 160,[14] whereas that of III has a base peak at m/e 188 and shows an insignificant fragment at m/e 160. Details of the mass spectra of these and related compounds will be reported elsewhere. From this experiment and from observations during the isolation of 1-(γ,γ-dimethylallyl)adenine, it is now obvious that in order to obtain 1-substituted adenines free from contamination with the corresponding 6-substituted-aminopurines it is necessary to avoid keeping the compounds in water solution at pH above 7 and to avoid heating the aqueous solutions.

Taylor and Loeffler[18] have converted several 1-substituted-7-methyladenines to the isomeric N^6-substituted-7-methyladenines by refluxing in water for a prolonged period of time and have postulated a reasonable mechanistic sequence for the conversion (of other heterocyclics as well) involving nucleophilic attack of the hydroxide ion at the 2-position. Since we have found the conversion I → II to proceed in very dilute aqueous solution and also on the acid side, we suggest the intervention of water as the base in a similar mechanistic sequence involving reversible steps leading to the more stable aromatic system (Scheme I).



SCHEME I

The rearrangement pathway involving ring opening and reclosure has been shown to be operative in related heterocyclic series.[22, 23] The heating of 1-(γ,γ-dimethyl-

TABLE 2

CYTOKININ ACTIVITY OF CRUDE AUTOCLAVED PRODUCT OF 1-(γ,γ-DIMETHYLALLYL)ADENINE AND OF PAPER CHROMATOGRAM SECTION ($R_f > 0.6$) OF A 10-MG SAMPLE AUTOCLAVED 1-(γ,γ-DIMETHYLALLYL)ADENINE IN THE TOBACCO BIOASSAY. ALL TEST SAMPLES FILTER STERILIZED (GROWTH PERIOD: JULY 15–AUGUST 19, 1965)

Sample	Conc.	Yield/Flask Fresh wt(gm)	Dry wt(mg)
Crude autoclaved product of I	0.01 μM/l	3.5	160
	0.05 "	8.9	370
	0.1 "	14.0	450
Chromatogram paper section, $R_f > 0.6$, of autoclaved I	1/50 per flask	13.2	550
	1/5 per flask	15.3	670
Kinetin control	0.14 μM/l	18.4	750
Basal control	0.0	0.5	43

allyl)adenine at 240° under nitrogen for 1 hr also resulted in its partial conversion to 6-(γ,γ-dimethylallylamino)purine, identified by direct comparison. Under these conditions it is not certain whether the compound acts as its own base in promoting the rearrangement by ring opening and reclosure, or whether extracyclic migration of the γ,γ-dimethylallyl group occurs. Argument for a process involving hydrolysis and realkylation might be invoked to account for the conversion of I to II under the autoclaving conditions at pH 5.8. However, when a solution of adenine and either γ,γ-dimethylallyl alcohol or α,α-dimethylallyl alcohol in equal proportion in aqueous phosphate buffer brought to pH 5.8 was autoclaved for 48 hr, no 6-substituted aminopurine was detectable by either column or paper chromatography. This route cannot account, therefore, for the major portion of 6-(γ,γ-dimethylallylamino)purine produced from I by autoclaving, in contrast to the finding of Hall and de Ropp[24] that the autoclaving of adenine with furfuryl alcohol at pH 4.0 for 30 min produces kinetin in 2 per cent yield. It should also be noted that 6-(γ,γ-dimethylallylamino)purine is stable and 100 per cent recoverable after 48 hr autoclaving at pH 5.8. In agreement with the chemical evidence, bioassays of the heated adenine alcohol mixtures gave no indication of cytokinin activity in excess of the low level attributable to the adenine itself. Mixtures of adenine and benzyl alcohol likewise were inactive.

From the isomerization experiments described above, it is clear that where 1-(γ,γ-dimethylallyl)adenine has been autoclaved alone or in the biological test medium the formation of 6-(γ,γ-dimethylallylamino)purine is responsible for the cytokinin activity. Since the conversion occurs readily at 100–120°, even a brief period of warming at 40–50° or prolonged standing at 28° in the growth medium could be sufficient to induce enough 1- to N^6-isomer rearrangement for cytokinin activity to be observed. Due to the extremely high activity of 6-(γ,γ-dimethylallylamino)purine, it can be detected biologially where it cannot be observed by ultraviolet light or by spotting techniques on paper. These chemical methods failed to detect the presence of the active isomer II when a solution of I at pH 5.8 was stirred for 21 days at room temperature, followed by the usual chromatographic procedures.

Exactly parallel chemical and biological experiments were conducted with 1-benzyladenine.[5, 6] Precautions similar to those used for the synthesis and isolation of pure I were applied to obtain *pure* 1-benzyladenine, mp 244–246°, R_f 0.57 (butanol, water, acetic acid —75:20:5), λ_{max}^{EtOH} 273 mμ (ϵ 12,200), λ_{min} 246 (4,200); λ_{max} (acidic) 260 (12,400), λ_{min} 234 (3,700); λ_{max} (basic) 274 (13,600), 268 (13,500, dual

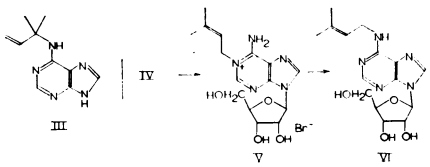
TABLE 3

CYTOKININ ACTIVITY OF 1-BENZYLADENINE IN THE TOBACCO BIOASSAY (GROWTH PERIOD: JULY 15–AUGUST 19, 1965)

Treatment	Conc. (μM/l)	Yield/Callus Piece Fresh wt(gm)	Dry wt(mg)
Autoclaved in the medium	0.01	0.62	35
	0.05	2.2	76
	0.1	3.6	110
Autoclaved apart from medium (at 5 μM/l)	0.01	1.1	59
	0.05	3.4	120
	0.1	5.1	160
Filter-sterilized	0.01	0.10	6
	0.05	0.08	5
	0.1	0.28	17
Kinetin control	0.14	6.1	250
Basal control	0.0	0.17	14

peak), 300 (sh) (3,400), λ_{min} 243 (4,300). 1-Benzyladenine thus obtained was sterilized by filtration and added to the growth medium after it had been autoclaved and allowed to cool nearly to the gelation point. In the tobacco bioassay it was found to be inactive in concentrations of 0.01, 0.05, and 0.1 μM/l (Table 3). Cytokinin activity was observed at the same concentrations when autoclaving was carried out. The compound, as with I, was more active when it was autoclaved apart from the medium than when autoclaved in the medium (Table 3, Fig. 1). The higher pH and higher concentration probably contribute to this effect. The conversion of 1-benzyladenine to the active cytokinin, 6-benzylaminopurine,[25–28] first effected under alkaline conditions,[5] has now been shown to occur on autoclaving in buffer of pH 5.8 at 120° and 14–16 psi for 48 hr, yielding the following: 6-benzylaminopurine (0.5%), starting material (19%), adenine (80%, total), and on refluxing in water, requiring slightly longer than I for a quantitative yield of rearranged product. Conversion of 1-benzyladenine to 6-benzylaminopurine was not detectable by chemical means when a solution of the former in aqueous buffer at pH 5.8 was stirred for 21 days at room temperature, but, as in the case of I→II, sufficient rearrangement could take place during any brief warming period (e.g., at 40–50° during addition to the medium just before pouring) or on prolonged standing at 28° for cytokinin activity to develop. Purely thermal rearrangement of 1-benzyladenine to 6-benzylaminopurine was realized at 255° under nitrogen.

The simplified conversion of 1-(γ,γ-dimethylallyl)adenine to 6-(γ,γ-dimethylallylamino)purine by refluxing a short time in aqueous solution[5, 18] suggested an improvement in the synthesis of 6-(γ,γ-dimethylallylamino)-9-β-D-ribofuranosylpurine (VI), which had been made earlier at the University of Illinois[21, 29] by treatment of adenosine (IV) with γ,γ-dimenthylallyl bromide to give 1-(γ,γ-dimethylallyl)-9-β-D-ribofuranosyladenine hydrobromide (V) followed by alkaline rearrange-

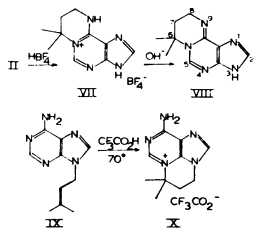


ment. Crude V (50%), obtained from the alkylation in anhydrous dimethylformamide at 20° followed by evaporation and treatment with acetone to effect partial solidification, was dissolved in water, brought to pH 7.5, and heated on the steam

bath (2.5 hr) while adding 0.1 *N* sodium hydroxide to maintain the pH at about 7.5. Extraction with ethyl acetate was followed by drying and evaporation to yield 76 per cent of rearranged product, 6-(γ,γ-dimethylallylamino)-9-β-D-ribofuranosylpurine (VI). Two recrystallizations from acetonitrile-ethanol (1:1) gave colorless prisms, mp 142–143°; λ^{H_2O} (pH 7) 269 mμ (ϵ 20,000), λ_{min} 232 (2,300); $\lambda^{H_2O}_{max}$ (0.1 *N* HCl) 265 (20,300), λ_{min} 232 (3,900); $\lambda^{H_2O}_{a}$ (0.1 *N* NaOH) 269 (19,800), λ_{min} 234 (3,200); $[\alpha]^{28}_{D}$ −103° (c 0.14, ethanol). (Anal. calcd. for $C_{15}H_{21}N_5O_4$: C, 53.72; H, 6.31; N, 20.89. Found: C, 53.48; H, 6.33; N, 20.73.) 6-(γ,γ-Dimethylallylamino)-9-β-D-ribofuranosylpurine ("N^6-2-isopentenyladenosine") (VI) assumes greater importance with its isolation from serine tRNA[11] and from yeast sRNA and calf liver sRNA.[12] Hall *et al.*[12] have also communicated its synthesis from 6-chloro-9-β-D-ribofuranosylpurine and γ,γ-dimethylallylamine. They have suggested that although VI has been isolated from sRNA, it is conceivable that in the sRNA molecule it could exist as the 1-isomer and undergo rearrangement under the conditions of enzymic hydrolysis of sRNA. This working hypothesis is equivalent to our synthetic route (IV→V→VI). The properties of the product which resulted from this rearrangement route correspond closely to those (mp, UV, $[\alpha]_D$) which Hall and his co-workers observed for VI, and we have found that VI possesses cytokinin activity,[21] although somewhat less than II in the tobacco bioassay.

In the process of reaching the conclusion that the development of cytokinin activity from 1-substituted adenines was due to their rearrangement to the N^6-isomers, we also examined the effect of additional substitution at the 1-position while N^6 was substituted with a group typically promoting activity.[21] For one variant in the 1,N^6-disubstituted adenine series we drew from our experience with the conversion of triacanthine (3-(γ,γ-dimethylallyl)adenine) hydrochloride to pyrotriacanthine chloride, a 3,9-cyclized product.[30] 6-(γ,γ-Dimethylallylamino)-purine (II) is similarly constituted for acid cyclization of the side chain to the 1-position, as observed also by Johnston, Fikes, and Montgomery[31] for the cyclization of the purin-6-yl nitrogen mustard. To a suspension of 2.40 gm (11.8 mmoles) of II in 45 ml of absolute ethanol was added 5.0 ml of 50 per cent aqueous fluoboric acid. The resulting solution was evaporated at 70° in a rotary evaporator during 10–15 min. The residue was shaken with 100 ml of ethanol, filtered, and the solid was dried, recrystallized from aqueous ethanol, and characterized as the fluoborate salt (VII) of 6,6-dimethyl-5a,6,7,8-tetrahydro-1,3,4,5a,9-pentaaza-3H-benz[*e*]-indene (VIII), yield 91 per cent, colorless needles, mp 278.5–279.5° d, NMR τ values (DMSO-d_6, TMS) 8.23 (6H, singlet, CH_3—C—CH_3), 7.62–7.84 (2H, multiplet, C—CH_2—C), 6.22–6.53 (2H, multiplet, N—CH_2), 1.59 and 1.20 (1H each, singlets, purine protons), N—H protons partially exchanged. (Anal. calcd. for $C_{10}H_{14}BF_4N_5$: C, 41.26; H, 4.85; N, 24.07. Found: C, 41.10; H, 4.78; N, 24.20.) The cyclization could also be effected by heating 6-(γ,γ-dimethylallylamino)purine in trifluoroacetic acid at 50° for 4 hr. The free base VIII was obtained by treating an aqueous solution of the fluoborate salt with Dowex-1 (HCO_3^- or OH^-). The colorless solid obtained on evaporation of this solution was recrystallized from ethanol as colorless prisms, mp 280.5–282° d (reported for a 1,N^6-compound, $C_{10}H_{13}N_5$,[12] 200–202° d, but structure not specified completely), characterizable as a 1,N^6-disubstituted adenine:[31–33] λ^{EtOH}_{max} 277.5 mμ (ϵ 14,000),

229.5 (20,200), λ_{min} 249 (3,600); λ_{max}^{EtOH} (0.1 *N* HCl) 263 (14,000), 210.5 (21,000), λ_{min} 232 (2,500); λ_{max}^{EtOH} (0.1 *N* NaOH) 280 (sh), 272.5 (18,000), λ_{min} 246 (5,500); m/e 148 ~ 90 per cent of base peak (203) in the mass spectrum. (Anal. calcd. for $C_{10}H_{13}N_5$: C, 59.09; H, 6.45; N, 34.46. Found: C, 58.83; H, 6.57; N, 34.56.) 6,6-Dimethyl-5a,6,7,8-tetrahydro-1,3,4,5a,9-pentaaza-3H-benz[*e*]indene (VIII, shown in one of the possible tautomeric forms, cf. 9H or 1H) showed no activity in the tobacco bioassay up to concentrations of 50 μM/l, in contrast to 6-(γ,γ-dimethylallylamino)purine (II) which showed a very large increase in final weights of tissue beginning at a concentration of 0.0005 μM/l.[7] N^6,1-Cyclization has thus inactivated compound II, suggesting that the 1-position must be free for cytokinin activity to be observed. In fact, a methyl group in the 1-position will practically remove activity from the active 6-benzylaminopurine.[21]



Returning to the chemical conversions, the treatment of 1-(γ,γ-dimethylallyl)-adenine (I) with fluoboric acid in ethanol as described for the N^6-isomer gave a mixture of starting material and adenine, and treatment of I with trifluoroacetic acid at 50° for 2 hr resulted in complete conversion to adenine. Whereas cleavage predominated in the case of the 1-isomer, it was possible to effect cyclization of 9-(γ,γ-dimethylallyl)adenine[30] (IX) to 8-amino-5,5-dimethyl-2a,3,4,5-tetrahydro-1,2a,5a,7-tetraazaacenaphthylenium trifluoroacetate (X) (49% yield) by treatment with trifluoroacetic acid at 70° for 10 hr, colorless fiber-like crystals from methanol-ethyl acetate, mp 209–210°, similar in ultraviolet properties to a cyclonucleoside:[34] λ_{max}^{EtOH} 275 mμ (ϵ 13,600), λ_{min} 236 (2,600); unstable in basic solution; NMR τ values (CF_3COOH, TMS) 8.03 (6H, singlet, CH_3—C—CH_3), 7.2–7.5 (2H, multiplet, C—CH_2—C), 5.1–5.3 (2H, multiplet, C—CH_2—N), 1.4–1.8 (1H, multiplet, NH), 1.00 (2H, broad singlet, purine H's), 0.5–0.8 (1H, multiplet, NH). (Anal. calcd. for $C_{12}H_{14}F_3N_5O_2$: C, 45.42; H, 4.45; N, 22.07. Found: C, 45.57; H, 4.73; N, 22.22.) Multiple cyclization of 6-geranylaminopurine[21, 35, 36] has been effected by acid,[36] and the cyclization is being extended to additional series of compounds.

Conclusion.—The development of cytokinin activity from 1-(γ,γ-dimethylallyl)-adenine and 1-benzyladenine under a variety of conditions has been shown to be due to their rearrangement to 6-(γ,γ-dimethylallylamino)purine and 6-benzylamino-purine, respectively. 6-(γ,γ-Dimethylallylamino)purine is inactivated by cyclization to the 1-position under certain acid conditions.

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