*International Journal of Pharmaceutics*, 24 (1985) 219–229
Elsevier



IJP 00820

# Degradation of mitomycin C in acidic solution

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(Received August 17th, 1984)
(Modified version received December 18th, 1984)
(Accepted January 3rd, 1985)

*Key words*: mitomycin C – degradation in acidic medium – high-performance liquid chromatography – degradation kinetics – degradation mechanism

## Summary

The degradation of mitomycin C in acidic medium has been investigated utilizing a stability indicating high-performance liquid chromatographic assay and ultraviolet spectrophotometry. Rate constants for the decomposition at 20°C are determined and a pH profile was constructed. The ratio between the two mitosene degradation products as function of pH is quantified. The degradation mechanism of mitomycin C in acidic solution is evaluated.

## Introduction

The mitomycins are a series of chemically related compounds produced by various *Streptomyces* strains (Hata et al., 1956; Wakaki et al., 1958; Herr et al., 1961). These antibiotics exhibit strong bactericidal and antitumour activity (Matsui et al., 1968). Mitomycin C (MMC) (Fig. 1) is the most prominent member of this class and has found clinical use for the palliative treatment of various malignancies (Crooke and Bradner, 1976).

Like its 1a-N-methyl derivative porfiromycin (PM) (Garrett, 1963), MMC is subject to degradative reactions in aqueous solutions. In alkali the 7-amino group is replaced by a hydroxyl function while the rest of the mitosane skeleton remains intact (Garrett and Schroeder, 1964; Beijnen et al., 1985a). The acid promoted

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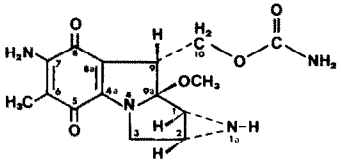
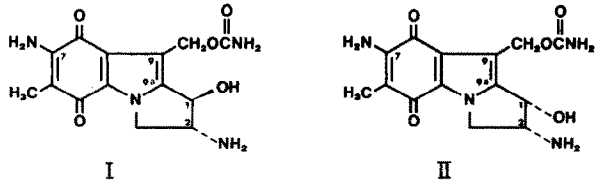


Fig. 1. Structure of mitomycin C.

degradation of mitomycins is much more complicated. Under mild acidic conditions the methoxy group is cleaved forming an unsaturated 9,9a bond and the 1,2-fused aziridine ring is opened to give two isomeric compounds with a hydroxyl function at the 1-position and an amino function at the 2-position (Taylor and Remers, 1974). The structures of the two resulting diastereomeric mitosenes (I, II) are illustrated in Fig. 2. The position of the 2-amino function is identical in I and II while the hydroxyl function possesses a *trans* position in I and a *cis* position in II (Taylor and Remers, 1974). In several laboratories (Underberg and Lingeman, 1983a and b; Taylor and Remers, 1975; Stevens et al., 1965; Cheng and Remers, 1977; Chiu and Kohn, 1983) studies have been undertaken to gain information on this decomposition process but the mechanism has not been unambiguously elucidated yet. A striking result in these studies was the prevalence of *cis* stereochemistry of the resulting 1,2-di-substituted mitosenes obtained after acidic hydrolysis. The *cis / trans* ratio was found to be in the order of about 4 : 1 (Cheng and Remers, 1977). This observation is opposite to what is known about ring opening of simple aziridines, such as cyclohexenimine (Paris and Fanta, 1952). Opening of the three-membered aziridine ring of these compounds almost exclusively yields the *trans* aminoalcohol derivative (Dermer and Ham, 1969). Another remarkable difference in comparison with simple aziridines is that the 1,2-fused aziridine ring of MMC opens much faster (Underberg and Lingeman, 1983a; Poochikian and Cradock, 1981). Underberg and Lingeman (1983a) proposed a mechanism for the initial degradation step of MMC on the basis of kinetic data. These investigators utilized ultraviolet spectrophotometry to study the degradation rate of MMC at 40°C. However, the spectrophotometric assay is relatively non-specific and provides neither qualitative nor quantitative information on the products formed during the degradation. This knowledge is essential to get a more clear insight into the mechanism underlying the degradation process of MMC in acidic medium. Therefore we developed a stability indicating high-performance liquid chromatographic (HPLC) assay in which MMC and its degradation products are separated and quantified simultaneously.



I II

Fig. 2. Structures of (I) *trans* and (II) *cis* 2,7-diamino 1-hydroxymitosene.

Kinetic data on the proton catalyzed degradation at 20°C and the ratio in which the isomeric mitosenes are formed, are presented in this paper. These data so far are not available in the literature.

## Experimental

*Materials*

MMC was kindly provided by Bristol Myers, (Weesp, The Netherlands). All other chemicals employed were of analytical grade and deionized water was used throughout.

*Buffer solutions*

In the pH region 3–6 0.001 M acetic acid–acetate-buffered solutions were used and at pH < 3 the solutions were acidified with aqueous perchloric acid. The pH values between pH 1 and 6 were measured at the temperature of study (20°C) using a glass-reference electrode and pH meter (Metrohm, Herisau E516 Titriskop). Extension of the $H_0$/pH scale below 1 was accomplished with the Hammett acidity function (Bates, 1973).

*Kinetic measurements*

Kinetic studies were carried out at 20 ± 0.2°C. The degradation was initiated by adding 3 μl of a stock solution of MMC in methanol to 3 ml buffered solution to give a final concentration of about $3 \times 10^{-5}$ M. These solutions had been equilibrated previously at 20°C and are stored in the dark to prevent possible photolytic degradation. At appropriate intervals, aliquots were withdrawn and analyzed for undegraded MMC as well as its decomposition products using the HPLC assay. The degradation at $H_0$ values < 0 was followed spectrophotometrically by monitoring the absorbance at 363 nm continuously, as described earlier (Underberg and Lingeman, 1983a). The degradation of these samples was too fast to be monitored by means of the HPLC assay.

*Apparatus and analytical procedures*

A Waters Assoc. model M-45 liquid chromatograph was used, equipped with a Model 440 UV detector (Waters Assoc., Milford, MA, U.S.A.) operating at 254 nm. The sensitivity was adjusted to 0.01 A.U.F.S. The column (30 cm × 3.9 mm i.d.) was self-packed with Lichrosorb RP 18 (10 μm) material (Merck, Darmstadt, F.R.G.). The mobile phase consisted of methanol–water (40 + 63, w/w) to which 0.5% (v/w) glacial acetic acid was added. The apparent pH (pH*) of this mobile phase was adjusted to 4.3 with sodium hydroxide. The chromatographic system was operated at ambient temperature and the flow rate was 1.0 ml · $min^{-1}$. Samples of 20 μl were injected into the chromatograph. Quantification of undegraded MMC and the degradation products I and II was based on integration of peak areas using a Spectra Physics (SP 4000) integrator. Standard solutions of MMC were chromato-

graphed and calibration curves were constructed on the basis of peak area measurements showing linear responses in the working concentration range of $3 \times 10^{-5}$ M to $2 \times 10^{-6}$ M.

As the molar extinction coefficients of I and II at 254 nm can be expected to be equal, the quotient of the peak areas of I and II yields the molar ratio of these compounds.

The spectrophotometric degradation analyses were carried out on a Shimadzu UV-140 Double Beam absorption spectrophotometer operating at 363 nm as described earlier (Underberg and Lingeman, 1983a). Ultraviolet-visible (UV-VIS) spectra were recorded on a Shimadzu UV-200 Double-Beam spectrophotometer.

Thin-layer chromatographic separation of the degradation products I and II was accomplished on silicagel plates (60 F254, Merck, thickness 0.25 mm) and with methanol as developing solvent in a saturated chamber.

The Field Desorption (FD) mass spectra were obtained with a Varian MAT 711 double-focussing mass spectrometer with a combined EI/FI/FD ion source and coupled to a spectra system MAT 100 data acquisition unit. 10 $\mu$m tungsten wire FD emitters containing carbon microneedles with an average length of 30 $\mu$m were used. The samples were dissolved in methanol and then loaded onto the emitters with the dipping technique. An emitter current of 12–20 mA was used to desorb the samples. The ion source temperature was generally 70°C.

Chemical ionization (CI) mass spectra were obtained using a Finnigan 3200 quadrupole mass spectrometer combined with a Finnigan 6000 data system. Isobutane was used as ionizing gas. The emission current was 0.20 mA, the multiplier voltage was 1800 V.

*Isolation of the degradation products I and II*

About 1 mg MMC was dissolved in 1.5 ml of a perchloric solution pH 2 and this solution was allowed to stand for 1 h at room temperature. During this period MMC is quantitatively converted into the products I and II. Next, solid sodium carbonate was added in order to increase the pH of the decomposition mixture to 9. This aqueous solution was evaporated to dryness under nitrogen. The brown-coloured residue was dissolved in methanol and applied as a narrow band on a thin-layer plate. After the development the bands corresponding to II ($R_f = 0.3$) and I ($R_f = 0.4$) were scraped off the plate and eluted with methanol. The purple-coloured methanolic solutions were used for mass spectrometry (MS), UV-VIS spectrophotometry and HPLC.

*Derivatization*

Derivatization of I and II was performed with a propionic anhydride–pyridine mixture (5 + 1, v/v) at ambient temperature for 1 h. The solvents were evaporated under nitrogen afterwards and the residue was purified by thin-layer chromatography on silicagel (DC-Fertigplatten Kieselgel 60, Merck, thickness 0.25 mm; eluent: chloroform–methanol 90 + 10, v/v). The resulting derivatives were used for isobutane CI-MS.

## Results and Discussion

*Degradation products*

The structure elucidation of the two mitosenes I and II is described extensively in the literature (Taylor and Remers, 1974, 1975; Stevens et al., 1965). In this study the distinction between the two isomeric substances I and II was accomplished by comparison of elution volumes and Rf values with those of authentic I and II which were prepared by the procedure of Stevens and coworkers (1965) and separated on silicagel (Taylor and Remers, 1974). Furthermore I and II were identified in this study as follows. I and II show identical UV spectra with $\lambda_{max}$ 309 nm and $\lambda_{min}$ 275 nm characteristic for the 7-amino mitosene skeleton. FD-MS for both compounds showed m/e values 321 ($MH^+$), 320 ($M^+$) and 277 ($M^+$-43). The last value may be due to fragmentation and loss of isocyanic acid from the carbamate side chain. CI-MS of I and II was only possible after derivatization. Propionation, like acetylation (Andrews et al., 1983) yields products in which propionyl groups are introduced at the oxygen atom attached to C-1 and the nitrogen atom at C-2. In the CI mass spectrum of propionated I and II no molecular ions were present. Only the fragments M-60 and M-117 could be observed.

*Chromatography*

The separation of MMC and its analogues, degradation products and metabolites can be obtained in several chromatographic ways (Aszalos, 1984). The reversed phase HPLC appears to be suitable for this task. Fig. 3 represents a HPLC chromatogram of partly degraded MMC. The mitosenes (I and II), having a primary amino group which is expected to be protonated at the pH of the eluting solvent, most likely show retention due to the existence of ion-pairs with acetate ions. Using a propionate buffer in the mobile phase instead of an acetate buffer, the capacity factor of MMC does not alter while the capacity factors of I and II increase.

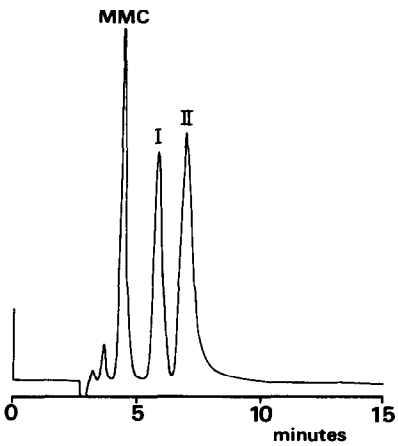



Fig. 3. HPLC chromatogram of a decomposition mixture of MMC in 0.001 M acetate buffer solution (pH 4.76). Detection 254 nm.

*Kinetics*

*Order of reaction*

The disappearance of MMC at each pH follows pseudo-first-order kinetics with respect to its concentration over more than three half-lives. The observed pseudo-first-order rate constants ($k_{obs}$) for the overall degradation was calculated by linear regression analysis of a plot of the natural logarithm of the residual concentration of MMC ($[MMC]_t$) versus time as depicted by Eqn. 1

$$\ln[MMC]_t = \ln[MMC]_0 - k_{obs} \cdot t \quad (1)$$

where $[MMC]_0$ is the initial MMC concentration.

The appearance of the degradation products also can be described by first-order kinetics. The rate constants of the formation of the individual mitosenes were determined by plotting the natural logarithm of the difference between the peak area ($PA_\infty$) after complete degradation of MMC, and the peak area ($PA_t$) at time t of these compounds versus time as illustrated by Eqn. 2

$$\ln(PA_\infty - PA_t) = \ln(PA_\infty) - k \cdot t \quad (2)$$

This graphical treatment yields straight lines ($r > 0.99$) with slopes $-k$.

*Influence of pH*

Buffer components such as phosphate and acetate ions have catalytic effects on the initial degradation step of MMC in acidic medium (Underberg and Lingeman, 1983a). Therefore in the presence of buffer species the expression for $k_{obs}$ should be:

$$k_{obs} = k_0^{MMC} + k_H^{MMC}[H^+] + k_{buffer}^{MMC}[buffer] \quad (3)$$

Eqn. 3 contains the terms $k_0^{MMC}$ which is the first-order rate constant for degradation in water only and $k_H^{MMC}$ the second-order rate constant for the proton-catalyzed degradation of MMC. Theoretically Eqn. 3 should also contain a term $k_{OH}^{MMC}[OH^-]$ for the hydroxyl ion-catalyzed degradation, but the contribution of this term to $k_{obs}$ (Eqn. 3) can be neglected because of the low hydroxyl concentration in the pH region under investigation. The term $k_{buffer}^{MMC}[buffer]$ represents the sum of second-order rate constants for the degradation catalyzed by each of the buffer components multiplied with its concentration. Taking into account the reported values $k_{Ac}^{MMC}$ and $k_{HAc}^{MMC}$ (respectively $8.7 \times 10^{-4}$ $mol^{-1} \cdot s^{-1}$ and 0 (Underberg and Lingeman, 1983a)), the contribution of $k_{buffer}^{MMC}[buffer]$ to $k_{obs}$ is negligible when MMC degrades in 0.001 M acetic acid–acetate solution because of the low [buffer]. Our kinetic experiments at $pH > 3$ were performed in 0.001 M acetate buffer solutions as under these conditions MMC only degrades to I and II. Degradation of MMC in acetate buffer solutions of higher molarity also yields, apart from I and II, acetylated mitosenes (Beijnen and Underberg, 1985). A pH profile was constructed, showing the influence of acidity on the MMC degradation (Fig. 4). The sigmoid inflection point at pH 1.5

is related to the $pK_a$ value of MMC (2.6) (Beijnen et al., 1985b). The straight line segments at $H_0 < 0.0$ and $pH > 3.0$ have a slope equal to unity indicating specific proton catalysis for the degradation of the protonated ($MMCH^+$) as well as the neutral MMC species (MMC). The expressions of the observed rate constants in various pH regions related with the pKa value of MMC (2.6) are:

$$H_0 < 0.6 \quad k'_{obs} = k_0^{MMCH^+} + k_H^{MMCH^+}[H^+] \tag{4}$$

$$0.6 < H_0/pH < 4.6 \quad k''_{obs} = \left(k_0^{MMCH^+} + k_H^{MMCH^+}[H^+]\right) \times \frac{[MMCH^+]}{[MMC]_t} + \left(k_0^{MMC} + k_H^{MMC}[H^+]\right) \times \frac{[MMC]}{[MMC]_t} \tag{5}$$

$$pH > 4.6 \quad k'''_{obs} = k_0^{MMC} + k_H^{MMC}[H^+] \tag{6}$$

The total MMC concentration $[MMC]_t = [MMC] + [MMCH^+]$. Plots of $k'_{obs}$ respectively $k'''_{obs}$ versus $[H^+]$ provide the rate constants $k_0^{MMCH^+}$ and $k_0^{MMC}$ as the intercepts and $k_H^{MMCH^+}$, $k_H^{MMC}$ as the slopes of the straight lines obtained. These rate constants are listed in Table 1.

The $k_{obs}$ values for the formation of I and II appear to be equal and, furthermore, do not significantly differ from the rate constant for the disappearance of MMC.

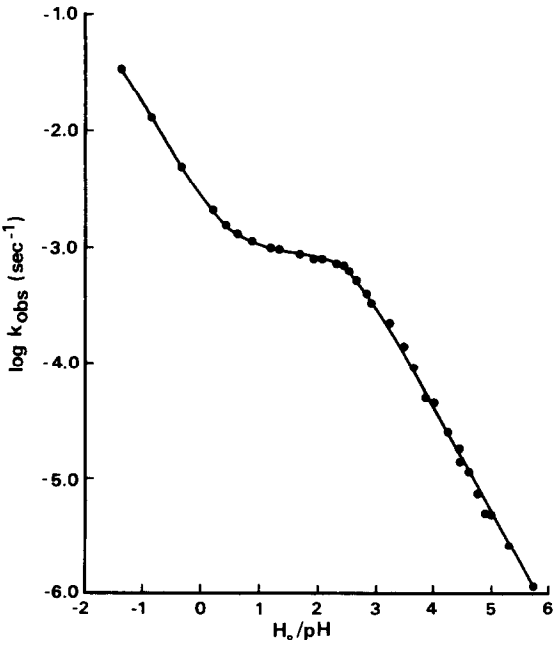



Fig. 4. $H_0/pH$ rate constant profile for MMC degradation at 20°C.

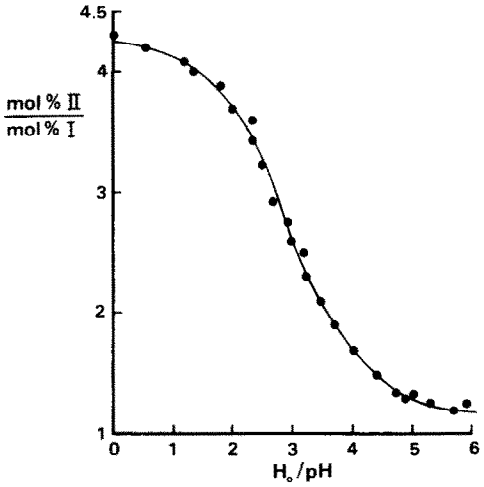



Fig. 5. pH dependence of the ratio between the mole fractions of II and I.

The $k_{obs}$ values for the formation of both mitosenes show the same pH dependence as $k_{obs}$ for the degradation of MMC. Examples are listed in Table 2. The ratio of I and II, formed during the MMC degradation varies as a function of pH, illustrated in Fig. 5. During the degradation process at a given pH no alteration of this ratio could be observed.

TABLE 1

RATE CONSTANTS FOR CATALYZED DEGRADATION REACTIONS OF MMC AT 20°C

| Rate constant | |
|---|---|
| $k_0^{MMCH^+}$ | $2\times10^{-3}\ s^{-1}$ |
| $k_H^{MMCH^+}$ | $1.3\times10^{-3}\ mol^{-1}\cdot s^{-1}$ |
| $k_0^{MMC}$ | $\sim 3\times10^{-6}\ s^{-1}$ |
| $k_H^{MMC}$ | $0.397\ mol^{-1}\cdot s^{-1}$ |

TABLE 2

OBSERVED RATE CONSTANTS ($k_{obs}$) FOR THE DEGRADATION OF MMC AND FOR THE FORMATION OF I AND II AT DIFFERENT ACIDITIES AT 20°C

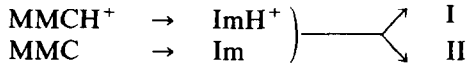
| $H_0$/pH | $k_{obs}$ ($s^{-1}$) | | |
|---|---|---|---|
| | MMC | I | II |
| 0.87 | $1.1\times10^{-3}$ | $1.3\times10^{-3}$ | $1.2\times10^{-3}$ |
| 1.95 | $7.9\times10^{-4}$ | $7.9\times10^{-4}$ | $7.9\times10^{-4}$ |
| 2.86 | $4.0\times10^{-4}$ | $3.8\times10^{-4}$ | $3.9\times10^{-4}$ |
| 3.84 | $4.8\times10^{-5}$ | $4.4\times10^{-5}$ | $4.8\times10^{-5}$ |
| 4.91 | $5.0\times10^{-6}$ | $6.0\times10^{-6}$ | $5.2\times10^{-6}$ |

*Degradation mechanism*

An overall scheme for the degradation of PM (Garrett, 1963) and proposals for the mechanism for the degradation of MMC in acidic media have been published (Underberg and Lingeman, 1983a; Stevens et al., 1965; Taylor and Remers, 1975). The quantification of the degradation products, as presented in this paper, provides a more clear insight to the mechanism.

As proposed by Underberg and Lingeman (1983a), the very fast opening of the fused aziridine ring system may be triggered by the cleavage of the 9a-methoxy group. The destabilizing effect of the 9a-methoxy function also appears from observations that the aziridine ring system is more stable in the absence of a 9a-methoxy group, since Kinoshita and coworkers (1971) found that 9a-demethoxy PM, a product arising from a reduction–reoxidation experiment with PM, was stable in acidic media. In comparison to MMC 7-aminomitosane-9a-sulfonate was found to be much more stable to acid (Hornemann et al., 1976). Preliminary results of our observations concerning the stability of mitomycin B (MMB) in acid media showed that this compound is more stable than MMC or PM (Beijnen et al., 1985). MMB possesses a hydroxyl group at the 9a-position. Cleavage of the 9a-function most likely starts with protonation of the oxygen atom of this group and this may be more facile in the case of a methoxyl substituent than a hydroxyl function because of the electron donating effect of a methyl substituent. The occurrence of proton catalysis in the degradation process is demonstrated by the pH–rate profile.

The prevalence of *cis* stereochemistry of the ring opened mitosenes was shown by several investigators. In most cases this phenomenon was observed when the degradation took place in 0.05 N HCl (Taylor and Remers, 1974, 1975; Cheng and Remers, 1977; Stevens et al., 1965), whereas no pH variations were studied. This study shows that the pH is a very important parameter influencing the stereochemistry of the resulting mitosenes. The kinetic data presented in this paper (Table 2) rule out a parallel reaction concerning the formation of I and II from MMC. An intermediate (Im) seems acceptable and the conversion of MMC into Im as the rate-limiting step. The reaction sequence for MMC and its protonated form ($MMCH^+$) is depicted in Scheme 1.




Scheme 1

The mechanism of the conversion of MMC in acidic media was published in detail earlier (Underberg and Lingeman, 1983a). Im, or its protonated form ($ImH^+$) arises from cleavage of the 9a-methoxy group and subsequent aziridine ring opening. In accordance to the degradation scheme (Underberg and Lingeman, 1985a) Im may have the structure of Fig. 6 (Chiu and Kohn, 1983). The C-1 carbonium ion is stabilized by the 9,9a-bond. The presence of an electrophilic centre at C-1, which is the target for incoming nucleophiles in the acid as well as reductively activated mechanism of action of MMC, is made plausible by a number of investigators (Underberg and Lingeman, 1983a; Tomasz and Lipman, 1979; Bean and Kohn,

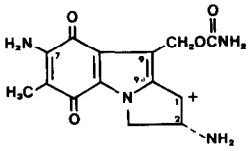


Fig. 6. Proposed intermediate (Im) in the acid-catalyzed degradation of MMC.

1983; Szybalski and Iyer, 1964; Chiu and Kohn, 1983; Hornemann et al., 1983). The intermediate (Fig. 6) may possess the key to the stereoselectivity of the mitosene degradation products which in turn is connected to the pH of the solution in which MMC degrades. The 9a-methoxy group in MMC appears to have no influence on the ratio in which I and II are formed (Cheng and Remers, 1977). The $pK_a$ value of the 2-amino group of I and II was determined to be in the order of 7 (Garrett, 1963). Comparing the 2-amino group of Im, its $pK_a$ value may be expected to be several units lower because of the adjacent carbonium ion. The presence of a protonated amino group in ethylenediamine lowers the second $pK_a$ value with 3 units ($pK_{a_1}$ = 10.1; $pK_{a_2}$ = 7.0) (Perrin, 1965). The inflection point of the sigmoid curve in Fig. 5 at pH 2.8 could agree with the $pK_a$ value of the 2-amino group in Im. At pH values < 1.8 the 2-amino group is almost quantitatively protonated and will direct an incoming nucleophile, such as a water molecule, by electrostatic attraction so that *cis* stereochemistry predominates. Whenever the 2-amino function of Im is not charged (presumably at pH > 4.8), the forcing electrostatic power of this group is absent and incoming nucleophiles may approach the carbonium ion C-1 at both sides. The fact that the ratio between I and II does not reach complete unity may be due to steric effects caused, for instance, by the 9-urethane side-chain which possibly influences the direction of incoming nucleophiles to a certain extend.

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