Evidence for the existence of some $B^+$ species intermediate between fully bound ferrioxamine B ($A^+$) and a tetradentate form ($BH^{2+}$) comes solely from the kinetic analysis of the very fast reaction. This species can only be present as a minor component. Since $B^+$ is an intermediate between $A^+$ and $BH^{2+}$, reasonable structures for this species are limited. The proposed structure shown in Scheme III involves addition of a coordinated water. Justification for this structure comes from our previously reported recognition of the importance of a coordinated $H_2O$ cis to the dissociating hydroxamate functional group[24] and the fact that seven-coordinate iron(III) complexes are known.[37] Species $B^+$ may also include some H-bonding interaction between coordinated $H_2O$ and the dissociating hydroxamate oxygen atom, as shown in structure $C^{2+}$ for the second hydroxamate dissociation.

Species $CH^{3+}$ is shown in Scheme III as having only one hydroxamate group bound to iron(III). This assignment is based on the visible absorption spectrum of $CH^{3+}$,[38] which is comparable to that observed for mono(hydroxamato)iron(III) complexes,[24] and the observation that $D^{3+}$ and $E^+$ do not absorb light in the visible region of the spectrum and therefore are assigned to $Fe(H_2O)_6^{3+}$ and $H_4DFB^+$, respectively. Further support of this structure for $CH^{3+}$ comes from the acid-dependent ($k_5$) and acid-independent ($k_6$) rate constants for the conversion of $CH^{3+}$ to $D^{3+}$ and $E^+$, which are comparable to the corresponding rate constants for mono(hydroxamato)iron(III) aquation.[24]

Species $C^{2+}$ and $C'H^{3+}$ represent two intermediates that occur between tetradentate- ($BH^{2+}$) and bidentate- ($CH^{3+}$) coordinated ferrioxamine B and that differ from each other by a proton. Within these limitations, the half-dissociated ligand species differing by a H-bonded proton, which are shown in Scheme III, are reasonable structures.

The first-order rate constants for the dissociation of successive hydroxamate groups from ferrioxamine B vary by a factor of $10^5$, with dissociation of the last hydroxamate group being the slowest. Since each dissociating group is a similar hydroxamate moiety that is being replaced by $H_2O$ ligands and since the chain length between hydroxamate groups is reasonably long, steric factors should be relatively small. This observation illustrates the significant difference in labilizing effect between coordinated $H_2O$ and $-C(O)-N(O^-)-$. This may be due to differences in coordinating ability as well as net charge at the metal center.

The final step of ferrioxamine B aquation proceeds by two parallel paths (reaction 23; Scheme III), which is typical of many iron(III) aquation processes in aqueous solution. Comparison of $k_5$ and $k_6$ to the corresponding constants for mono(hydroxamato)iron(III) complexes shows that both acid-dependent and acid-independent aquation rate constants are smaller for ferrioxamine B than for the synthetic hydroxamic acids investigated in our laboratory.[14,24] *Thus, the natural product deferriferrioxamine B has a structure that minimizes the aquation rate of ferrioxamine B by both acid-dependent and acid-independent paths to enhance both the thermodynamic and kinetic stability of the iron complex.* The rate constants and equilibrium constant for the final step of the aquation reaction, and the literature value for the $Fe(H_2O)_6^{3+}$ hydrolysis constant,[34] allow us to compute values for the rate constants of the ferrioxamine B formation reaction. The computed rate constants for reaction with $Fe(H_2O)_6^{3+}$ and $Fe(H_2O)_5OH^{2+}$ at 25 °C, $I = 2.0$, are $2 \times 10^{-1}$ $M^{-1}$ $s^{-1}$ and $2 \times 10^2$ $M^{-1}$ $s^{-1}$, respectively. These formation rate constants are a factor of 4–20 smaller than the corresponding formation rate constants for the synthetic monohydroxamic acids[24] but are consistent with the trends established by them. These data taken in total suggest a variable degree of hydroxamic acid ligand participation in the transition state, which is consistent with the associative-interchange character previously proposed for the mono(hydroxamato)iron(III)-formation reactions.[24] However, it is possible that charge and/or steric effects may also influence the kinetics of ferrioxamine B complex formation.

**Acknowledgment.** Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for support of this research. We also thank the Ciba Geigy Corporation for their generous gift of the methanesulfonate salt of deferriferrioxamine B (DESFERAL).

**Registry No.** $[Fe(HDFB)]ClO_4$, 82265-74-5; $(H_3DFB)MeSO_3H$, 138-14-7; $Fe(HDFB)^+$, 82265-75-6; Fe, 7439-89-6.

**Supplementary Material Available:** Absorbance and rate data, Tables I–III (4 pages). Ordering information is given on any current masthead page.

(37) See, for example: Spijkermann, J. J.; Hall, L. H.; Lambert, J. L. *J. Am. Chem. Soc.* **1968**, *90*, 2039.

(38) The inset of Figure 3 shows that $CH^{3+}$ has a $\lambda_{max} \sim 480$ nm; $\epsilon_{CH} = 1020$ (250) $M^{-1}$ $cm^{-1}$ at 425 nm from dynamic-equilibrium measurements (Scheme I).

# Treflorine, Trenudine, and *N*-Methyltrenudone: Novel Maytansinoid Tumor Inhibitors Containing Two Fused Macrocyclic Rings

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**Abstract:** Treflorine ($C_{36}H_{48}ClN_3O_{12}$), trenudine ($C_{36}H_{48}ClN_3O_{13}$), and *N*-methyltrenudone ($C_{37}H_{48}ClN_3O_{13}$), isolated from *Trewia nudiflora* (Euphorbiaceae) seed, are the first representatives of a new class of maytansinoid tumor inhibitors containing two fused macrocyclic rings. In addition to the 19-membered ring characteristic of all previously known maytansinoids, these new compounds have a 12-membered ring joining C-3 and the amide nitrogen at C-18. The structures of these new maytansinoids were established by 470-MHz $^1H$ NMR, $^{13}C$ NMR, and mass spectrometry and chemical degradation.

The seed of *Trewia nudiflora* L. (Euphorbiaceae), a tree native to parts of India, is a rich source of maytansinoids including trewiasine (**1**), dehydrotrewiasine (**2**), and demethyltrewiasine (**3**).[1] There is considerable current interest in maytansinoids because of their unique ansa macrolide structure together with their exceptionally potent antitumor activity[2] and a range of other bio-

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(1) Powell, R. G.; Weisleder, D.; Smith, C. R., Jr. *J. Org. Chem.* **1981**, *46*, 4398–4403.

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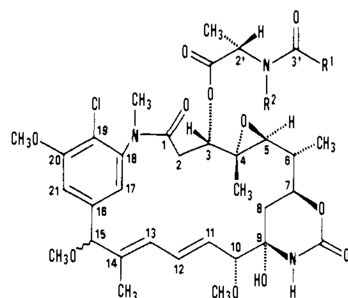
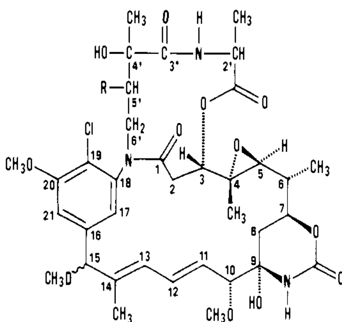


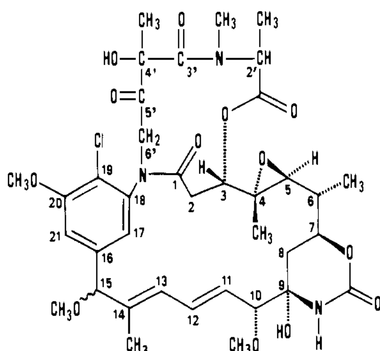
1 Trewiasine $R^1 = CH(CH_3)_2$; $R^2 = CH_3$
2 Dehydrotrewiasine $R^1 = C(CH_3)=CH_2$; $R^2 = CH_3$
3 Demethyltrewiasine $R^1 = CH(CH_3)_2$; $R^2 = H$

logical effects, including toxic or antifeedant activity against several insect pests.[3] These compounds have generated intensive synthetic efforts by several groups of investigators.[4,5] In this paper, we present the characterization of three unique maytansinoids, treflorine (**4**), trenudine (**5**), and *N*-methyltrenudone (**6**). These new



4 Treflorine R = H
5 Trenudine R = OH

maytansinoids differ from previously recognized members of the series in that they contain an additional macrocyclic ring linking C-3 and the aromatic amide nitrogen.



6 *N*-Methyltrenudone

## Results and Discussion

Fractionation of the ethanolic extract of *T. nudiflora* seed was guided by assay against KB cell culture and against PS leukemia in mice.[6] This extract was processed by a solvent partitioning scheme modified somewhat from the one used in our previous paper[1] (see Experimental Section). Isolation of six maytansinoids in pure form was then achieved by repeated column chromatography of the chloroform-soluble fraction on silica followed by repeated preparative HPLC of the active fractions on reversed phase ($C_{18}$) columns. Characterization of three of these compounds (**1**–**3**) was described previously. Three additional maytansinoids (**4**–**6**) were provided in sufficient quantities for structural elucidation.

Comparison of the $^1H$ and $^{13}C$ NMR spectra of treflorine (**4**), $C_{36}H_{48}ClN_3O_{12}$, with those of **1** (Table I and II) indicated that these two compounds were similar in most respects. It was particularly clear that the maytansinoid ring system of **1**, including the C-15 methoxyl group,[1] was present in **4** as all appropriate NMR signals were readily observed. It was also apparent that an ester group was attached at C-3 (H-3 appears at $\delta$ 4.75 in **1**) and that both the C-2′ proton and the C-2′ methyl signals were retained in **4**. However, other features of the $^1H$ and $^{13}C$ NMR spectra of **4**, as well as its mass spectrum, indicated fundamental differences which set **4** apart from previously characterized maytansinoids. No *N*-methyl proton singlets (occurring near $\delta$ 2.88 and 3.16 in spectra of **1** and **2**) were observed in the $^1H$ spectrum of **4**, and the $^{13}C$ spectrum lacked signals associated with *N*-methyl groups (quartets centered at $\delta$ 30.4 and 35.2 in the spectrum of **1**). The $^1H$ NMR spectrum of **4** showed a doublet at $\delta$ 7.06 which was coupled ($J$ = 10.7 Hz) to the C-2′ proton signal; this downfield proton exchanged slowly in $D_2O$. The absence of an *N*-methyl group adjacent to C-2′ is further evidenced by the $^{13}C$ shift of C-2′ near $\delta$ 46.5 for **4**, which is upfield compared to $\delta$ 52.4 for this carbon in **1**. These observations suggested that **4** contained a proton on the nitrogen attached to C-2′, as in demethyltrewiasine (**3**),[7] and accounted for one of the "missing" *N*-methyl groups of **4**. Compound **4** exhibited one less C-methyl signal than **1**; doublets at $\delta$ 1.06 and 1.12 in the $^1H$ spectrum of **1**, associated with two methyl groups attached at C-4′, were replaced by a singlet at $\delta$ 1.40. Proof that C-4′ is oxygenated in **4** is found in both the $^1H$ NMR shift of the 4′ methyl ($\delta$ 1.40) and by the presence of an additional oxygenated carbon in the $^{13}C$ spectrum ($\delta$ 73.4). A complex series of multiplets was observed at $\delta$ 1.4–4.5 which were unresolved in $^1H$ spectra of **4** at 90 MHz. Two unassigned triplets, $\delta$ 35.6 and 43.8, remained in the $^{13}C$ spectrum of **4**; the latter was consistent with a methylene group attached to nitrogen. The electron-impact mass spectrum of **4** exhibited an $M^+$ – a ion[8,9] with the composition $C_{35}H_{45}ClN_2O_{10}$ as shown by accurate mass determination. In contrast, **1** gives an $M^+$ – a ion with the composition $C_{36}H_{49}ClN_2O_9$ corresponding to a molecular formula of $C_{37}H_{52}ClN_3O_{11}$. These formulations require that **4** contain one more ring or double bond equivalent than **1** and NMR spectra eliminate the possibility of an additional double bond. Significantly, no $M^+$ – (a + b) ion was observed

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(6) Cytotoxic and antitumor activities were assayed under auspices of the National Cancer Institute by the procedures described by: Geran, R. I.; Greenberg, N. H.; MacDonald, M. M.; Schumacher, A. M.; Abbott, B. J. *Cancer. Chemother. Rep., Part 3*, **1972**, *3*, 1. Treflorine (**4**), trenudine (**5**), and *N*-methyltrenudone were respectively cytotoxic ($ED_{50}$) at $2.7 \times 10^{-4}$, $6.4 \times 10^{-4}$, and $<1.0 \times 10^{-2}$ µg/mL. In addition, **4** gave T/C 150–157 at 4.0–16.0 µg/kg, **5** T/C 144–155 at 2.0–15.5 µg/kg, and **6** T/C 153 at 4 µg/kg against PS leukemia. Trenudine was also active, T/C 137–212 in the dosage range 1.0–16.0 µg/kg, against Bl melanoma.

(7) The corresponding values for **3**, omitted from our previous paper,[1] are $\delta$ 6.87 ($J$ = 10.5 Hz) for NH coupled to a C-2′ proton.

(8) Kupchan, S. M.; Komoda, Y.; Branfman, A. R.; Sneden, A. T.; Court, W. A.; Thomas, G. J.; Hintz, H. P. J.; Smith, R. M.; Karim, A.; Howie, G. A.; Verna, A. K.; Nagao, Y.; Dailey, R. G., Jr.; Zimmerly, V. A.; Summer, W. C., Jr. *J. Org. Chem.* **1977**, *42*, 2349–2357.

(9) Behavior of maytansinoids in electron impact mass spectrometry has been documented extensively.[1,8] Characteristically, molecular ions are not found, but instead a distinctive $M^+ - 61$ [$M^+ - (H_2O + HNCO)$, abbreviated as $M^+$ – a] ion appears. Elimination of the ester side chain b at C-3 gives another characteristic maytansinoid ion $M^+$ – (a + b). Other distinguishing ions normally include $M^+$ – (a + Cl), $M^+ - (a + CH_3)$, b – OH, and b – $CO_2H$. In compounds with an *N*-methyl group in the ester side chain, a prominent ion arising from fragmentation of b appears at $m/z$ 58 $[CH_3CH{=}NHCH_3]^+$ and in compounds lacking an *N*-methyl group in the side chain a similar ion appears at $m/z$ 44 $[CH_3CH{=}NH_2]^+$.

Table I. $^1$H NMR of *Trewia nudiflora* Components and Reaction Products[a]

| proton assignt | compound | | | | | |
|---|---|---|---|---|---|---|
| | 1[b] | 4 | 5 | 6 | 7b | 8b |
| $2_A$ | 2.18 (dd, *J* = 14.3, 3.0) | 2.21 (dd, *J* = 14.9, 3.9) | 2.22 (dd, *J* = 13.6, 3.7) | 2.09 (dd, *J* = 14.7, 3.6) | 5.56 (d, *J* = 15.4) | 5.62 (d, *J* = 15.6) |
| $2_B$ | 2.55 (dd, *J* = 14.3, 12.2) | 2.50 (dd, *J* = 14.9, 12.0) | 2.53 (dd, *J* = 13.6, 12.0) | 2.62 (dd, *J* = 14.7, 12.0) | | |
| 3 | 4.75 (dd, *J* = 12.2, 3.0) | 4.51 (dd, *J* = 12.0, 3.9) | 4.63 (dd, *J* = 12.0, 3.7) | 4.51 (dd, *J* = 12.0, 3.6) | 6.29 (d, *J* = 15.4) | 6.42 (d, *J* = 15.6) |
| 4-$CH_3$ | 0.76 (s) | 0.72 (s) | 0.80 (s) | 0.82 (s) | 1.03 (s) | 1.05 (s) |
| 5 | 3.01 (d, *J* = 9.6) | 3.07 (d, *J* = 9.8) | 2.98 (d, *J* = 9.8) | 3.06 (d, *J* = 9.8) | 2.62 (d, *J* = 9.8) | 2.63 (d, *J* = 9.9) |
| 6-$CH_3$ | 1.27 (d, *J* = 6.2) | 1.29 (d, *J* = 6.4) | 1.30 (d, *J* = 6.4) | 1.29 (d, *J* = 5.3) | 1.35 (d, *J* = 6.4) | 1.34 (d, *J* = 6.6) |
| 7 | 4.28 (m) | 4.22 (m) | 4.21 (m) | 4.18 (m) | 4.35 (m) | 4.34 (m) |
| 10 | 3.51 (d, *J* = 9.1) | 3.52 (d, *J* = 9.0) | 3.55 (d, *J* = 8.8) | 3.54 (d, *J* = 8.8) | 3.46 (d, *J* = 9.3) | 3.48 (d, *J* = 9.0) |
| 11 | 5.72 (dd, *J* = 15.3, 9.1) | 5.59 (dd, *J* = 15.2, 9.0) | 5.68 (dd, *J* = 15.4, 8.8) | 5.68 (dd, *J* = 15.4, 8.8) | 5.69 (dd, *J* = 15.0, 9.3) | 5.63 (dd, *J* = 15.4, 10.0) |
| 12 | 6.46 (dd, *J* = 15.3, 11.1) | 6.48 (dd, *J* = 15.2, 10.9) | 6.52 (dd, *J* = 15.4, 10.8) | 6.52 (dd, *J* = 15.4, 10.7) | 6.46 (dd, *J* = 15.0, 11.0) | 6.44 (dd, *J* = 15.4, 10.0) |
| 13 | 6.98 (d, *J* = 11.1) | 6.60 (d, *J* = 10.9) | 6.72 (d, *J* = 10.8) | 6.67 (d, *J* = 10.7) | 6.38 (d, *J* < 12) | 6.27 (d, *J* = 10.0) |
| 14-$CH_3$ | 1.52 (s) | 1.57 (s) | 1.55 (s) | 1.53 (s) | 1.58 (s) | 1.58 (s) |
| 15 | 4.86 (s) | 4.95 (s) | 4.88 (s) | 4.87 (s) | 4.82 (s) | 4.71 (s) |
| 17 | 6.54 (d, *J* = 1.5) | 7.26 (d, *J* = 1.6) | 7.26 (d, *J* = 1.6) | 7.25 (d, *J* = 1.5) | 6.97 (d, *J* = 1.5) | 6.73 (d, *J* = 1.5) |
| 21 | 7.22 (d, *J* = 1.5) | 7.47 (d, *J* = 1.6) | 7.27 (d, *J* = 1.6) | 7.54 (d, *J* = 1.5) | 7.25 (d, *J* = 1.5) | 7.22 (d, *J* = 1.5) |
| 10-$OCH_3$[c] | 3.35 (s) | 3.39 (s) | 3.42 (s) | 3.40 (s) | 3.37 (s) | 3.37 (s) |
| 15-$OCH_3$[c] | 3.37 (s) | 3.41 (s) | 3.43 (s) | 3.42 (s) | 3.40 (s) | 3.38 (s) |
| 20-$OCH_3$ | 3.99 (s) | 4.02 (s) | 4.00 (s) | 3.99 (s) | 4.01 (s) | 4.01 (s) |
| 18-$NCH_3$ | 3.16 (s) | | | | | |
| 2′ | 5.37 (m) | 4.79 (m) | 4.95 (m) | 5.57 (q) | 4.28 (m) | 4.53 (m) |
| 2′-$CH_3$ | 1.28 (d, *J* = 6.8) | 1.33 (d, *J* = 6.9) | 1.34 (d, *J* = 7.0) | 1.29 (d, *J* = 7.0) | 1.43 (d, *J* = 7.1) | 1.47 (d, *J* = 7.2) |
| 2′-$NCH_3$ | 2.88 (s) | | | 2.75 (s) | | |
| 4′ | 2.76 (m) | | | | | |
| 4′-$CH_3$ | 1.06 (d)<br>1.12 (d) | 1.40 (s) | 1.48 (s) | 1.53 (s) | 1.40 (s) | 1.53 (s) |
| $5'_A$ | | 1.45 (m, *J* = 14.2, 3.0) | 3.93 (m, *J* = 2.5, 2.4) | | 1.77 (dt, *J* = 14.8, 4.8, 4.8) | 4.06 (m, *J* = 7.0, 2.3) |
| $5'_B$ | | 2.78 (m, *J* = 14.2, 3.0) | | | 2.46 (m, *J* = 14.8, 9.5, 4.8) | |
| $6'_A$ | | 3.03 (m, *J* = 14.2, 3.0) | 3.55 (dd, *J* = 15.0) | 4.17 (d, *J* = 14.6) | 3.28 (dt, *J* = 14.2, 4.8, 4.8) | 3.43 (dd, *J* = 14.4, 7.0) |
| $6'_B$ | | 4.46 (m, *J* = 14.2, 3.0) | 4.49 (dd, *J* = 15.0) | 4.51 (d, *J* = 14.6) | 4.25 (m, *J* = 14.2, 9.5, 4.8) | 4.21 (m, *J* = 14.4, 2.3) |
| 9-NH | 6.23 (s) | 6.22 (s) | 6.25 (s) | 6.20 (s) | 6.37 (s) | 6.34 (s) |
| 2′-NH | | 7.06 (d, *J* = 10.7) | 7.67 (d, *J* = 9.9) | | 7.51 (d, *J* = 7.4) | 7.58 (d, *J* = 8.0) |
| $CO_2CH_3$ | | | | | 3.80 (s) | 3.77 (s) |

[a] Chemical shifts (δ) are expressed in ppm from internal tetramethylsilane, and coupling constants (*J*) are expressed in Hz. Extensive decoupling was used to verify assignments. Spectra were recorded in deuteriochloroform solution on a Nicolet NT-470 spectrometer. In all of the above, the C-6 and C-8 proton signals occur at approximately δ 1.3 and are obscured by other signals in this region. [b] Values for 1 are quoted from our previous paper.[1] [c] These assignments may be reversed.

Table II. $^{13}$C NMR of *Trewia nudiflora* Maytansinoids[a]

| carbon assignt | compound | | | |
|---|---|---|---|---|
| | 1[b] | 4 | 5 | 6 |
| 2 | 32.4 (t) | 32.5 (t) | 33.1 (t) | 32.4 (t) |
| 3 | 78.2 (d) | 78.5 (d) | 78.5 (d) | 78.8 (d) |
| 4 | 60.0 (s) | 59.4 (s) | 59.5 (s) | 59.5 (s) |
| 5 | 67.7 (d) | 67.1 (d) | 66.9 (d) | 66.7 (d) |
| 6 | 38.9 (d) | 37.8 (d) | 37.9 (d) | 38.1 (d) |
| 7 | 74.1 (d) | 74.0 (d) | 74.0 (d) | 71.4 (d) |
| 8 | 36.3 (t) | 36.1 (t) | 36.2 (t) | 36.2 (t) |
| 9 | 80.7 (s) | 80.8 (s) | 80.8 (s) | 81.0 (s) |
| 10 | 85.5 (d) | 88.7 (d) | 88.6 (d) | 88.6 (d) |
| 11 | 129.9 (d) | 128.9 (d) | 129.3 (d) | 129.3 (d) |
| 12 | 132.5 (d) | 132.6 (d) | 132.4 (d) | 132.6 (d) |
| 13 | 128.0 (d) | 126.5 (d) | 127.2 (d) | 127.3 (d) |
| 14 | 142.1 (s) | 141.9 (s) | 141.9 (s) | 141.7 (s) |
| 15 | 86.7 (d) | 87.0 (d) | 86.6 (d) | 86.9 (d) |
| 16 | 141.3 (s) | 140.7 (s) | 141.5 (s) | 141.4 (s) |
| 17 | 120.3 (d) | 121.9 (d) | 120.7 (d) | 120.7 (d) |
| 18 | 139.0 (s) | 139.1 (s) | 140.3 (s) | 140.4 (s) |
| 19 | 118.9 (s) | 118.2 (s) | 118.2 (s) | 118.7 (s) |
| 20 | 156.3 (s) | 156.2 (s) | 156.0 (s) | 156.3 (s) |
| 21 | 109.0 (d) | 109.0 (d) | 109.0 (d) | 108.6 (d) |
| C=O | 176.7 (s) | 176.1 (s) | 175.0 (s) | 171.4 (s) |
| C=O | 170.9 (s) | 171.8 (s) | 174.9 (s) | 171.1 (s) |
| C=O | 168.8 (s) | 170.9 (s) | 170.8 (s) | 169.5 (s) |
| C=O | 152.4 (s) | 152.5 (s) | 152.6 (s) | 152.5 (s) |
| $OCH_3$ | 56.3–56.7 (3 q) | 56.3–56.8 (3 q) | 56.4–56.9 (3 q) | 56.7–56.9 (3 q) |
| $CH_3$ | 14.6 (q) | 15.7 (q) | 16.2 (q) | 14.4 (q) |
| $CH_3$ | 13.1 (q) | 14.3 (q) | 14.2 (q) | 12.9 (q) |
| $CH_3$ | 12.0 (q) | 12.1 (q) | 12.0 (q) | 12.3 (q) |
| $CH_3$ | 10.0 (q) | 9.8 (q) | 9.9 (q) | 10.1 (q) |
| 18-$NCH_3$ | 35.2 (q) | | | |
| 2'-$NCH_3$ | 30.4 (q) | | | 30.2 (q) |
| 2' | 52.4 (d) | 46.3 (d) | 46.7 (d) | 52.6 (d) |
| 4' | 30.4 (d) | 73.4 (s) | 72.2 (s) | 78.5 (s) |
| 4'-$CH_3$ | 19.4 (q) 18.8 (q) | 28.9 (q) | 28.5 (q) | 23.6 (q) |
| 5' | | 35.6 (t) | 79.1 (d) | 204.0 (s) |
| 6' | | 43.8 (t) | 52.4 (t) | 57.8 (t) |

[a] Chemical shifts (δ) are expressed in ppm from internal tetramethylsilane. Proton-decoupled and off-resonance-decoupled spectra were recorded in deuteriochloroform solution on a Fourier tranform Bruker WH-90 spectrometer. [b] Values for 1 are quoted from our previous paper.[1]

in the mass spectrum of **4**, suggesting that the side chain remained attached to the maytansinoid ring even after cleavage of the C-3 ester linkage. The mass spectrum of **4** also contained two ions not previously observed in other known maytansinoid spectra; these occurred at $m/z$ 577 ($M^+ - (a + OCH_3 + Cl + CO_2H)$) and at $m/z$ 188.

Trenudine (**5**, $C_{36}H_{48}ClN_3O_{13}$) contains one more oxygen than **4**; this additional oxygen was correlated with a $^{13}$C NMR doublet at δ 79.1 consistent with a carbon bearing a secondary hydroxyl group. We allocated this to C-5′, which appeared as a triplet at δ 35.6 in the spectrum of **4** and was absent in the $^{13}$C spectrum of **5**. The $^{13}$C resonance of C-6′ also shifted downfield 8.6 ppm compared to **4** because of the introduction of a hydroxyl at C-5′. With these exceptions, the $^{13}$C NMR spectra of **4** and **5** were nearly identical. The 90-MHz $^1$H spectrum of **5** was also nearly identical with that of **4** except for several unresolved peaks in the δ 3.5–4.5 region and a shift of the 2′-NH signal from δ 7.06 to δ 7.67. The mass spectrometric behavior of **5** was analogous to that of **4**, since an $M^+ - (a + b)$ ion associated with loss of an ester side chain was again notably absent. An $M^+ - a$ ion was prominent in the mass spectrum of **5** ($C_{35}H_{45}ClN_2O_{11}$), and other significant ions were observed at $m/z$ 593 ($M^+ - (a + OCH_3 + Cl + CO_2H)$) and 44 ($C_2H_6N$).

The structure of *N*-methyltrenudone (**6**, $C_{37}H_{48}ClN_3O_{13}$) was deduced within the same frame of reference used for **4** and **5**. The $^{13}$C NMR doublet of **5** at δ 79.1 was replaced in the spectrum of **6** by a singlet at δ 204.0 associated with a new carbonyl function; this we ascribed to C-5′. In addition, the C-2′ $NCH_3$ group was

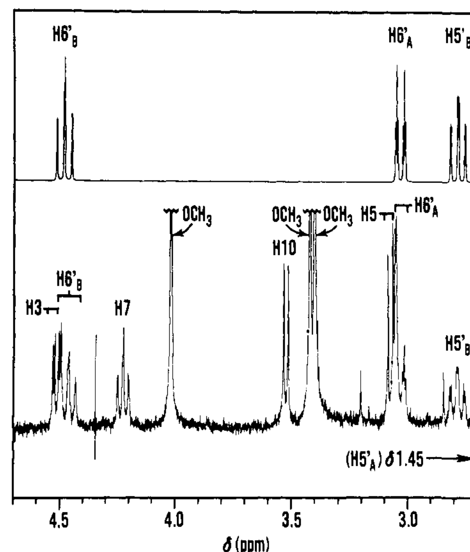



**Figure 1.** Portions of the 470-MHz $^1$H NMR spectrum of treflorine (**4**) in $CDCl_3$. The upper trace is the final iterated theoretical spectrum obtained by using parameters of Table I.

"restored" as signified by a quartet in the $^{13}$C spectrum at δ 30.2 and also by a three proton singlet in the $^1$H spectrum at δ 2.75. It was also clear that **6** contained an isolated methylene attached to nitrogen or oxygen (δ 4.17 and 4.51 (AB quartet, $J$ = 14.6 Hz)). The mass spectrum of **6** exhibited an $M^+ - a$ ion ($C_{36}H_{45}ClN_2O_{11}$), but again no $M^+ - (a + b)$ was observed. The mass spectrum of **6** also contained significant ions at $m/z$ 605 ($M^+ - (a + OCH_3 + Cl + CO_2H)$), 542, and 58 ($C_3H_8N$).

On the basis of these observations, we developed a working hypothesis for the structures of **4–6** in which an additional ring was formed; by formal abstraction of two hydrogens, we envisioned that a covalent bond had been established between the "missing" C-4′ methyl and the C-18 *N*-methyl groups of **1**.

Our structural assignments for **4–6** were strengthened by high-field $^1$H NMR spectroscopy. At 470 MHz, it was clear that four unassigned protons were present in the spectrum of **4**. Double-resonance experiments and analysis by the ITRCAL iterative spin-simulation program[10] revealed that there are four mutually coupled protons (δ 1.45, 2.78, 3.03, 4.46) due to nonequivalent protons on two adjacent methylene groups; these we ascribe to C-5′ and C-6′. Similarly, **5** has three mutually interacting protons (δ 3.93, 3.55, 4.49) which we assign to a methine proton at C-5′ and a methylene at C-6′ on the basis of double-resonance and simulation experiments. Portions of the 470-MHz NMR spectra of **4** and **5** together with spectra simulated by ITRCAL are shown in Figures 1 and 2. The large coupling observed for vicinal protons $5'_B$ and $6'_B$ ($J$ = 14.8 Hz in **4**) requires a very large dihedral angle. A gauche conformation of the groups on C-5′ and C-6′ accommodates these couplings and also provides for the equal couplings observed for $J(5'_A,6'_A)$ and $J(5'_B,6'_A)$. The presence of 12-membered rings in these compounds is supported by comparison with bicyclic *Senecio* alkaloids which include examples of both 11- and 12-membered rings.[11] In these 12-membered rings, a considerably larger chemical shift difference between geminal protons adjacent to a bridgehead is noted than in the 11-membered rings.

Alkaline hydrolysis of **4** and **5** provided conclusive evidence in support of the structures we have adopted. When treated with sodium carbonate in 50% aqueous methanol at ambient temperature, previously investigated maytansinoids have yielded the C-3 acyl moiety as a free acid with concurrent formation of a 2,3 double bond.[8] We have applied this procedure to **1** with similar results.[1] However, when **4** and **5** were treated under these con-

(10) "1180 ITRCAL", Nicolet Instrument Co., 1977.
(11) Culvenor, C. C. J.; Woods, W. G. *Aust. J. Chem.* **1965**, *18*, 1625–1637.

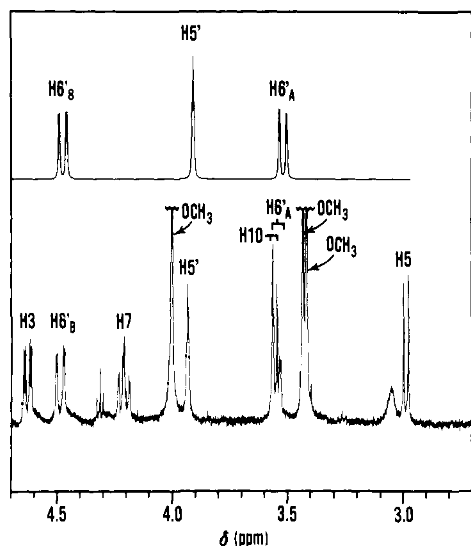
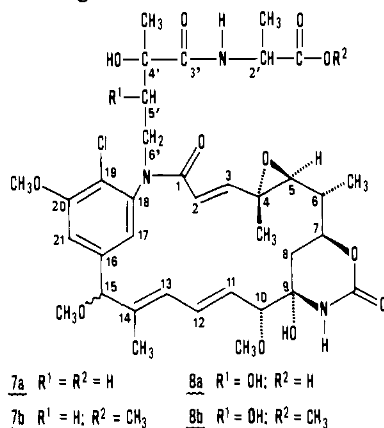



**Figure 2.** Portions of the 470-MHz $^1H$ NMR spectrum of trenudine (**5**) in $CDCl_3$. The upper trace is the final iterated theoretical spectrum obtained by using parameters of Table I.

ditions, the reaction proceeded at a much slower rate; after 3.5 h, there was no appreciable reaction. When the reaction time was extended to 3–4 days, **4** and **5** yielded complex mixtures of products which were methylated with diazomethane and then separated by preparative TLC. In this manner, we isolated **7b** and **8b** in which a side chain bearing the carbomethoxy groups remained attached to the 19-membered maytansinoid ring via the aromatic amide nitrogen.[12]




High-resolution MS of ester **7b** (derived from **4**) gave an $M^+ - a$ ion at $m/z$ 702.2880 ($C_{36}H_{47}ClN_2O_{10}$ requires $m/z$ 702.2919) corresponding to a molecular formula of $C_{37}H_{50}ClN_3O_{12}$ for **7b**. As expected, $M^+ - (a + Cl)$ at $m/z$ 667 and 44 were also prominent ions in the spectrum. Most instructive was an intense ion at $m/z$ 202.1115 ($C_9H_{16}NO_4$ requires $m/z$ 202.1079) which must represent the entire ester side chain severed from the aromatic amide nitrogen.[13] Two other prominent ions in the mass spectrum of **7b** are presumed to arise from cleavage between C-3′ and C-4′ ($m/z$ 572) and between C-4′ and C-5′ ($m/z$ 528). In the MS of **8b**, the $M^+ - a$ ion occurs at $m/z$ 718.2859 ($C_{36}H_{47}ClN_2O_{11}$ requires $m/z$ 718.2868) corresponding to a molecular formula of $C_{37}H_{50}ClN_3O_{13}$. The $M^+ - (a + Cl)$ ion is present at $m/z$ 683, and $m/z$ 44 is the base peak. Fragment ions are present at $m/z$ 588 (cleavage between C-3′ and C-4′), $m/z$ 570 (588 − $H_2O$), and $m/z$ 544 (cleavage between C-4′ and C-5′).

The 470-MHz $^1H$ NMR spectrum of **7b** differed from that of the parent compound **4** in that the C-3 ester linkage had been eliminated with resultant double-bond formation between C-2 and C-3 (δ 5.56 and 6.29 ($J$ = 15.4 Hz)). The spectrum of **8b** exhibited a similar double bond (δ 5.62 and 6.42 ($J$ = 15.6 Hz)). Both compounds possessed methyl ester singlets (δ 3.80 in **7b** and 3.77 in **8b**). There were other relatively minor differences in chemical shifts and coupling constants between **7b** and **8b** and their precursors (**4** and **5**), primarily with respect to protons on C-2′, C-5′, and C-6′; these differences may result from relief of conformational restraints in these ring-opened derivatives.

The mass spectra of **4–6** and derivatives **7a** and **8a** are in full agreement with conclusions as to the structures of these compounds based on $^1H$ and $^{13}C$ NMR spectra.[14] It is noteworthy that biological activity in the maytansinoid series is fully retained in compounds with additional macrocyclic rings linking C-3 and the aromatic amide nitrogen.

## Experimental Section

Melting points were determined with a Fisher-Johns block[15] and are uncorrected. Analytical and preparative TLC was accomplished by using silica gel 60 F-254 plates (0.25 mm thick (E. Merck)). Plates were developed with $CH_2Cl_2$–MeOH (19:1), unless specified otherwise, and visualized under UV light. Hi Flosil, 60/200 mesh (Applied Science), was used for column chromatography. HPLC was performed with a Waters Associates, Inc., preparative 500 LC system (using silica or $C_{18}$-bonded silica cartridges, as appropriate) or a Model ALC/PC-201 instrument equipped with a 7.8 × 300 mm $C_{18}$ μ-Bondapak column and an RI detector and operated at a flow rate of 2 mL/min. IR spectra were recorded on a Perkin-Elmer Model 700 instrument with 1% $CHCl_3$ solutions, and optical rotations were determined with a Perkin-Elmer Model 241 polarimeter. High-field (470-MHz) $^1H$ spectra were recorded on a Nicolet NT-470 instrument, and extensive decoupling was used to verify assignments. Portions of the $^1H$ NMR spectra of **4** and **5** (Figures 1 and 2) were analyzed by the ITRCAL iterative spin-simulation program.[10] $^1H$ (90-MHz) and $^{13}C$ NMR (22.63-MHz) spectra were determined with a Bruker WH-90 instrument; $CDCl_3$ solutions were used with tetramethylsilane as an internal standard. Mass spectra were obtained with both Nuclide 12-90-DF and/or Kratos MS-30 instruments.

**Extraction Procedures.** *Trewia nudiflora* seed (PR53256 and PR53445; 114 kg)[16] was ground in a Wiley mill, divided into 16 batches, and defatted with hexane in a pilot-plant-scale Soxhlet extractor, yielding 21 kg of oil. Each batch was then further extracted with 95% ethanol, yielding a combined total of 7.25 kg of ethanol-soluble material. Ethanol solubles were divided into six equal portions, and each portion was partitioned between 6 L of ethyl acetate and 6 L of water; aqueous phases were then backwashed two times with 3-L portions of ethyl acetate. Combined ethyl acetate extracts were evaporated, and the resulting thick syrup was partitioned between 13 L of hexane and 13 L of 10% aqueous methanol; the hexane layer was backwashed with 6.5 L of additional aqueous methanol. The combined aqueous methanol-soluble material was evaporated to a thick syrup and partitioned between 13-L each of $CHCl_3$ and $H_2O$. The aqueous layer was then backwashed twice with 6.5-L portions of $CHCl_3$, and the combined $CHCl_3$ extracts were evaporated to dryness, yielding 710 g of highly cytotoxic material.

**Chromatographic Separations.** The $CHCl_3$-soluble fraction, 710 g, was divided into 13 portions, and each was subjected to column chromatography on a 6-cm i.d. column packed with 300 g of silica per run. Elution was carried out by successive application of 1.5-L each of the following solvents: $CHCl_3$, $CHCl_3$–MeOH (19:1), $CHCl_3$–MeOH (3:2), and MeOH. Similar fractions were combined on the basis of TLC analysis,

(12) For convenience, we have numbered the side-chain positions in **7** and **8** the same as in the precursors (**4** and **5**).

(13) The $m/z$ 188 ion observed in mass spectrum of treflorine (**4**) must be derived from thermal elimination at C-3, yielding free acid **7a**, followed by an identical cleavage between C-6′ and the aromatic amide nitrogen. It follows that the ion at $m/z$ 540, in the mass spectrum of **6**, must arise by elimination of the C-3 ester followed by a preferred cleavage between C-4′ and C-5′.

(14) Recently published $^{13}C$ NMR shifts for ansamitocins PHO-3 and *epi*-PHO-3, which have a C-15 hydroxyl group (cf. Izawa, M.; Wada, Y.; Kasahara, F.; Asai, M.; Kishi, T. *J. Antibiot.* **1981**, *34*, 1591–1595), suggest that *Trewia* maytansinoids have the *R* (or β) configuration at C-15. Shifts reported by Izawa et al. for β-carbons C-21 and C-14 methyl are close to those of the *Trewia* maytansinoids, whereas the corresponding signals for *epi*-PHO-3 are considerably downfield. For **4–6**, relative configurations at C-4′ and C-5′ have not been assigned. All these compounds are strongly levorotatory, indicating that configurations at all other assymetric centers correspond to those reported for maytansine: cf. Bryan, R. F.; Gilmore, C. J.; Haltiwanger, R. C. *J. Chem. Soc., Perkin Trans. 2* **1973**, 897–902.

(15) The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

(16) We thank Dr. James Duke, USDA, Beltsville, MD, for supplying seed material in accordance with the program developed by the National Cancer Institute.

and the most active material (167 g), as determined by bioassay, was collected near the middle of each run or during elution with $CHCl_3$–MeOH (9:1). Further chromatography of this material was carried out in six portions on a 6-cm i.d. column, 280 g of silica per run, by using 1.5 L of each of the following solvents: hexane–ethyl acetate (1:4), ethyl acetate, ethyl acetate–MeOH (19:1), ethyl acetate–MeOH (9:1), and ethyl acetate–MeOH (1:1). Similar fractions were again combined on the basis of TLC analysis, and 17 g of highly cytotoxic material was selected for further study. The concentrate was split into four portions, and each was then chromatographed on a reversed phase column. Each sample was deposited on approximately 90 $cm^3$ of $C_{18}$ silica packed in a precolumn, which was placed in a preparative HPLC instrument ahead of a $C_{18}$ cartridge. Columns were then eluted with 3.5 l of MeOH–$H_2O$ (7:3), and the complex of *Trewia* maytansinoids emerged as a broad peak at column volumes of 1.5–3.5. Samples were arbitrarily collected across the peak, yielding 12 fractions and a total of 8.4 g of material, mainly mixtures of **1–6**. Individual compounds were obtained by semipreparative HPLC on a $C_{18}$ $\mu$-Bondapak column in 15-mg portions eluting with MeOH–$H_2O$ (65:35) and operating at 2 mL/min; each fraction was further purified by preparative TLC. Elution times and yields obtained under these conditions were as follows: **5**, 12.5 min, 743 mg (($6.5 \times 10^{-4}$)%); **6**, 15.0 min, 127 mg (($1.1 \times 10^{-4}$)%); **4**, 25.5 min, 207 mg (($1.8 \times 10^{-4}$)%); **2**, 27.5 min, 191 mg (($1.6 \times 10^{-4}$)%); **3**, 31.0 min, 25 mg (($2.0 \times 10^{-5}$)%); **1**, 33.0 min, 3700 mg (($3.2 \times 10^{-3}$)%).

**Treflorine (4).** After preparative TLC and recrystallization from $CH_2Cl_2$–hexane, **4** had the following properties: mp 205–208 °C dec; IR ($CHCl_3$) 3600, 3440, 1760, 1715, 1675, 1640, 1610, 1590 $cm^{-1}$; UV max (EtOH) 233 nm ($\epsilon$ 24 000), 243 (sh, 18 500), 253 (19 850), 282 (5060), 288 (5060); $[\alpha]^{23}_D$ −138° (*c* 0.045, $CHCl_3$); $^1H$ and $^{13}C$ NMR values, Tables I and II; mass spectrum (70 eV), *m/z* (relative intensity) 688 ($M^+$ − a, 2.7), 188 (5.7), 149 (4.2), 69 (14.7), 58 (32.1), 55 (13.4), 44 (100); $C_{35}H_{45}ClN_2O_{10}$ requires *m/z* 688.2762, found ($M^+$ − ($H_2O$ + HNCO)) found 688.2751.

**Conversion of 4 to 7a and Characterization as the Methyl Ester 7b.** A solution of **4** (162 mg) and sodium carbonate (120 mg) in 16 mL of 50% aqueous methanol reacted at room temperature for 96 h. The reaction mixture was acidified with 40 mL of 5% aqueous tartaric acid solution and extracted with $CHCl_3$, yielding 160 mg of $CHCl_3$-soluble material. This product was partially purified by preparative TLC on silica plates developed with $CHCl_3$–MeOH (4:1) to provide 54 mg of crude **7a**. Crude **7a** was treated with diazomethane, and the methylated product was subjected to preparative TLC on plates developed with $CH_2Cl_2$–MeOH (9:1) which yielded methyl ester **7b**: 3.5 mg; mp 135–140 °C; $^1H$ NMR spectrum of **7b**, summarized in Table I; mass spectrum (70 eV), *m/z* (relative intensity) 702 ($M^+$ − a, 1.2), 667 (1.8), 572 (2.7), 528 (1.0), 511 (1.6), 202 (45), 149 (11.0), 111 (18.6), 97 (24.1), 85 (25.3), 83 (39.0), 75 (28.0), 72 (45), 69 (46), 57 (69), 55 (67), 44 (100), 43 (56); $C_{36}H_{47}ClN_2O_{10}$ requires *m/z* 702.2919, found 702.2880 ($M^+$ − ($H_2O$ + HNCO)).

**Trenudine (5).** After preparative TLC and recrystallization from $CH_2Cl_2$–hexane, **5** gave the following properties: mp 200–205 °C dec; IR ($CHCl_3$) 3600, 3450, 3360, 1760, 1715, 1665, 1610, 1590 $cm^{-1}$; UV max (EtOH) 233 nm ($\epsilon$ 26 400), 248 (sh, 21 500), 253 (22 600), 282 (6130), 288 (6130; $[\alpha]^{23}_D$ −114° (*c* 0.24, $CHCl_3$); $^1H$ and $^{13}C$ NMR values, Tables I and II; mass spectrum (70 eV), *m/z* (relative intensity) 704 ($M^+$ − a, 3.9), 672 (2.4), 669 (2.6), 593 (2.6), 204 (3.2), 185 (3.1), 171 (5.5), 95 (10.2), 85 (10.5), 83 (11.7), 81 (11.5), 69 (16.9),, 55 (23.9), 44 (100); $C_{35}H_{45}ClN_2O_{11}$ requires *m/z* 704.2711, found 704.2711 ($M^+$ − ($H_2O$ + HNCO)).

**Conversion of 5 to 8a and Characterization as the Methyl Ester 8b.** A solution of **5** (80 mg) and sodium carbonate (60 mg) in 8 mL of 50% aqueous methanol reacted at room temperature for 72 h. The reaction mixture was acidified with 40 mL of 2% aqueous tartaric acid solution and extracted with $CHCl_3$, yielding 63 mg of $CHCl_3$-soluble material. This material was partially purified by preparative TLC on silica plates developed with $CHCl_3$–MeOH (4:1), yielding 9.6 mg of crude **8a**. Crude **8a** was treated with diazomethane, and the methylated product was subjected to preparative TLC on silica plates developed with $CH_2Cl_2$–MeOH (19:1): yield of methyl ester **8b**, 2.7 mg; mp 150–160 °C; $^1H$ NMR spectrum of **8b**, summarized in Table I; mass spectrum (70 eV), *m/z* (relative intensity) 718 ($M^+$ − a, 0.5), 683 (0.3), 588 (0.2), 570 (0.3), 544 (0.2), 149 (31.3), 111 (31.3), 97 (45), 83 (36), 71 (59), 69 (52), 57 (67), 55 (55), 44 (100), 43 (61); $C_{36}H_{47}ClN_2O_{11}$ requires *m/z* 718.2868, found 718.2859 ($M^+$ − ($H_2O$ + HNCO)).

***N*-Methyltrenudone (6).** After preparative TLC and recrystallization from dichloromethane–hexane, **6** gave the following properties: mp 192–197 °C dec; IR ($CHCl_3$) 3600, 3450, 1760, 1720, 1675, 1640, 1590 $cm^{-1}$; UV max (EtOH) 233 nm ($\epsilon$ 27 000), 247 (sh, 21 300), 252 (21 900), 282 (5270), 289 (5470); $[\alpha]^{23}_D$ −110° (*c* 0.183, $CHCl_3$); $^1H$ and $^{13}C$ NMR values, Tables I and II; mass spectrum (70 eV), *m/z* (relative intensity) 716 ($M^+$ − a, 12.3), 681 (4.4), 605 (2.6), 542 (7.5), 292 (10.0), 109 (15.1), 75 (47.7), 71 (18.3), 58 (100), 55 (27.7), 44 (74.6), 43 (48.1); $C_{36}H_{45}ClN_2O_{11}$ requires *m/z* 716.2711, found 716.2662 ($M^+$ − ($H_2O$ + HNCO)).

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**Registry No. 1**, 78987-26-5; **2**, 78987-27-6; **3**, 78987-28-7; **4**, 82390-93-0; **5**, 82390-94-1; **6**, 82400-19-9; **7b**, 82390-95-2; **8b**, 82390-96-3; **7a**, 82390-97-4; **8a**, 82390-98-5.

# Thiamin Biosynthesis in Yeast. Origin of the Five-Carbon Unit of the Thiazole Moiety

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**Abstract:** Radioactivity from D-[1-$^{14}C$]-, D-[2-$^{14}C$]-, and D-[6-$^{14}C$]glucose, from D-[1-$^{14}C$]fructose, and from [1-$^{14}C$]glycerol is incorporated nonrandomly into the $C_5$ chain of the thiazole moiety of thiamin in *Saccharomyces cerevisiae*. The incorporation pattern leads to the inference that the $C_5$ chain is derived from a 2-pentulose, which is generated from the hexose precursors by the oxidative as well as by the nonoxidative pentose phosphate pathway. A chemically rational scheme for the biogenesis of the thiazole moiety of thiamin is presented.

The ultimate steps in the biosynthesis of thiamin (**1**) (vitamin $B_1$), and, in particular, the mode of construction of its skeleton, by condensation of the intact pyrimidine unit (**29**) with the intact thiazole unit (**3**), are well documented.[1] The routes leading to

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