on which the reaction is dependent is perturbed by 0.34 pK units to pK_a = 5.03 in D_2O, and the rate constant is decreased by 3.35-fold in D_2O.

The following indirect method was used to obtain the difference spectrum of *trans*-cinnamoylpapain *vs.* papain, since this acyl-enzyme cannot be prepared quantitatively. A difference spectrum of a solution of the acyl-enzyme and enzyme (freed from excess substrate by Sephadex filtration) *vs.* enzyme was obtained using a Cary 14 PM spectrophotometer. The spectrum was obtained immediately after filtration and was repeated at 7 min. intervals until 3 to 4 half-lives had elapsed. By combining the spectral data and data on the concentration of cinnamic acid at various times, it was possible to calculate the difference spectrum of *trans*-cinnamoylpapain, using eq. 1, where ϵ_λ^{CP} is the (difference) molar absorptivity of

$$\epsilon_\lambda^{CP} - \epsilon_\lambda^{C} = \frac{A_\lambda^{t} - A_\lambda^{\infty}}{[C]^{\infty} - [C]^{t}} \qquad (1)$$

the acyl-enzyme *vs.* enzyme at any wave length. Values of A_λ^t, the absorbance at time t and wave length λ, known to $\pm 0.6\%$, were taken from the early spectra. Values of A_λ^∞ were calculated from plots of the absorbance *vs.* time data from the repeated spectra.[7] Values of $[C]^t$, the concentration of cinnamic acid at time t, were found by spectrophotometric determination of cinnamic acid in aliquots of the reaction mixture; the cinnamic acid was separated from the acyl-enzyme and enzyme by Sephadex filtration and its concentration at various times was determined. ϵ_λ^C is the molar absorptivity of cinnamic acid at wave length λ.

The λ_{max} for the difference spectrum of *trans*-cinnamoylpapain *vs.* papain calculated on this basis, 326 mμ, is considerably higher than those of *trans*-cinnamoyl derivatives of three serine proteinases (Table I), indicating that *trans*-cinnamoylpapain is a different

TABLE I

DIFFERENCE SPECTRA OF SOME CINNAMOYL-ENZYMES AND CINNAMOYL ESTERS[a]

trans-Cinnamoyl-	λ_{max}, mμ	ϵ_{max}	$\lambda_{max} - \lambda_{max}$(model) m$\mu$	Δkcal.
Papain[d]	326[g]	26,500[c]	20	5.73
α-Chymotrypsin[2]	292[h]	17,700	10.5	3.65
Trypsin[i]	296	19,400	14.5	4.98
Subtilisin[b]	289	21,000	7.5	2.65
N-Acetylserinamide[e]	281.5	24,300	..	..
Cysteine[f]	306	22,600	..	..

[a] pH 4, 1.6% (v./v.) acetonitrile–water, 25°. [b] Unpublished observations of Dr. M. L. Begué. [c] Probable error in absorptivity is $\pm 3\%$. [d] 0% CH_3CN, pH 3.43. [e] 10% CH_3CN. [f] pH 2.1. [g] 309 mμ after treatment with 4.8 M guanidinium chloride. [h] 282 mμ at 51°.[j] [i] M. L. Bender and E. T. Kaiser, *J. Am. Chem. Soc.*, **84**, 2556 (1962). [j] J. Mercuroff and G. P. Hess, *Biochem. Biophys. Res. Commun.*, 11, 283 (1963).

chemical species than the other three cinnamoyl-enzymes. Ruling out *trans*-cinnamoylimidazole because of the pH dependence of the deacylation (Fig. 1), the closest model compound of *trans*-cinnamoylpapain is the thiol ester S-cinnamoylcysteine,[8] which is found to have a λ_{max} of 306 mμ. Thus, the λ_{max} of *trans*-cinnamoylpapain is significantly higher than the λ_{max} of its model compound just as the λ_{max} of *trans*-cinnamoyl-α-chymotrypsin and the other acyl-serine proteinases are higher than the λ_{max} of the model compound, N-acetylserinamide. In energy terms, the electronic transition of the conjugated system is 4.2 ± 1.5 kcal./mole less in the cinnamoyl-enzyme than in the cinnamoyl ester, for all enzymes investigated. This common relationship supports the spectral assignment of *trans*-cinnamoylpapain as a thiol ester. This conclusion is supported by the fact that denaturation of *trans*-cinnamoylpapain in 4.8 M guanidinium chloride gives a λ_{max} of 309 mμ, almost identical with that of the model, S-*trans*-cinnamoylcysteine. Although an acylimidazole would be expected to acylate a thiol (papain) faster than an alcohol (α-chymotrypsin), this result is not found, presumably because of differing specificities of these two enzymes.

The common relationship between the absorption maxima of *trans*-cinnamoyl-enzymes of different chemistry and their model compounds (Table I) may result from a common interaction of the conjugated system and the protein.

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(7) F. J. Kézdy, J. Jaz, and A. Bruylants, *Bull. Soc. Chim. Belges*, **67**, 687 (1958).

(8) Synthesized by the method of L. Zervas, I. Photaki, and N. Ghelis, *J. Am. Chem. Soc.*, **85**, 1337 (1963).

Daunomycin. I. The Structure of Daunomycinone

Sir:

Daunomycin, a metabolite of *Streptomyces peucetius*,[1] is an antibiotic which exhibits strong inhibition of the growth of a variety of experimental tumors.[2]

Daunomycin hydrochloride, $C_{27}H_{29}O_{10}N \cdot HCl$,[3] m.p. 188–190° dec., $[\alpha]_D$ +253° (c 0.15, methanol), displays an ultraviolet spectrum closely related to that of 1,4,5-trihydroxyanthraquinones,[4] the maxima in the visible region being less defined and shifted 5–10 mμ toward longer wave lengths when compared directly with the spectrum of helminthosporin. The quinoid chromophore is also proved by the ready reversible reduction on treatment with reducing agents and by polarographic behavior. Mild acid hydrolysis (0.2 N hydrochloric acid for 1 hr. at 90°) affords a red aglycone, daunomycinone, and a new amino sugar, daunosamine.[5]

Daunomycinone, $C_{21}H_{18}O_8$, m.p. 213–214°, $[\alpha]_D$ +193° (c 0.1, dioxane), one OCH_3, same electronic spectrum as the parent glycoside, yields tetracene, identified by its ultraviolet spectrum, on zinc dust distillation, thus suggesting the tetracyclic structure already found in the anthracyclines.[6] The presence of an aliphatic ketonic group is shown by the infrared absorption at 1718 cm.$^{-1}$ and by the ready formation of a 2,4-

(1) A. Grein, C. Spalla, A. Di Marco, and G. Canevazzi, *Giorn. Microbiol.*, **11**, 109 (1963).

(2) A. Di Marco, M. Gaetani, P. Orezzi, B. Scarpinato, R. Silvestrini, M. Soldati, T. Dasdia, and L. Valentini, *Nature*, **201**, 706 (1964).

(3) Satisfactory elemental and functional group analyses together with consistent spectroscopic properties were obtained for all compounds. Melting points were taken at the Kofler microscopic hot stage and are uncorrected. Optical rotations were measured at 20 ± 3°. Infrared spectra (KBr) were determined with a Perkin Elmer Model 21 double-beam spectrophotometer.

(4) J. H. Birkinshaw, *Biochem. J.*, **59**, 485 (1955).

(5) F. Arcamone, G. Cassinelli, P. Orezzi, G. Franceschi, and R. Mondelli, *J. Am. Chem. Soc.*, **86**, 5335 (1964).

(6) H. Brockmann, *Fortschr. Chem. Org. Naturstoffe*, **21**, 121 (1963).

dinitrophenylhydrazone. The nuclear magnetic resonance spectrum[7] (CF_3COOH) shows one OCH_3 (singlet, δ 4.04), one $COCH_3$ (singlet, δ 2.69), broad absorptions at δ 3.27 (2 H), 2.67 (2 H), 7.1–7.9 (3 aromatic H), and a signal at 5.50 (1 H, broad), attributed to a proton on a benzylic carbon bearing a hydroxyl group because of its downfield shift (6.40) on acetylation.[8]

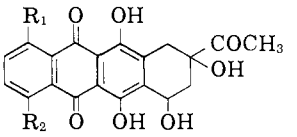
By refluxing with dimethyl sulfate in acetone in the presence of potassium carbonate daunomycinone is converted to a trimethyl ether, $C_{24}H_{24}O_8$, m.p. 193°, four OCH_3, $[\alpha]D$ +181° (*c* 0.1, dioxane), hydroxyl band at 3350 cm.$^{-1}$. This compound shows four sharp singlets at δ 4.00 (6 H, two aromatic OCH_3), 3.89 (3 H, aromatic OCH_3), 3.56 (3 H, aliphatic OCH_3), and 2.40 (3 H, $COCH_3$). A free hydroxyl (singlet, δ 5.02) is clearly recognized by the upfield shift with dilution and downfield shift with acid. A signal at δ 4.92 (1 H, four lines), showing the Ar-CH-O proton, is the X part of an ABX spectrum,[9] the AB part of which consists of two pairs[10] of symmetric doublets centered approximately at 1.87 (1 H) and 2.42 (1 H); a first-order analysis gives J_{AB} = 15 ± 0.2, J_{AX} = 3.5 ± 0.2, and J_{BX} = 2.5 ± 0.2 c.p.s. The magnitude of the J_{AB}, showing geminal coupling, and the shifts of H_A and H_B suggest a methylene β to an aromatic ring. Two doublets (2 H, J = 18.5 ± 0.2 c.p.s.) centered at δ 3.02 and 3.22 (AB pattern) indicate two geminal protons α to the aromatic system, without vicinal hydrogens. A complex multiplet (δ 7–8, 3 H, ABC pattern) suggests three aromatic protons on one ring. This is in agreement with the recovery of salicylic acid by alkaline fusion of either daunomycinone or its trimethyl ether.

The presence of four hydroxyls in daunomycinone is proved by the conversion, on treatment with acetic anhydride and pyridine at 60°, to a tetraacetate $C_{29}H_{26}O_{12}$, one OCH_3, m.p. 225° (from methanol), $[\alpha]D$ −95.5° (*c* 0.11, $CHCl_3$), phenolic (1776 cm.$^{-1}$) and alcoholic (1740 cm.$^{-1}$) acetate bands, no hydroxyl absorption in the infrared.[11] Treatment of daunomycinone with either acids or alkalis gives a bisanhydro derivative, $C_{21}H_{14}O_6$, m.p. 325–330°, conjugated ketone absorption (1685 cm.$^{-1}$), which in turn yields a diacetate, $C_{25}H_{18}O_8$, m.p. 240–243°, one OCH_3, phenolic acetate absorption (1765 cm.$^{-1}$), thus showing the presence of two phenolic and two alcoholic hydroxyls in daunomycinone, the last two being involved in the dehydration reaction.

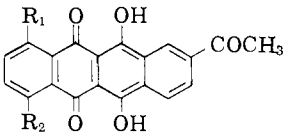
Hydrogenolysis of the benzylic hydroxyl of daunomycinone with Pd on $BaSO_4$ in dioxane affords deoxydaunomycinone, $C_{21}H_{18}O_7$, m.p. 229–231°, $[\alpha]D$ −91° (*c* 0.11, $CHCl_3$), one OCH_3. The n.m.r. spectrum shows OCH_3 (δ 4.05), $COCH_3$ (2.35), two strongly hydrogen-bonded phenolic OH (13.3 and 13.75), one free alcoholic OH (3.75), two benzylic CH_2 (broad, *ca.* 3), CH_2 β to the aromatic system (*ca.* 2), and three aromatic protons (*ca.* 8). Deoxydaunomycinone yields a triacetate, $C_{27}H_{24}O_{10}$ m.p. 126–128°.

Sodium borohydride reduction of daunomycinone, followed by periodate oxidation, affords acetaldehyde, isolated as the 2,4-dinitrophenylhydrazone, in good yield, thus proving the acetyl side chain and its attachment to a hydroxylated carbon atom. Oxidative fission with permanganate of bisanhydrodaunomycinone affords almost quantitatively 1,2,4-benzenetricarboxylic acid (trimellitic acid), m.p. 216–219°, and 3-methoxyphthalic acid, m.p. 168–171°, both identical in all respects with authentic samples.

On the basis of these findings structure I (a or b) and II (a or b), aside from stereochemistry, can be written for daunomycinone and for bisanhydrodaunomycinone, respectively.



Ia, R_1 = H, R_2 = OCH_3
b, R_1 = OCH_3, R_2 = H



IIa, R_1 = H, R_2 = OCH_3
b, R_1 = OCH_3, R_2 = H

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Daunomycin. II. The Structure and Stereochemistry of Daunosamine

Sir:

We wish to present evidence which assigns structure I to daunosamine, the amino sugar moiety of the antitumor antibiotic daunomycin.[1] Daunosamine [hydrochloride (I) $C_6H_{13}O_3N \cdot HCl$,[2] m.p. 168° dec., $[\alpha]D$ at equilibrium −54.5° (H_2O)] is a reducing (positive Fehling, Tollens) amino (positive Elson-Morgan, ninhydrin) trideoxyhexose, yielding ammonia on treatment with hot alkalis. Acetylation of I with acetic anhydride and pyridine gives a crystalline mixture of the anomeric triacetates (II), m.p. 168–170°, $[\alpha]D$ −71° (acetone).

The recovery of malonic dialdehyde, identified by the reaction with thiobarbituric acid,[3] and of acetaldehyde, isolated as the 2,4-dinitrophenylhydrazone, among the products of the periodate oxidation[4] of I indicates the presence of the deoxy group at C-2 (or C-3) and of the methyl group at C-5. The latter is also supported by the fact that I gives a positive iodoform test.

I is readily converted by treatment with 0.3 *N* methanolic HCl to methyl daunosaminide (III), m.p.

(7) N.m.r. spectra were taken with a Varian A60 spectrometer; chemical shifts are in p.p.m. (δ), relative to tetramethylsilane as internal standard.

(8) The spectrum ($CDCl_3$) of the monotrifluoroacetate of daunomycinone, formed in the trifluoroacetic acid solution on standing, shows the same proton at δ 6.55, two strongly hydrogen-bonded hydroxyls (sharp singlets at δ 13.0 and 13.6), and one free OH (broad, *ca.* δ 4.1).

(9) J. A. Pople, W. G. Schneider, and H. J. Bernstein, "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., Inc., New York, N. Y., 1959, p. 132.

(10) One of them is further split by a long-range coupling (*ca.* 1 c.p.s.).

(11) The n.m.r. spectrum confirms the information obtained by the spectra of the other compounds.

(1) F. Arcamone, G. Franceschi, P. Orezzi, G. Cassinelli, W. Barbieri, and R. Mondelli, *J. Am. Chem. Soc.*, **86**, 5334 (1964).

(2) See footnote 3 in ref. 1.

(3) V. S. Waravdecar and L. D. Saslaw, *J. Biol. Chem.*, **234**, 1945 (1959).

(4) R. W. Jeanloz and E. Forchielli, *ibid.*, **188**, 361 (1951).