tion and X and Y are in the symmetry planes. If the wave functions are taken to be real, $m^z_{n\pi,0}$ is pure imaginary, so that the indicated factor $i = \sqrt{-1}$ yields a real rotatory strength.

Since $i\theta^{xy}_{0,n\pi}m^z_{n\pi,0}$ is itself an observable,[5] the expression gives the absolute configuration of the composite system in terms of parameters measurable in the independent subsystems. Approximating the n–π^* transition as $p_y \leftarrow p_x$ on the oxygen (and thus further specifying the orientation of the coordinate system), the factor is calculated to be negative and large in atomic units. It will not be sensitive in sign to refinement of the transition description. With this result, eq 2 gives the empirically verified sign of rotatory strength.

Equation 2 as given applies only to nonpolar isotropic perturbers, or a tetrahedral arrangement of equivalent bonds approximating the methyl group, or any nonpolar groups which attain virtual isotropy through free rotation about bonds. With a persistent anisotropy of the perturber, the simple XYZ octant behavior is modified by terms with more complicated coordinate dependence. If the perturber is dipolar or charged, terms which must be added to eq 2 obtain within the same theoretical framework.

In the case of a charged perturber, the added contribution that arises is identical in coordinate dependence with that which Schellman obtained by general symmetry arguments.[7] A previously proposed perturbation,[8,9] the incompletely screened nucleus which acts like a static charge, also should give XY dependence for its leading terms. However, limitation of the wave function basis set and neglect of multicenter integrals can circumvent such terms, causing the first nonvanishing ones to have XYZ dependence.

It has been suggested that the magnitude of incomplete screening and hence the concomitant differential overlap of subsystems has been considerably overestimated.[10] Equation 2, on the other hand, with parameters appropriate to 3-methylcyclopentanone,[8] gives a magnitude completely satisfactory in its agreement with experiment. Using polarizabilities measured at zero frequency and taking the transition as $p_y \leftarrow p_x$, the partial molecular rotation at the D line is calculated to be about 120°. The observed total molecular rotation is 130°.[8]

As presented here, vicinal action arises from additive van der Waals interactions. The development is essentially an extension of the Kirkwood polarizability theory of optical activity[11] considering a transition ignored in that work. Electric transition moment "borrowing" that is implicit in this approach readily accounts for the modification of ordinary absorption intensity laid to charge-transfer processes, the basis of yet another interpretation of the octant rule.[12] Nevertheless the fundamental question remains, which only extensive calculation can answer, as to how large and important overlap may be in α- or β-substituted halo ketones and the like.

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(5) The term is the *difference* of components of a second-order absorption mechanism.[6]

(6) See, for example, G. Araki, *Phys. Rev.*, 74, 472 (1948); H. F. Hameka, "Advanced Quantum Chemistry," Addison-Wesley Publishing Co., Inc., Reading, Mass., 1965, p 123.

(7) J. A. Schellman, *J. Chem. Phys.*, 44, 55 (1966).

(8) W. J. Kauzmann, J. E. Walter, and H. Eyring, *Chem. Rev.*, 26, 339 (1940).

(9) A. Moscowitz, *Advan. Chem. Phys.*, 4, 67 (1962).

(10) M. V. Vol'kenshtein and M. P. Kruchek, *Opt. Spectry*, 9, 243 (1960).

(11) J. G. Kirkwood, *J. Chem. Phys.*, 5, 479 (1937).

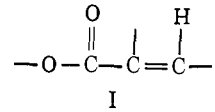
(12) S. F. Mason, *Quart. Rev.* (London), 17, 20 (1963), and references cited therein.

The Isolation and Structural Elucidation of Two Novel Sesquiterpenoid Tumor Inhibitors from *Elephantopus elatus*[1,2]

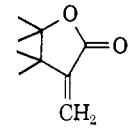
Sir:

In a search for tumor inhibitors from plant sources, alcoholic extracts of dried *Elephantopus elatus* Bertol. (Compositae)[3] showed significant inhibitory activity *in vitro* against cells derived from human carcinoma of the nasopharynx (KB).[4] We report herein the isolation and structural elucidation of two novel tumor-inhibitory sesquiterpene dilactones of the germacrane type from *E. elatus*.

Fractionation involving successive partitions and chromatography yielded elephantin[5] (IVa), $C_{20}H_{22}O_7$, mp 242–244°, $[\alpha]^{27}D$ −380°, λ_{max}^{MeOH} 215 mμ (ε 25,200), λ_{max}^{KBr} 5.64, 5.68, 5.84, and 6.07 μ, and nmr[6] signals at τ 1.95 (2.42 in $DCCl_3$, 1 H, s, I),[7,8] 3.85 and 4.22 (2 H, doublets, J = 3 cps, II), 4.24 (1 H, s, vinyl H), 4.50



I



II

(m, >CH–O or vinyl H), 5.80 (2 H, m, >CH–O), 7.97 and 8.12 (6 H, doublets, J = 1 cps, vinyl methyls), and 8.83 (3 H, s, tertiary CH_3). Alkaline hydrolysis of elephantin (IVa) gave elephantol (IIIc, $C_{15}H_{16}O_6$, mp 282–284°, $[\alpha]^{27}D$ +274°, λ_{max}^{MeOH} 209 mμ (ε 18,300), λ_{max}^{KBr} 2.94, 5.68, 5.73, and 6.06 μ) and dimethylacrylic acid.

Further chromatography yielded a dilactone, elephantopin[5] (IVb), $C_{19}H_{20}O_7$, mp 262–264°, $[\alpha]^{25}D$

(1) Tumor Inhibitors. XVI. Part XV: S. M. Kupchan, S. Kubota, E. Fujita, S. Kobayashi, J. H. Block, and S. A. Telang, *J. Am. Chem. Soc.*, in press.

(2) Supported by grants from the National Cancer Institute (CA-04500), the American Cancer Society (T-275), the National Science Foundation (G-B2878), and a contract with the C.C.N.S.C. (PH 43-64-551).

(3) Specimens were gathered in Florida, Sept. 1963. The authors acknowledge with thanks receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U. S. Department of Agriculture, Beltsville, Md., in accordance with the program developed with the U. S. Department of Agriculture, by the C.C.N.S.C.

(4) Cytotoxicity and *in vivo* inhibitory activity were assayed under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, by the procedures described in *Cancer Chemotherapy Rept.*, 25, 1 (1962).

(5) Elephantin and elephantopin showed significant inhibitory activity against Walker carcinosarcoma 256 in rats at 50–100 mg/kg and cytotoxicity (ED_{50}) against KB (human carcinoma of the nasopharynx) cell culture at 0.28–2.0 μg/ml.[4]

(6) Determined in hexadeuteriodimethyl sulfoxide.

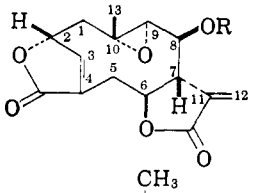
(7) H. Immer, J. Polonsky, R. Toubiana, and H. D. An, *Tetrahedron*, 21, 2117 (1965).

(8) W. Herz, H. Watanabe, M. Miyazuki, and Y. Kishida, *J. Am. Chem. Soc.*, 84, 2601 (1962).

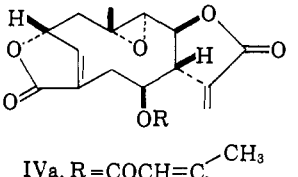
$-398°$, λ_{max}^{MeOH} 210 mμ (ϵ 27,000), λ_{max}^{KBr} 5.68, 5.73, 5.86, 6.08, and 6.12 μ, and nmr signals at τ 1.93 (1 H, s), 3.82 and 4.19 (2 H, doublets, J = 3 cps), 3.84 and 4.21 (2 H, singlets), 4.49 (1 H, m), 5.83 (2 H, m), 8.12 (3 H, s), and 8.82 (3 H, s). Alkaline hydrolysis of elephantopin (IVb) gave elephantol (IIIc) and methacrylic acid.

Hydrogenation of IVb (Pd–C) gave tetrahydroelephantopin (VIa), $C_{19}H_{24}O_7$, mp 290–292°, $[\alpha]^{25}D$ $-370°$, λ_{max}^{MeOH} 210 (ϵ 10,600), λ_{max}^{KBr} 5.64, 5.78, 5.83, and 6.08 μ, and nmr signals at τ 5.42 (1 H, m) and 5.97 (1 H, t, J = 8 cps). Alkaline hydrolysis of tetrahydroelephantopin (VIa) gave dihydroelephantol (Vb), $C_{15}H_{18}O_6$, mp 288–290°, $[\alpha]^{30}D$ $+38°$, λ_{max}^{MeOH} 211 mμ (ϵ 9600), λ_{max}^{KBr} 2.90, 5.62, and 5.75 μ, and nmr signals at 4.75 (1 H, d, J = 5 cps, –OH) and 6.02 (1 H, m).

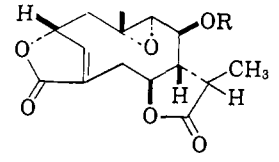
Elephantol *p*-bromobenzoate (IIIb), $C_{22}H_{19}BrO_7$, mp 302–304°, $[\alpha]^{25}D$ $+125°$, λ_{max}^{MeOH} 246 mμ (ϵ 25,600), λ_{max}^{KBr} 5.69, 5.74, 5.78, and 6.23 μ, crystallized from methanol in the orthorhombic system, space group $P2_12_12_1$, with four molecules of $C_{22}H_{19}BrO_7$ in a cell of dimensions a = 10.64, b = 30.34, c = 6.41 A. The atoms were located by evaluating three-dimensional electron density distributions with Fourier coefficients weighted according to the method proposed by Sim.[9] Two Fourier syntheses were used in the elucidation of the molecular structure and the approximate coordinates were then refined by least-squares calculations. The present value of R for 1690 independent X-ray reflections is 11.9%. The results establish that the *p*-bromobenzoate has the structure and stereochemistry IIIb. The absolute configuration was determined by Bijvoet's method,[10] based on the anomalous dispersion by the bromine atom of the Cu Kα radiation. The absolute stereochemistry shown in IIIb yields a value of R for the estimated reflections $h\bar{k}l$ of 11.9%, whereas for the opposite configuration the value is 12.2%.



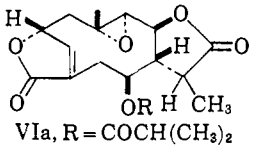
IIIa, R = COC=CH$_2$ (CH$_3$)
b, R = COC$_6$H$_4$-*p*-Br
c, R = H



IVa, R = COCH=C(CH$_3$)$_2$
b, R = COC=CH$_2$ (CH$_3$)
c, R = H



Va, R = COCH(CH$_3$)$_2$
b, R = H



VIa, R = COCH(CH$_3$)$_2$
b, R = H

Acylation of elephantol (IIIc) with methacrylic anhydride gave an isomer of elephantopin (IVb), elephantol methacrylate (IIIa), $C_{19}H_{20}O_7$, mp 238–240°, $[\alpha]^{26}D$ $+218°$, λ_{max}^{MeOH} 209 mμ (ϵ 21,400), λ_{max}^{KBr} 5.65, 5.78, 5.86, 6.09, and 6.15 μ. Similar treatment of dihydroelephantol (Vb) gave an isomer of VIa, dihydroelephantol isobutyrate (Va), $C_{19}H_{24}O_7$, mp 201–203°, $[\alpha]^{26}D$ $+210°$, λ_{max}^{MeOH} 211 mμ (ϵ 10,300), λ_{max}^{KBr} 5.64, 5.72, 5.82, and 6.05 μ, and nmr signals at τ 5.02 (1 H, t, J = 9 cps) and 5.85 (1 H, m).

In dihydroelephantol isobutyrate (Va) the signal at τ 5.02 (t, J = 9 cps) corresponds to the proton at ester-bearing C-8 while the multiplet at τ 5.85 corresponds to the proton (spin coupled to three protons) at lactone-bearing C-6. In contrast, the spectrum of tetrahydroelephantopin (VIa) shows a multiplet at τ 5.42, assigned to the proton at ester-bearing C-6, while the lactone proton signal appears as a clean triplet at τ 5.97, indicating attachment to C-8. The latter results indicated that a lactone rearrangement[11] had taken place during alkaline hydrolysis. In accord with this, *acid* hydrolysis of tetrahydroelephantopin (VIa) gave dihydroelephantolide (VIb, isomeric with Vb), $C_{15}H_{18}O_6$, mp 300–302°, $[\alpha]^{25}D$ $-273°$, λ_{max}^{MeOH} 211 mμ (ϵ 8700), λ_{max}^{KBr} 2.85, 5.75, and 6.10 μ, and nmr signals at τ 4.46 (1 H, d, J = 5 cps, –OH) and 6.02[12] (1 H, t, J = 9 cps). Treatment of VIb with isobutyric anhydride gave tetrahydroelephantopin (VIa), and treatment of VIb with base gave Vb.

High-resolution mass spectra confirmed the elemental composition of all compounds mentioned. Comparison of the mass spectra of elephantopin (IVb) and tetrahydroelephantopin (VIa) with those of elephantol methacrylate (IIIa) and dihydroelephantol isobutyrate (Va) strongly supports the assigned structures. All compounds exhibit prominent loss of the ester side chain from the molecular ions (mol wt 360 for IVb and IIIa, 364 for VIa and Va) to yield ions of *m/e* 275 and 274 (in the case of IVb and IIIa) or *m/e* 277 and 276 (for VIa and Va). In the case of compounds IVb and VIa, further elimination of 58 mass units (C_3H_6O) gives rise to the intense peaks at *m/e* 216 ($C_{12}H_8O_4$, as determined from the high-resolution mass spectrum, for IVb) and 218 ($C_{12}H_{10}O_4$, for VIa), respectively. This process plays a minor role for IIIa and Va, where the loss of the ester side chain is followed by elimination of 96 mass units ($C_5H_4O_2$, representing one of the lactone rings) and gives rise to intense peaks at *m/e* 178 ($C_{10}H_{10}O_3$, for IIIa) and 180 ($C_{10}H_{12}O_3$, for Va), which are virtually absent in the spectra of IVb and VIa. This difference is explicable by considering that loss of the elements of methacrylic acid (from IVb and IIIa) or isobutyric acid (from VIa and Va) may result in the formation of a C-6,7 double bond (for IVb and VIa) or one at C-7,8 (for IIIa and Va). For VIa and IVb, subsequent transfer of a C-5 hydrogen to the epoxide with concomitant ring opening may be followed by a McLafferty rearrangement (involving the C-2 hydrogen atom) to eliminate a fragment of composition C_3H_6O and yield ions of *m/e* 216 for IVb or 218 for VIa. For compounds IIIa and Va, however, simple cleavage of the C-5,6 and C-1,2 bonds should be

(9) G. A. Sim, *Acta Cryst.*, 12, 813 (1959); 13, 511 (1960); "Computing Methods and the Phase Problem in X-ray Crystal Analysis," R. Pepinsky, J. M. Robertson, and J. C. Speakman, Ed., Pergamon Press, Oxford, 1961, p 227.

(10) J. M. Bijvoet, A. F. Peerdeman, and A. J. van Bommel, *Nature*, 169, 271 (1951).

(11) *Cf. e.g.*, D. H. R. Barton, O. C. Bockman, and P. de Mayo, *J. Chem. Soc.*, 2263 (1960); W. Herz, Y. Kishida, and M. V. Lackshmikantham, *Tetrahedron*, 20, 979 (1964).

(12) Compare multiplicity of corresponding signal in Vb.

favored, leading directly to the prominent fragments of *m/e* 178 (IIIa) or 180 (Va) and 96.

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A Procedure for the Addition of Amino Acid Residues to the Amino Groups of Insulin. Trimethionyl-insulin

Sir:

Amino acid residues have been added to the amino groups of insulin through the reaction of carbamino anhydrides of amino acids (Leuchs' anhydrides) with aqueous solutions of insulin.[1,2] The resulting products retained a considerable fraction of the biological activity of insulin. Although the anhydrides reacted to some extent with both of the α-amino groups at the N terminus of the two chains of insulin as well as with the ϵ-amino group of lysine, in no case did all of the amino groups completely react. This was true even when large excesses of reagent were used, resulting in incorporation of up to 7.3 amino acid residues per mole of insulin.[1] Such products must be considered as mixtures of molecules in which some of the original amino groups are unsubstituted while others may be substituted by single or poly amino acid residues. The significance of biological activity in such mixtures is understandably subject to some ambiguities in interpretation.

We wish to report a procedure by which new amino acid residues can be attached to all of the amino groups in insulin in such a fashion as to put only one amino acid residue on each amino group and thereby give essentially one product. The procedure, which is based on recent developments in peptide chemistry,[3-5] involves the reaction of *t*-butyloxycarbonyl (*t*-BOC) amino acid *p*-nitrophenyl esters with insulin in dimethylformamide, followed by removal of the *t*-BOC groups in anhydrous trifluoroacetic acid. The success of this method rests in part on the marked stability of insulin in acidic media.

Insulin hydrochloride[6] (60 mg, 10 μmoles) was dissolved in 5.0 ml of dimethylformamide and then triethylamine (10.0 mg, 100 μmoles) was added along with the *p*-nitrophenyl ester of *t*-BOC-methionine[7] (110 mg, 300 μmoles). The reaction was allowed to proceed for 18 hr at room temperature and the insulin derivative was precipitated and washed with ether. The resulting product, which was ninhydrin negative, was dried thoroughly over P_2O_5 under high vacuum and then dissolved in 2.0 ml of anhydrous trifluoroacetic acid. The solution was kept for 1 hr at room temperature by which time the ninhydrin color had reached a maximum value. The insulin derivative was precipitated, washed with ether, and dried. The residue was subjected to two isoelectric precipitations by dissolving it in 7 ml of 0.5 *M* acetic acid (containing 1 mg of zinc acetate) and precipitating at pH 5.9 with ammonium hydroxide. The final precipitate was collected by centrifugation, washed with water, acetone, and ether, and dried under vacuum; yield 65 mg, 93%.

Acid hydrolysis and amino acid analysis[8] revealed the incorporation of 3.0 methionine residues/mole of insulin. The trimethionyl-insulin was further characterized by a modified Edman degradation.[9] The ethylene chloride soluble thiazolinone was hydrolyzed in alkali.[10] Amino acid analysis of the hydrolysate showed the presence of three residues of methionine/mole of derivative and no other amino acids. Acid hydrolysis of the ethylene chloride insoluble residue, followed by amino acid analysis, afforded an amino acid composition identical with that of insulin. In another characterization, the trimethionyl-insulin derivative was treated with TPCK-trypsin.[11] No alanine was formed and a nonapeptide with the composition $Gly_{1.02}Phe_{1.93}Tyr_{0.98}Thr_{0.94}Pro_{0.99}Lys_{0.90}Met_{1.05}Ala_{1.00}$ was isolated. The action of trypsin on native insulin releases a heptapeptide, Gly-Phe-Phe-Tyr-Thr-Pro-Lys, and alanine.[12] The fact that no alanine was liberated on treating trimethionyl-insulin with trypsin,[13] along with the isolation of a methionine-containing nonapeptide, constitutes good evidence for locating one methionine residue attached to the ϵ-amino group of lysine at position 29 of the B chain. The remaining two methionine residues which were liberated by the Edman degradation must be attached to the N terminals of the A and B chains. These results indicate that the reagent was quite specific in that it reacted only with the free amino groups of insulin.

In the mouse convulsion assay the trimethionyl-insulin possessed a biological activity of 12.3 ± 1.7 units/mg. A control sample of insulin which had been put through all of the manipulations involved in this synthesis with the exception of deletion of the acylating reagent was recovered in crystalline form with essentially no loss in biological activity, 20.4 ± 4.5 units/mg.

The method described here should be applicable to the addition of a variety of amino acid residues to insulin and may be generally applicable to other proteins as a specific procedure for amino group modification. Experiments are in progress involving a correlation of biological activity and conformational changes with the addition of neutral, basic, and acidic amino acid residues on the insulin molecule.

Acknowledgment. We wish to thank Mrs. Joan E. Hafeez for aid with the amino acid analysis and Dr. E. L. Grinnan of Eli Lilly and Co. for generous supplies of insulin and for arranging for the biological assays.

(1) H. Fraenkel-Conrat, *Biochim. Biophys. Acta*, 10, 180 (1953).
(2) T. K. Virupaksha and H. Tarver, *Biochemistry*, 3, 1507 (1964).
(3) L. A. Carpino, *J. Am. Chem. Soc.*, 81, 955 (1959).
(4) M. Bodanszky, *Nature*, 175, 685 (1955).
(5) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, 44, 1991 (1961).
(6) F. H. Carpenter, *Arch. Biochem. Biophys.*, 78, 539 (1958).
(7) E. Scoffone, F. Marchiori, A. M. Tamburro, and R. Rocchi, *Gazz. Chim. Ital.*, 94, 695 (1964).
(8) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, 30, 1190 (1958).
(9) R. F. Doolittle, *Biochem. J.*, 94, 742 (1965).
(10) B. Africa and F. H. Carpenter, *Biochem. Biophys. Res. Commun.*, in press.
(11) V. Kostka and F. H. Carpenter, *J. Biol. Chem.*, 239, 1799 (1964).
(12) F. H. Carpenter and W. E. Baum, *ibid.*, 237, 409 (1962).
(13) When the ϵ-amino group of lysine is acylated, the α-peptide bond is resistant to the action of trypsin: C. B. Anfinson, M. Sela, and H. Tritch, *Arch. Biochem. Biophys.*, 65, 156 (1956).