hemagglutinating antibodies for rat erythrocytes at the serum concentrations used in our studies. Additional studies (*11*) have shown marked enhancement of C-dependent bone resorption by antibodies obtained by immunization of rabbits with rat erythrocytes or rat bone sonicates. However, non-tissue-related antigen-antibody complexes, which activate either the classical or alternate C pathways (*12*), were ineffective in enhancing the release of ^{45}Ca in cultures containing C-sufficient serum. Moreover, preparations containing the active complement fragments C3a and C5a did not enhance bone resorption (*13*), which is not surprising in view of the C6 requirement for the effect. These findings all indicate that C activation must take place on a cell membrane.

The mechanism by which C activation increases PGE synthesis is not known. Conceivably, alteration of the cell membrane by C activation might provide a signal for increased PGE synthesis. Alternatively, C activation on a membrane might result in the release of fatty acids from membrane phospholipids which could then serve as precursors of prostaglandins. Fatty acids may also stimulate bone resorption directly but only at concentrations of $10^{-4}M$ or higher (*14*).

Prostaglandins have been detected in inflamed gingival tissue and exudates as well as in supernatants of rheumatoid synovial cultures (*15*). The present studies indicating a relationship between C activation and PGE synthesis could help explain these findings as well as the associated pathologic breakdown of adjacent bone in such disorders as rheumatoid arthritis and periodontal disease.

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References and Notes

1. P. H. Stern and L. G. Raisz, *Exp. Cell Res.* **46**, 106 (1967); E. Hausman, R. Genco, N. Weinfeld, R. Sacco, *Calcif. Tissue Res.*, in press.
2. H. B. Fell and L. Weiss, *J. Exp. Med.* **121**, 551 (1965); H. B. Fell, R. R. A. Coombs, J. T. Dingle, *Int. Arch. Allergy Appl. Immunol.* **30**, 146 (1966); P. J. Lachmann, R. R. A. Coombs, H. B. Fell, J. T. Dingle, *ibid.* **36**, 469 (1969).
3. H. B. Fell and M. E. J. Barratt, *Int. Arch. Allergy Appl. Immunol.* **44**, 441 (1973).
4. L. G. Raisz, *J. Clin. Invest.* **44**, 103 (1965); ——— and I. Niemann, *Endocrinology* **85**, 446 (1969).
5. J. E. Horton, L. G. Raisz, H. A. Simmons, J. J. Oppenheim, S. E. Mergenhagen, *Science* **177**, 793 (1972).
6. U. Rother and K. Rother, *Z. Immunitaetsforsch. Exp. Ther.* **121**, 224 (1961).
7. Functionally purified human and guinea pig C6 and guinea pig C2 were obtained from the Cordis Company together with appropriate vehicles containing gelatin. These preparations were reconstituted, dialyzed against BGJ, and added to the culture medium at a final concentration of 25 percent.
8. D. C. Klein and L. G. Raisz, *Endocrinology* **86**, 1436 (1970); A. H. Tashjian, E. F. Voelkel, L. Levine, P. Goldhaber, *J. Exp. Med.* **136**, 1329 (1972); J. M. Goodson and L. G. Raisz, unpublished observations.
9. J. R. Vane, *Nat. New Biol.* **231**, 232 (1971).
10. Media were acidified with citric acid and extracted with ethyl acetate; the extracts dried under nitrogen. Bones were extracted with ethyl alcohol. Samples were then dissolved in benzene–ethyl acetate (60 : 40) and chromatographically purified by the method of R. M. Zusman, B. V. Caldwell, L. Speroff [*Prostaglandins* **2**, 41 (1972)], which separates PGE from fatty acids, PGA, PGB, and PGF (prostaglandins A, B, and F, respectively). After conversion of the PGE to PGB by base hydrolysis, the samples were assayed with a commercial antibody to PGB_2 according to the method of L. Levine and H. Van Vunakis [*Biochem. Biophys. Res. Commun.* **41**, 1171 (1970)]. This assay measures both PGE_1 and PGE_2, but was expressed in terms of PGE_2 equivalents uncorrected for recovery (recovery of PGE_2 averaged 55 percent).
11. A. L. Sandberg and L. G. Raisz, in preparation.
12. A. L. Sandberg, A. G. Osler, H. S. Shin, B. Oliveira, *J. Immunol.* **104**, 329 (1970); A. L. Sandberg and A. G. Osler, *ibid.* **107**, 1268 (1971).
13. L. G. Raisz, R. A. Snyderman, S. E. Mergenhagen, unpublished observations.
14. B. Samuelsson, E. Granstrom, K. Green, M. Hamberg, *Ann. N.Y. Acad. Sci.* **180**, 138 (1971); P. H. Stern, *Experientia* **27**, 1061 (1971).
15. J. M. Goodson, F. Dewhirst, A. Brunetti, *J. Dent. Res.* **52**, 182 (1973) (special issue); D. R. Robinson, H. Smith, L. Levine, *Arthritis Rheum.* **16**, 129 (1973).
16. Supported by PHS grants AM 05205 and DE 03556.
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Selective Alkylation: A Biomimetic Reaction of the Antileukemic Triptolides?

Abstract. *The potent antileukemic plant principles triptolide and tripdiolide contain a characteristic hydrogen-bonded 9,11-epoxy-14β-hydroxy system. They alkylate propanethiol in a process which involves opening of the epoxide function with neighboring hydroxyl assistance. The reaction may mimic the inhibition of tumor growth via selective alkylation of the thiol groups of key enzymes concerned with growth regulation.*

Triptolide (**1**) and tripdiolide (**2**) have recently been characterized as the novel and highly active antileukemic principles of the plant *Tripterygium wilfordii* Hook (*1*). The compounds at 0.1 mg/kg show impressive life-prolonging effects (that is, $T/C \geq 230$) in mice afflicted with the L-1210 lymphoid leukemia (*2*). Biological and chemical data are presented in support of the importance of intramolecular catalysis (by a neighboring hydroxy group on the opening of an epoxide by nucleophiles) for the mode of action of the antileukemic triptolides. The hypothesis is discussed in light of earlier proposals that other plant-derived tumor inhibitors may act via selective alkylation of the thiol groups of key enzymes concerned with growth regulation (*3–7*).

The nuclear magnetic resonance (NMR) spectra of the antileukemic triptolides **1** and **2** display resonances at τ 7.16 (doublet, $J = 11$ hertz, 14-OH, disappears upon D_2O addition) and τ 6.50 (doublet of doublets, $J = 11$ hertz and $J = 1$ hertz, 14-H, collapses to a singlet upon D_2O addition). The highly distinctive J_{HCOH} coupling constant (11 hertz) is attributable to the rigid *trans* orientation of the coupled protons resulting from strong hydrogen bonding between the 14-hydroxyl and the 9,11-epoxide groups (*8*). Free rotation J_{HCOH} coupling constants are typically 3 to 6 hertz (*9*). The co-occurring triptolide **3** ("triptonide") differs structurally from **1** solely at C-14, which bears a ketonic function rather than a β-oriented hydroxyl. Accordingly, the NMR spectrum of **3** does not show the resonances attributable to the hydrogen-bonded hydroxy-epoxide system. Triptonide shows no antileukemic activity in doses up to 0.4 mg/kg. These facts led us to hypothesize that the 9,11-epoxy-14β-hydroxy system is necessary for the antileukemic activity of the triptolides. Furthermore, intramolecular catalysis by the 14-hydroxyl group may assist selective alkylation of biological macromolecules by the 9,11-epoxide. Subsequent testing of the minor variants 14-epitriptolide [**4**, with

α-oriented 14-hydroxyl (*10*)], and the thiol adducts **5** and **6** (lacking the 9,11-epoxide) revealed that these triptolide derivatives, as well, show no antileukemic activity at doses up to 0.4 mg/kg.

The importance of the 9,11-epoxy-14β-hydroxy system for the biological activity of the triptolides was also indicated by their relative cytotoxicities against KB cells in culture (*2*). The median effective dose, ED_{50}, values for the series are (in micrograms per milliliter): **1**, 0.0017; **2**, 0.0042; **3**, 0.021; **4**, 0.076; **5**, > 1.0; and **6**, >1.0.

A number of naturally occurring α-methylene γ-lactones have been shown to inhibit tumor proliferation in vitro and in vivo (*11, 12*). Comparison of the relative cytotoxicities of these sesquiterpene lactones and their derivatives has demonstrated that the (electrophilic) α-methylene γ-lactone function is essential for maximum activity (*3, 12*). Several lines of evidence indicate that the nucleophilic addition of thiols to the α,β-unsaturated system may be involved in the tumor inhibitory properties of the sesquiterpene lactones and other plant-derived compounds. A study of the reactions of the α-methylene γ-lactones with model biological nucleophiles revealed that thiols were the most reactive of the nucleophiles investigated and that biological activity decreased, markedly, with successive addition of cysteine to bis-unsaturated lactones (*3*). Subsequently, the tumor inhibitory α-methylene γ-lactones were shown to inhibit phosphofructokinase (*4*) and glycogen synthase (*5*), with concurrent loss of selected enzyme sulfhydryl groups. The electrophilicity of the tumor inhibitory quinone methides taxodone and taxodione, and their inhibition of phosphofructokinase upon reaction with selected SH groups (*4*), suggested that these compounds may also act by alkylation of biologically important macromolecular thiols. Jatrophone, a novel macrocyclic tumor inhibitor, reacts rapidly with simple thiols and with the macromolecular thiol bovine serum albumin, and inhibits DNA-dependent RNA polymerase from *Escherichia coli* with concurrent partial loss of enzyme SH groups (*6*).

The tumor inhibitory α-methylene γ-lactones have half-lives of about 1 to 50 minutes at reactant and thiol concentrations of $10^{-4}M$ (*12*). In contrast, tripdiolide (**2**, $1.5 \times 10^{-2}M$) is unchanged (thin-layer chromatography) after a week in a sealed tube at 37°C in the presence of propanethiol ($7.5 \times 10^{-2}M$) in $0.067M$ phosphate buffer (*p*H 7.4). However, treatment of **2** for 2 weeks under the conditions described above and with a 100-fold excess of propanethiol gave a 79 percent yield of **6** (*13*). The thiol adduct **6** was isolated and purified by thin-layer chromatography on silica gel F-254 (methanol and chloroform, 1 : 9). The NMR spectrum of **6** showed a signal for the 11-H at τ 6.23, which corresponded to a proton on methine bearing OH (*14*). Furthermore, there was an increase in the 11-H, 12-H coupling constant [to 5 hertz (*13*)], and a decrease in the 14-H coupling constant [to 7 hertz (*13*)] from those of tripdiolide (**2**). The increase in the 11-H, 12-H coupling constant is consistent with the *trans* relationship between the relevant protons. The diminished 14-H coupling constant indicates that the rigid hydrogen bonding relationship between the 14-hydroxyl and the 9,11-epoxide of triptolide has been removed, as a consequence of thiol attack at C-9 from the α side to produce the thiol adduct **6**. The ultraviolet absorption at 214 nm (log ε = 4.06) indicates that the α,β-unsaturated lactone is still present in the thiol adduct **6**.

Treatment of **1** ($10^{-2}M$ in a mixture of methanol and phosphate buffer, *p*H 7.4, 1 : 1) with a 100-fold excess of propanethiol for 1 week as above gave **5** (*10*), melting point 240° to 242°C, in 78 percent yield. In contrast to the reactivity of **1**, **4** was recovered unchanged (92 percent recovery) after treatment under analogous reaction conditions. The estimated half-lives of the pseudo first-order reaction were ≤ 70 hours and ≥ 1400 hours, for **1** and **4**, respectively. The increased reactivity of the antileukemic triptolides, **1** and **2**, relative to the inactive 14-epimeric derivative, **4**, may be attributable to hydroxyl-group participation in the epoxide opening of the former compounds, as depicted in **7** (*15*).

The results of the biological and chemical studies are in accord with the hypothesis that the hydroxyl-assisted attack by nucleophiles on the 9,11-epoxide may mimic the mechanism by which the antileukemic triptolides exert their biological activity.

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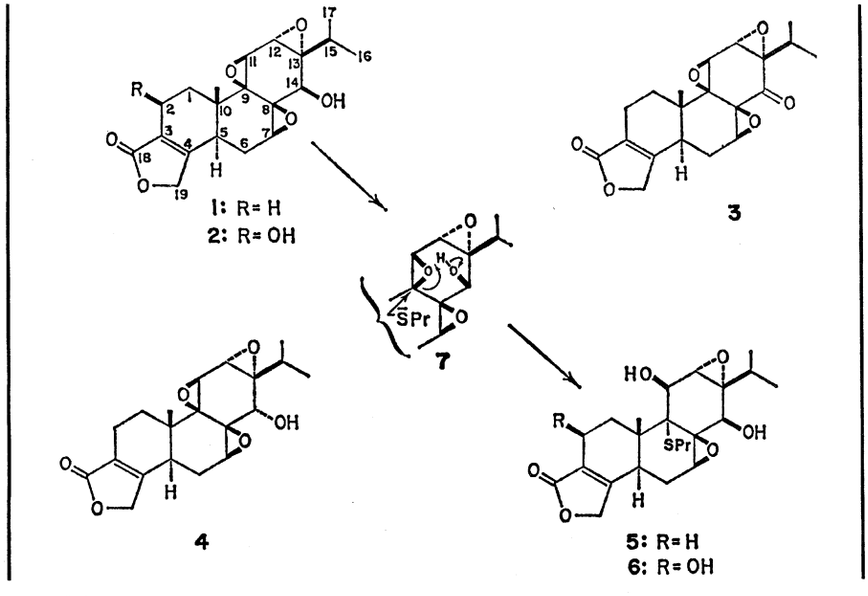

References and Notes

1. S. M. Kupchan, W. A. Court, R. G. Dailey, Jr., C. J. Gilmore, R. F. Bryan, *J. Am. Chem. Soc.* **94**, 7194 (1972).
2. Antileukemic activity and cytotoxicity against KB cells in culture were assayed under the auspices of the National Cancer Institute as described in *Cancer Chemother. Rep.* **25**, 1 (1962). Evaluation of assay results on a statistical basis in sequential testing is such that a material is considered active if it causes an increase in survival of treated animals (*T*) over controls (*C*) resulting in a $T/C \geq 125$ percent.
3. S. M. Kupchan, D. C. Fessler, M. A. Eakin, T. J. Giacobbe, *Science* **168**, 376 (1970).
4. R. L. Hanson, H. A. Lardy, S. M. Kupchan, *ibid.*, p. 378.
5. C. H. Smith, J. Larner, A. M. Thomas, S. M. Kupchan, *Biochim. Biophys. Acta* **276**, 94 (1972).
6. J. R. Lillehaug, K. Kleppe, C. W. Sigel, S. M. Kupchan, *ibid.* **327**, 92 (1973).
7. S. M. Kupchan, *Intra-Sci. Chem. Rep.*, in press.
8. N. L. Bauld and Y. S. Rim, *J. Org. Chem.* **33**, 1303 (1968); R. D. Stolow and A. A. Gallo, *Tetrahedron Lett.* (1968), p. 3331; E. F. Kiefer, W. Gericke, S. T. Amimoto, *J. Am. Chem. Soc.* **90**, 6246 (1968).
9. R. J. Ouellette, *J. Am. Chem. Soc.* **86**, 4378 (1964); C. R. Rader, *ibid.* **88**, 1713 (1966); J. J. Uebel and H. W. Goodwin, *J. Org. Chem.* **31**, 2040 (1966); R. J. Ouellette, D. L. Marks, D. Miller, *J. Am. Chem. Soc.* **89**, 913 (1967).
10. 14-Epitriptolide (**4**) was the main product

obtained from the reduction of **3** with sodium borohydride. The compound melted at 246° to 247°C. Elemental analysis and ultraviolet, infrared, NMR, and mass spectral data were concordant with the assigned structures for **4** and **5**.
11. S. M. Kupchan, *Trans. N.Y. Acad. Sci.* **32**, 85 (1970).
12. ———, M. A. Eakin, A. M. Thomas, *J. Med. Chem.* **14**, 1147 (1971).
13. The physical constants for **6** are: melting point (with decomposition) 249° to 250°C; ultraviolet absorption maximum (ethanol) (log ϵ), 214 (4.06) nm; infrared absorption maximum (Nujol), 2.90, 5.70, 5.93 μm; NMR ($CDCl_3$) τ 9.12 (doublet, $J = 7$ hertz, 16-CH_3), 8.97 (doublet, $J = 7$ hertz, 17-CH_3), 8.94 (triplet, $J = 7$ hertz, Pr-CH_3), 8.69 (singlet, 20-CH_3), 7.27 (triplet, $J = 7$ hertz, S-CH_2), 7.00 (doublet, $J = 7$ hertz, 14-H; collapses to a singlet on D_2O addition), 6.82 (doublet, $J = 5$ hertz, 12-H), 6.65 (doublet, $J = 5$ hertz, 7-H), 6.23 (doublet, $J = 5$ hertz, 11-H), 5.38 (multiplet, 2-H), 5.22 (triplet, $J = 1$ hertz, 19-CH_2); mass spectrum *m/e* 452; elemental analysis, found: C, 61.02 percent; H, 7.28 percent.
14. R. M. Silverstein and G. C. Bassler, *Spectrometric Identification of Organic Compounds* (Wiley, New York, ed. 2, 1967), p. 137.
15. While this work was in progress, it was reported that neighboring hydroxyl-group participation in the opening of 4α,5α-epoxycholestane derivatives by azide leads to significant rate enhancement. The estimated half-lives for the pseudo first-order reaction were 5 hours and 90 hours for the 7α-hydroxy and 7β-hydroxy derivatives, respectively [D. H. R. Barton and Y. Houminer, *J. Chem. Soc. Chem. Commun.* (1973), p. 839].
16. This communication is part 94 in the series entitled "Tumor Inhibitors"; part 93 is by S. M. Kupchan (*Revista Latinoamericana de Química*, in press). Supported by grant CA-11718 from the National Cancer Institute, grant CI-102J from the American Cancer Society, and an NIH postdoctoral fellowship to R.M.S.

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Aglomerularism in Antarctic Fish

Abstract. *Urine formation in antarctic bony fish does not involve glomerular filtration. Evidence for aglomerularism came from both direct observation of kidney serial sections by light microscopy and the low concentrations of inulin labeled with carbon-14 that were excreted into the urine when this renal clearance tracer was injected into the bloodstream via a cannula implanted in the caudal vein. Aglomerularism most likely prevents urinary loss of glycoproteins with biological antifreeze properties.*

Certain of the bony fish lack glomeruli as functional units in their renal morphology and consequently have a radically different mode of urine formation from that of the other vertebrates. In its most proximal region, the nephron of these fish ends as a blind tubule with no Bowman's capsule or associated glomus; as a result no filtration process occurs. The urine is formed mainly by active secretion of material across the renal tubular epithelium into the nephron lumen. There is some reabsorption from the formative urine to conserve materials which have moved by passive diffusion from the blood into the tubule lumen, but by far the major process is active secretion (*1*).

Many species of antarctic bony fish avoid freezing because they possess a series of glycoproteins with antifreeze properties (*2–4*). The blood, coelomic fluid, and pericardial fluid all contain these glycoproteins and show a difference of as much as 1.5°C between their freezing and melting points, a phenomenon referred to as thermal hysteresis (*4*). These glycoproteins account for 4 percent of the blood (weight to volume) and they differ only in size (*2*). The concentration (by weight) of the small glycoproteins (molecular weights 8000, 3500, and 2600) is five times as great as that of the larger ones (molecular weights 10,500 to 33,000) (*5*). In the vertebrate kidney molecules with molecular weights less than approximately 40,000 are generally filtered into the formative urine at the glomerulus (*6, 7*), and in view of the high concentration of glycoprotein antifreeze molecules in the blood and their small sizes one would expect them also to be present in the urine of the antarctic fish. However, examination of antarctic teleost urine reveals that it freezes and thaws at the same temperature (approximately -1.0°C), indicating that the glycoprotein antifreeze molecules are not excreted in the urine.

There are two possible explanations for the absence of glycoprotein antifreeze in the urine of these fish: (i) the glycoproteins are filtered at the glomerulus and actively reabsorbed in the proximal tubule of the nephron in much the same manner as glucose or (ii) the kidney of the antarctic fish is aglomerular, preventing the movement of glycoproteins into the formative urine.

In the study reported here whole kidneys were dissected from live animals caught in McMurdo Sound, Antarctica, and fixed for light microscopy in Bouin's solution. The renal tissue was embedded in paraffin, serially sectioned, and stained by standard histological techniques. The kidneys of *Trematomus borchgrevinki, T. bernacchii, T. hansoni, T. lepidorhinus, T. loennbergii,* and *Gymnodraco acuticeps* were completely aglomerular (Fig. 1). Because of its large size the kidney of *Dissostichus mawsoni* could be sampled only in selected regions; however, no glomeruli were found in any of the sections.

The excretion of ^{14}C-labeled inulin (New England Nuclear) was monitored as a physiological test for the aglomerular condition. Clearance of this polysaccharide (molecular weight 5000 to 5500) is commonly employed as a measure of glomerular filtration rate because it is freely filterable at the glomerulus and is neither secreted nor reabsorbed by the tubular epithelium (*6, 8*). Specimens of *T. hansoni, T. bernacchii,* and *D. mawsoni* were fitted with urinary catheters for continuous urine collection and [^{14}C]-inulin was introduced into the bloodstream via a cannula in the caudal vein. Specimens of *T. hansoni* and *T. bernacchii* weighing approximately 200 g each were injected with 20 μc of [^{14}C]inulin, and a 10-kg specimen of *D. mawsoni* was injected with 100 μc. Urine was collected continuously and 100-μl blood samples were collected periodically. Fifty-microliter samples of urine or blood plasma were mea-

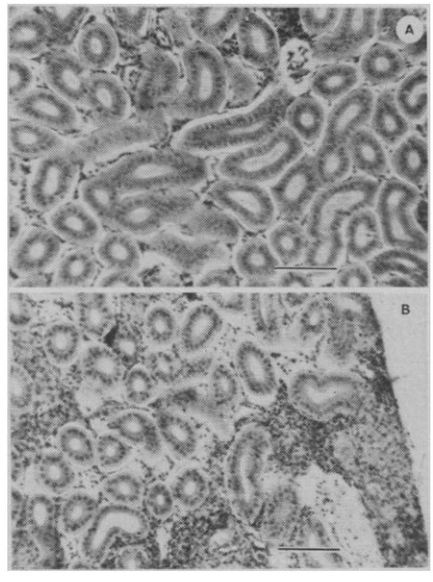

Fig. 1. (A) Photomicrograph of a representative histological section of the renal tubules of *Trematomus hansoni* in the posterior kidney region. (B) Photomicrograph of renal tissue in the trunk kidney of *Trematomus bernacchii*. The intertubular region contains hemopoietic tissue resembling that found in the head kidney. Sections in (A) and (B) were 7-μm thick and were stained with hematoxylin and eosin (scale bars, 100 μm).