Table V—Comparison between the Observed and the Calculated Fraction of Disopyramide Derivatives Bound to Plasma Protein in Table III

Compound[a]	Fraction of Drug Bound, %[b]		
	Observed	Calculated	
		Eq. 11	Eq. 12
VIII	8.23	5.46	50.92
IX	10.48	18.59	46.31
X	30.03	36.41	17.35
XI	45.03	43.91	16.61
XII	87.85	83.12	51.20
XIII	46.35	44.17	39.15

[a] SC-13127, SC-13173, SC-13209, SC-13733, SC-13489, and SC-13486, respectively. [b] Fraction of drug bound (%) = $[D_b/(D_b + D_f)] \times 100$.

plasma binding was dependent, in a different degree, on the magnitude of pKb and lipophilicity as demonstrated by the difference in their slopes (−0.448 and −0.726, respectively).

If the influence of lipophilicity, log (p.c.), is neglected and only the pKb values are related to the extent of plasma binding for these two subgroups of disopyramide derivatives, the importance of pKb can be clearly shown. The linear relationship (Fig. 1) is defined by the following expression:

$$\log (D_b/D_f) + \log (K_b + [H^+]) = 6.528 - 0.621(\pm 0.090)\text{pKb} \quad \text{(Eq. 12)}$$

n	R	s^2
13	0.901	0.279

Because of the neglect of the lipophilicity term in Eq. 12, the data points from Table III were noticeably displaced from the slope (Fig. 1). The deviation of the calculated from the observed values was significantly greater for the chemicals in Table III (comparing column 2 with column 4 in Table V) than for the chemicals in Table II (comparing column 2 with column 4 in Table IV). The results demonstrate that the series of disopyramide derivatives in Table II will be better expressed by Eq. 10 and that the extent of plasma binding is linearly correlated with the magnitude of pKb with a slope of −0.448. On the other hand, the extent of plasma binding for the disopyramide derivatives in Table III is a linear function of their lipophilicity and inversely proportional to the magnitudes of pKb. This relationship is best defined by Eq. 11.

In conclusion, the extent of interaction between plasma protein and disopyramide derivatives is significantly influenced by the magnitude of the pKb of the diisopropylaminoethyl group.

REFERENCES

(1) Y. W. Chien, H. J. Lambert, and T. K. Lin, *J. Pharm. Sci.*, **64**, 961(1975).

(2) L. Z. Benet and J. E. Goyan, *ibid.*, **56**, 665(1967).

(3) T. V. Parke and W. W. Davis, *Anal. Chem.*, **26**, 642(1954).

(4) T. Fujita, *J. Med. Chem.*, **15**, 1049(1972).

(5) J. T. Edsall and J. Wyman, "Biophysical Chemistry," vol. 1, Academic, New York, N.Y., 1958, p. 610.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 6, 1974, from the *Biopharmaceutics Section, Development Department, Searle Laboratories, Division of G. D. Searle & Company, Skokie, IL 60076*

Accepted for publication February 11, 1975.

Appreciation is expressed to Miss D. M. Jefferson for technical assistance and to Miss Susan Justi for manuscript preparation.

* Present address: Alcon Laboratories, Fort Worth, Tex.

x To whom inquiries should be directed.

Preparation, Isolation, and Identification of 4-Dedimethylamino-11-methoxyanhydrotetracycline

L. J. STOEL *, E. C. NEWMAN ‡, G. L. ASLESON, and C. W. FRANK x

Abstract □ The reaction of diazomethane with 4-dedimethylaminoanhydrotetracycline produced a number of mono-, di-, and trimethylated products. The isolation of one monomethylated product (4-dedimethylamino-11-methoxyanhydrotetracycline) and its subsequent identification utilizing mass spectral and NMR data are described.

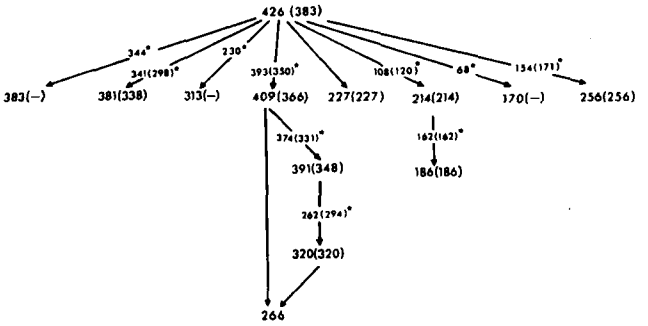
Keyphrases □ 4-Dedimethylamino-11-methoxyanhydrotetracycline—isolation and identification after diazomethane methylation of 4-dedimethylaminoanhydrotetracycline □ Methylation of 4-dedimethylaminoanhydrotetracycline with diazomethane—isolation and identification of one reaction product □ Mass spectrometry—identification, 4-dedimethylamino-11-methoxyanhydrotetracycline

During investigations of metal-ion complexation with tetracyclines, it was necessary to prepare, isolate, and identify tetracyclines with blocked or removed functional groups. One method employed was the preparation of methyl ethers of the acidic hydroxy proton sites of anhydrotetracycline by reaction with diazomethane. While preparation of the methyl ethers by this method was relatively simple, the difficulty remained in separating and identifying the reaction products. This paper describes the isolation and structural identification of one product of the methylation reaction.

EXPERIMENTAL

NMR spectra were obtained using high-resolution NMR spectrometers[1]. The chemical shift data were measured relative to tetramethylsilane as an internal standard and reported as parts per million. All NMR spectra were recorded using d_6-dimethyl sulfoxide as the solvent. The mass spectra were obtained on a medium

[1] Varian A-60 and Varian HA-100.



*Scheme I—Mass spectral fragmentation pathways of anhydrotetracycline. The fragmentation pathway of I is indicated in parentheses. * = metastable; (–) = not observed in I.*

resolution mass spectrometer[2] utilizing the direct inlet at approximately 250°. An ionization energy of 70 ev was used for all spectra reported.

Significant methylation of the C-4 dimethylamine group was reported for the reaction of diazomethane with tetracycline (1). Therefore, this functional group was first removed from the starting material (tetracycline hydrochloride[3]).

Preparation of 4-Dedimethylaminotetracycline—The synthesis of 4-dedimethylaminotetracycline from tetracycline with tetracycline methiodide as an intermediate product, as reported by McCormick *et al.* (2), was used for this preparation. TLC on silica gel H, using acetone–ethyl acetate–water (20:10:3) as the eluting solution revealed a long streaking spot of R_f 0.33. This spot turned pink when exposed to air for about 15–20 min. Nonaqueous titration of the sample in dimethylformamide resulted in an equivalent weight of 411 ± 15 (calc. 401). Mass spectral analysis revealed a parent ion at *m/e* 401.

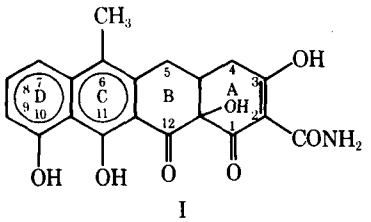
Preparation of 4-Dedimethylaminoanhydrotetracycline—4-Dedimethylaminotetracycline (0.5096 g) was dissolved in 150 ml of a 50% chloroform–isopropanol mixture to which was added 25 ml of concentrated hydrochloric acid. The resulting solution was refluxed for 2 hr and filtered. The volume of the solution was decreased by vacuum, and the remainder was cooled to 0°. The resulting 4-dedimethylaminoanhydrotetracycline (3, 4) was filtered, washed with distilled water, and dried at 40° for 12 hr in a vacuum oven.

The product (0.4314 g), mp 235° dec, had a mass spectral parent ion at *m/e* 383. Nonaqueous titration in dimethylformamide resulted in an equivalent weight of 382 ± 3 (calc. 383).

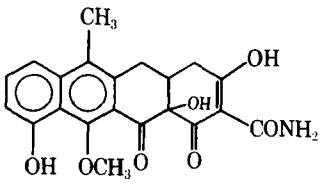
Anal.—Calc. for $C_{20}H_{17}NO_7$: C, 62.66; H, 4.47; N, 3.65. Found: C, 63.06; H, 4.43; N, 3.65.

Preparation and Separation of Methyl Ethers—An ethereal solution of diazomethane was prepared, using *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide as the precursor, by the methods outlined by the manufacturer[4]. A solution of 4-dedimethylaminoanhydrotetracycline dissolved in a minimum amount of chloroform was treated with the ethereal diazomethane solution. After standing at room temperature for 2 hr, the sample was evaporated to dryness under vacuum and stored under nitrogen.

The methylated mixture was redissolved in chloroform and separated on preparative silica gel H TLC plates. Acetone–ethyl acetate–water (20:10:3) was used as the eluting solvent. Bands were observed at R_f values of 0.47, 0.54, 0.64, and 0.81. The band at R_f 0.54 was extracted into chloroform and subsequently purified by



I



II

separation on 10% $CaCl_2$-treated silica gel preparative TLC plates using the previously mentioned eluting solution. The isolated band was extracted from the silica gel by treatment with a heterogeneous mixture of 0.1 *N* HCl and chloroform. The chloroform layer was dried with anhydrous sodium sulfate and evaporated to dryness under vacuum. The sample was dried at 60° in a vacuum oven, mp 210° dec.

The band at R_f 0.47 was identified as 4-dedimethylaminoanhydrotetracycline by mass spectrometry and NMR spectroscopy. Isolation and examination of the other TLC bands indicated that di-, tri-, and, possibly, tetramethylated products are present.

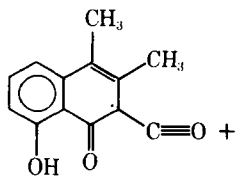
DISCUSSION

The structure of the methylated product of 4-dedimethylaminoanhydrotetracycline (R_f 0.54) was determined from the mass spectral and NMR data. The 70-ev mass spectral fragmentation patterns of some tetracyclines were reported, and several common fragments were assigned in these spectra (5). As in steroid systems (6), the dimethylamino group was observed to have a major effect on the fragmentation pattern of tetracyclines. In cases where the dimethylamino group had been removed, the carbonyl groups exhibited a dominating influence on the fragmentation pathways. In all cases, the initial fragmentation involved the loss of water to form a corresponding anhydro analog. The subsequent fragmentation patterns were reported to be the same as those of the corresponding 5a,6-anhydrotetracycline (5).

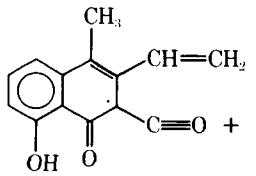
A fragmentation pathway involving the loss of NH_3 (*m/e* 17) and H_2O (*m/e* 18) and the subsequent single-step loss of CH_3NCH_2 and CO (combined *m/e* 71) was observed in the mass spectrum of anhydrotetracycline (5). This fragmentation sequence was confirmed by metastable peaks (*m/e* 393, 374, and 262, respectively) and is outlined in Scheme I. A similar fragmentation pattern was observed for 4-dedimethylaminoanhydrotetracycline (I).

A fragment corresponding to the loss of $CONH_3$ (*m/e* 45) from the parent ion was observed at *m/e* 381 in the mass spectrum of anhydrotetracycline and at *m/e* 338 in the mass spectrum of 4-dedimethylaminoanhydrotetracycline. Metastable peaks at *m/e* 341 and 298, respectively, confirmed these fragmentations. These resulting fragments retained the tetracyclic structure of the parent compound.

Somewhat similar fragmentation patterns were observed in the mass spectrum of the methylated product of 4-dedimethylaminoanhydrotetracycline; however, possibly as a result of methyl substitution, ring cleavage was the favored fragmentation pathway for this compound. The mass spectrum of the methylated 4-dedimethylaminoanhydrotetracycline (Fig. 1) contained a parent ion peak at *m/e* 397. The loss of NH_3 (*m/e* 17) from the parent ion resulted in a peak at *m/e* 380 and was confirmed by an observed metastable peak at *m/e* 364. Peaks at *m/e* 362 and 334 indicated the subsequent loss of H_2O and CO, respectively, as was previously observed



III: *m/e* 214



IV: *m/e* 227

[2] Hitachi Perkin-Elmer RMU 6E.
[3] Pfizer, Inc.
[4] Aldrich Chemical Co.

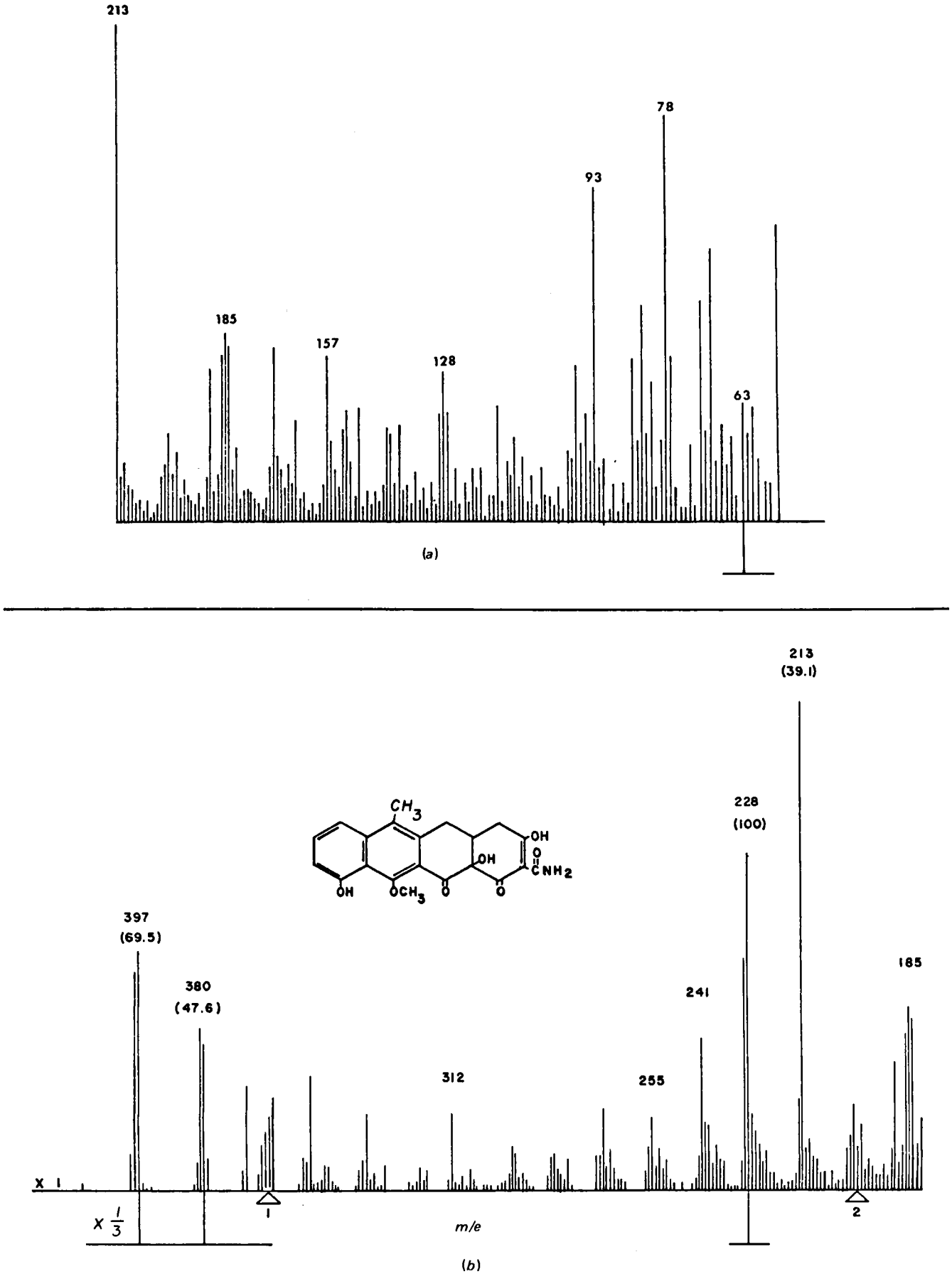
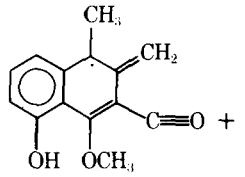


Figure 1—*Mass spectrum of 4-dedimethylamino-11-methoxyanhydrotetracycline* (a) *below* m/e *185 and* (b) *above* m/e *185. The* Δ_1 *and* Δ_2 *designate metastable ions at* m/e *341 and 199, respectively.*



V: *m/e* 228

for 4-dedimethylaminoanhydrotetracycline. A peak at *m/e* 352 was also observed for the loss of $CONH_3$ from the parent ion. The presence of these peaks indicated that only one methyl ether group was present in the methylated product and was retained on these fragments.

Because the C and D rings are aromatic, most ring cleavages of anhydrotetracyclines were reported to occur in the A or B rings. Peaks observed at *m/e* 313, 270, and 256 in the mass spectrum of anhydrotetracycline were reported to be a result of A ring fragmentation (5). Fragmentation of the A ring of 4-dedimethylaminoanhydrotetracycline was observed at *m/e* 298, 270, 266, 256, and 241. Corresponding peaks were observed at *m/e* 312, 284, 279, 269, and 255 in the mass spectrum of the monomethyl ether of 4-dedimethylaminoanhydrotetracycline (II) and indicated that the methyl ether was not on the A ring of this tetracycline.

Cleavage of the B ring was observed by fragments at *m/e* 227 and 214 in the mass spectra of both anhydrotetracycline and 4-dedimethylaminoanhydrotetracycline. Possible structures (III and IV) for these fragments are related to the intact C and D rings (5).

The 70-ev mass spectrum of the methylated product contained peaks at *m/e* 241 and 228; the *m/e* 228 fragment (V) was the dominant peak. A possible structure for this fragment, analogous to III, was derived from the intact C and D rings, since both can be generated by a similar fragmentation process. The presence of these fragments indicated that the methyl ether function was located on the C or D ring.

The *m/e* 228 fragment subsequently decomposed, losing CH_3 (*m/e* 15) and CO (*m/e* 28). This stepwise loss of C_2H_3O was observed and reported for the mass spectra of a number of aryl methyl ethers (7, 8). A metastable peak was observed at *m/e* 199, confirming the decomposition path.

The addition of methylene to the C-10 or C-11 hydroxy of 4-dedimethylaminoanhydrotetracycline was confirmed by the use of deuterodiazomethane (CD_2N_2). The 70-ev mass spectrum of the isolated product contained a dominant peak at *m/e* 230, and the subsequent decomposition of this fragment to the *m/e* 213 fragment was noted. This stepwise loss of CD_2H and CO was the same as was previously observed for the monomethyl ether of 4-dedimethylaminoanhydrotetracycline and indicated that the CH_3 fragment was from the methyl ether functional group. The loss of this methyl group made further assignment of the smaller fragments very difficult; however, it was concluded that the methyl ether function was located at either the C-10 or C-11 position.

The differentiation between methylation at the C-10 or C-11 position was accomplished using NMR spectroscopy. A number of exchangeable protons were observed downfield from 8 ppm in the NMR spectra of tetracyclines in d_6-dimethyl sulfoxide (9). Based on observations from model compounds, these signals were assigned to the hydrogen-bonded hydroxy protons of the C-10, C-11, and C-3 positions and the amide protons. The NMR spectrum of 4-dedimethylaminoanhydrotetracycline contained the signals of five exchangeable protons downfield from 8 ppm. The signals at 8.9 and 9.0 ppm were assigned to the amide protons of the carboxamido group, while the signals at 9.9, 15.3, and 18.2 ppm were assigned to the hydroxy protons of the C-10, C-11, and C-3 positions, respectively (9).

The NMR spectrum of methylated 4-dedimethylaminoanhydrotetracycline contained signals at 8.9, 9.1, 10.1, and 18.3 ppm. The absence of a signal in the 15-ppm region indicated that the C-11 hydroxy proton had been replaced by a methyl group. An additional singlet at 3.95 ppm, corresponding to three protons, was observed for these methoxy protons.

REFERENCES

(1) F. A. Hochstein, C. R. Stephens, L. H. Conover, P. P. Regna, R. Pasternack, P. N. Gordon, F. J. Pilgrim, K. J. Brunings, and R. B. Woodward, *J. Amer. Chem. Soc.*, **75**, 5455(1953).

(2) J. R. D. McCormick, S. M. Fox, L. L. Smith, B. A. Bitler, J. Reichenthal, V. E. Origoni, W. H. Müller, R. Winterbottom, and A. P. Doerschuk, *ibid.*, **79**, 2849(1957).

(3) A. Green, R. G. Wilkinson, and J. H. Boothe, *ibid.*, **82**, 3946(1960).

(4) H. Muxfeldt and A. Kreutzer, *Chem. Ber.*, **94**, 881(1961).

(5) D. R. Hoffman, *J. Org. Chem.*, **31**, 792(1966).

(6) W. Vetter, P. Longevialle, F. Khoung-Huu-Laine, Q. Khoung-Huu, and R. Goutarel, *Bull. Soc. Chim. Fr.*, **3**, 1324(1963).

(7) C. S. Barnes, D. J. Collins, J. J. Hobbs, P. I. Mortimer, and W. H. F. Sasse, *Aust. J. Chem.*, **20**, 699(1967).

(8) R. H. Shapiro and C. Djerassi, *J. Org. Chem.*, **30**, 955(1965).

(9) G. L. Asleson, L. J. Stoel, E. C. Newman, and C. W. Frank, *J. Pharm. Sci.*, **63**, 1144(1974).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 14, 1974, from the *Department of Chemistry, University of Iowa, Iowa City, IA 52242*

Accepted for publication February 13, 1975.

Supported in part by Pfizer, Inc.

Appreciation is expressed to Pfizer, Inc., New York, N.Y., for donation of tetracycline hydrochloride and to Dr. V. Nair for assistance and guidance with the manipulation of ethereal diazomethane solutions.

* Present address: Department of Chemistry, Augustana College, Rock Island, Ill.

‡ Present address: 3M Company, 3M Center, Minneapolis, Minn.

x To whom inquiries should be directed.