The structure of the stylocheilamide degradation product (**8**) was deduced from a single-crystal X-ray diffraction experiment. Preliminary X-ray photographs showed orthorhombic symmetry. Accurate cell constants were determined by a least-squares fit of 15 moderate (35–45°) 2θ values. These were $a = 20.600$ (4), $b = 8.908$ (1), and $c = 6.403$ (1) Å. Systematic extinctions and the known optical activity uniquely indicated $P2_12_12_1$ as the space group. A measured and calculated density ($Z = 4$) of 1.29 g/cm^3 indicated that one molecule of composition $C_{11}H_{16}O_5$ formed the asymmetric unit. All unique diffraction maxima with $2\theta \leq 114°$ were collected on a Syntex $P2_1$ diffractometer with Cu Kα radiation (1.541 78 Å). A total of 971 reflections were explored and after correction for Lorentz, polarization, and background effect, 863 (89%) were considered observed ($F_o^2 \geq 3\sigma(F_o^2)$). There was no indication of crystal decomposition based on periodic examination of three standard reflections every hour and no correction for absorption was made.

A trial structure was arrived at by a multiple solution weighted tangent formula approach.[17] Full-matrix least-squares refinements with anisotropic nonhydrogen atoms and isotropic hydrogens smoothly converged to a standard crystallographic residual of 0.031 for the observed reflections. Fractional coordinates are given in Table I and the observed and calculted structure factors are given in Table IV.[19] The derived metric results, bond distances and bond angles, are given in Tables II and III, respectively.[19] A final difference synthesis displayed no unacceptably high electron density and there were no anomalously short intermolecular contacts.

Acknowledgments. We should like to thank Dr. William Fenical for the 220-MHz ^{1}H NMR spectrum of **3**, Mr. Jim Loo for ^{13}C NMR and high-resolution mass spectral data, and the National Science Foundation for partial financial support. Jon Clardy thanks the Camille and Henry Dreyfus Foundation for a Teacher and Scholar Grant (1972–1977).

Supplementary Material Available: Tables of fractional coordinates, structure factors, and bond distances and angles (7 pages). Ordering information is given on any current masthead page.

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Oxidation of Dihydronicotinamides by Flavopapain

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Abstract: Flavopapain **6** is prepared by the modification of the active site of papain with 7α-bromoacetyl-10-methylisoalloxazine (**5**). In its reactions with dihydronicotinamides this semisynthetic enzyme shows normal flavoenzyme behavior, exhibiting saturation kinetics at low substrate concentrations and modest rate accelerations when compared to appropriate model systems. Evidence has been obtained for the formation of a transient intermediate in the anaerobic redox reaction of flavopapain **6** with 1-benzyl-1,4-dihydronicotinamide. Although the nature of the intermediate remains to be established, a reasonable hypothesis is that it corresponds to a species in which the active site flavin function has moved from the site it occupies in the Michaelis complex to a new site where reaction with the dihydronicotinamide takes place.

Introduction

Much current research is concerned with the development of model catalysts simulating the action of enzymes. Major advances have been made in this area of research, and in particular, investigations of the chemistry of models such as the cyclodextrins, which are capable of forming inclusion complexes with substrate molecules and which can undergo subsequent catalytic steps, are being actively pursued in several laboratories.[1-6] Among the features of the cyclodextrins which are attractive for their use as models are their considerable water solubility, their ability to complex a large variety of organic substrates, and the possibility of chemically modifying them to generate new and different types of catalytic species. The use of the cyclodextrins as models, however, is not without difficulty. Thus, selective modification is not always facile, nor is the identification of the nature of the modified species always straightforward. Also, the degree of enantiomeric specificity observed for the cyclodextrins in catalytic reactions has been rather limited.[7-9]

We have been engaged recently in a rather different approach to developing new model catalysts. We have embarked on an investigation of the conversion of simple enzymes which are hydrolytic catalysts into modified enzyme species which can catalyze a range of synthetically important reactions including oxidation–reduction, transamination, decarboxylation, etc. Our experimental methodology involves the covalent at-

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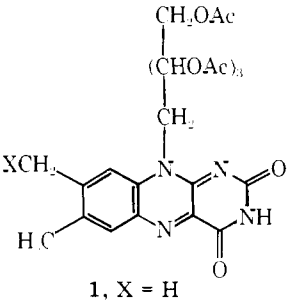
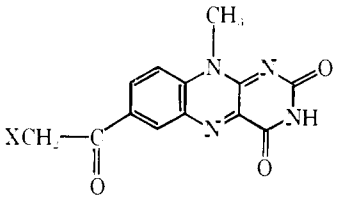
tachment of coenzyme analogues at or on the periphery of the active sites of hydrolytic enzymes in a manner which still permits the binding of substrates to occur. If this binding of the substrates brings them in close proximity to the coenzyme functions, then it may be possible to catalyze many new reactions with the modified enzymes. The motivation for the choice of hydrolytic enzymes for modification is that a significant number of them are very available and easily purified and that not only their primary but also their tertiary structures are often known. Thus, chemical modification can be carried out in a rational manner, and the structural consequences of such modification can be examined in the kind of detail which is desirable for the development of model catalysts.

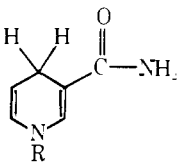
In earlier preliminary communications[10,11] we reported the alkylation of the sulfhydryl group of Cys-25 in the active site of the cysteine proteinase papain (E.C. 3.4.4.10) by 8α-



1, X = H
2, X = Br
3, X = *S*-papain



4, X = H
5, X = Br
6, X = *S*-papain
7, X = *S*-glutathione



8, R = $CH_2CH_2CH_3$ (NPrNH)
9, R = CH_2Ph (NBzNH)
10, R = ADPR (NADH)

bromo-2′,3′,4′,5′-tetra-*O*-acetylriboflavin (**2**) and 7α-bromoacetyl-10-methylisoalloxazine (**5**) to produce the semisynthetic oxido-reductases flavopapains **3** and **6**, respectively. In the present article we describe the reactions of the modified papain **6** acting as a flavoenzyme in the binding and oxidation of the dihydronicotinamide substrates **8**–**10**.

Experimental Section

General. Proton magnetic resonance (^{1}H NMR) spectra were determined using either Varian A-60A or Bruker HS-270 spectrometers. Chemical shifts are expressed as δ values (parts per million downfield from Me_4Si). Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. All spectrophotometric determinations were performed on either a Beckman Acta MVI spectrophotometer, a Beckman DU spectrophotometer with Gilford attachments, or a Durrum-Gibson stopped-flow spectrophotometer. These spectrophotometers were all equipped with thermostatic baths that held the temperature to 25.0 ± 0.3 °C. Fluorescence emission spectra were recorded on an Aminco-Bowman spectrofluorimeter.

Materials. *p*-Chloroacetophenone was purchased from Aldrich and distilled before use. Adams catalyst (40 mesh) was obtained from Ventron. The papain used throughout this work was obtained from Worthington Biochemical Co. as a suspension of twice crystallized enzyme in 0.05 M acetate buffer, pH 5.0. The following reagents were obtained from Sigma and used without further purification: *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester (Cbz-Gly-PNP); *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPA); glutathione (GSH); cysteine; glucose oxidase; and catalase. NADH was purchased from PL Biochemicals; tris(hydroxymethyl)aminomethane (Tris) from Schwarz-Mann; Sepharose 4B, Sephadex G-25, and DEAE Sephadex A-25 from Pharmacia. Buffers were prepared with doubly distilled deionized water (Continental Demineralizer); all other solvents and reagents were of the highest purity available and were used without further purification.

Purification of Papain. Commercially purchased papain was purified initially by affinity chromatography on a column of agarose-Gly-Gly-Tyr(OBz)-Arg (20 × 80 mm), as described by Blumberg et al.[12] The affinity column employed was purchased from Miles-Yeda. In the later stages of this work papain was prepared as the mercury derivative by the method of Sluyterman and Wijdenes[13] with the exception that Me_2SO and 1-butanol were eliminated from the elution buffers. For activation prior to modification, solutions of mercuripapain in 0.05 M acetate, pH 5.0, containing 0.1 M KCl and 0.05 mM $HgCl_2$ were made 50 mM in cysteine or β-mercaptoethanol and 1 mM in EDTA. The resulting solutions were allowed to stand at room temperature for 5–8 h and then dialyzed against three changes of deionized water at 4 °C for 24 h. Protein concentrations were determined spectrophotometrically based on $E^{1\%}_{280nm}$ = 25.0 and a molecular weight of 23 400.[14,15] Papain activity was determined spectrophotometrically by assays using Cbz-Gly-PNP[16] and BAPA.[17]

Modification of Papain. Freshly purified papain in deionized water was mixed with a fivefold excess of 7α-bromoacetyl-10-methylisoalloxazine, the brominated flavin derivative, **5**, in Me_2SO at room temperature. After incubation for 5 h, a fivefold molar excess of cysteine was added, followed by a second addition of a fivefold excess of the brominated flavin. To achieve a total reduction of more than 99% of the original papain activity, another addition of cysteine and flavin was required. The modified papain was separated from excess flavin by gel filtration on Sephadex G-25 or by dialysis against deionized water at 4 °C.

The free sulfhydryl content of the modified enzyme was determined by S-carboxymethylation with iodoacetic acid followed by amino acid analysis using a Beckman Spinco Amino Acid Analyzer Model 121, as described by Price et al.[18]

The concentrations of solutions of modified papain were determined either by the alkaline hydrolysis method of Fruchter and Crestfield[19] or by spectrophotometric measurements using ϵ_{427} 10 900 M^{-1} cm^{-1} (ϵ_{427} for **4**). The concentrations determined by these two methods agreed to better than 15%.

Dihydronicotinamides. 1-Propyl-1,4-dihydronicotinamide (**8**) was synthesized by the method of Suelter and Metzler.[20] 1-Benzyl-1,4-dihydrocotinamide (**9**) was synthesized as described by Mauzerall and Westheimer.[21] The identity of all dihydronicotinamides was confirmed by UV and NMR spectroscopy.

4-Chloro-3-nitroacetophenone (11). To a stirred solution of 80 mL of concentrated sulfuric acid and 20 mL of concentrated nitric acid cooled below 0 °C in an ice-salt bath was added 10.4 g (0.068 mol) of *p*-chloroacetophenone dropwise over a period of 30 min. The solution was stirred for an additional 15 min and then poured over 500 mL of ice water. The resulting crystals were collected, washed with several portions of ice water, and recrystallized from methanol: yield 12.3 g (0.061 mol, 95%); mp 98–100 °C (lit.[22] 99–100 °C); NMR ($CDCl_3$) δ 2.65 (3 H, s), 7.65 (1 H, d, J = 9 Hz), 8.10 (1 H, 2 d, J = 9, 2 Hz), 8.43 (1 H, d, J = 2 Hz).

4-Methylamino-3-nitroacetophenone (12). To a stirred solution containing 2.0 g (0.01 mol) of **11** in 15 mL of ethanol was added 4.0 g (0.057 mol) of a 40% solution of methylamine in water. After refluxing for 3 h, 25 mL of 4 N ammonium hydroxide was added, and the turbid solution was allowed to stand at 4 °C for several hours. The product which precipitated was collected and recrystallized from methanol to give 1.54 g (0.0079 mol, 79%) of product: mp 167–168 °C (lit.[23] 167–168 °C); NMR ($CDCl_3$) δ 2.58 (3 H, s), 3.13 (3 H, d, J = 6 Hz), 6.93 (1 H, d, J = 9 Hz), 8.10 (1 H, 2 d, J = 9, 1.5 Hz), 8.78 (1 H, d, J = 1.5 Hz).

7-Acetyl-10-methylisoalloxazine (4). Compound **12** (502.1 mg, 2.59 mmol) was dissolved in 150 mL of ethyl acetate and catalytically hydrogenated over 125 mg of Adams catalyst. When the reduction was complete as judged by TLC (silica gel, $CHCl_3$ eluent), the solution was filtered and evaporated to dryness in vacuo. The residue was dissolved in 100 mL of absolute ethanol, 417.4 mg (2.61 mmol) of alloxan monohydrate in 5 mL of concentrated HCl was added, and the solution was refluxed for 15 min. The product which crystallized upon standing at 4 °C was collected and washed with ether, yield 486.6 mg (1.80 mmol, 69%). After recrystallization from Me_2SO, compound **4** had mp 292–295 °C dec; NMR (CF_3COOH) δ 3.03 (3 H, s), 4.67 (3 H, s), 8.50 (1 H, d, J = 9 Hz), 9.03 (1 H, 2 d, J = 9, 1.5 Hz), 9.10 (1 H, d, J = 1.5 Hz); λ_{max} ($\epsilon_{M^{-1}\,cm^{-1}}$) in 0.05 M phosphate buffer,

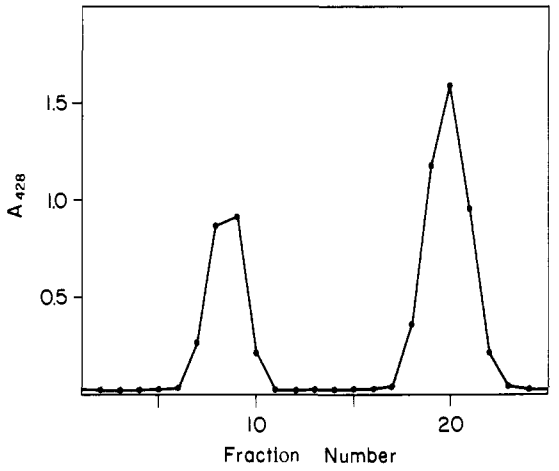


Figure 1. Purification of **7**. The reaction mixture was applied to a DEAE Sephadex A-25 ion exchange column (14 × 300 mm) preequilibrated with 0.1 M Tris-HCl, pH 7.5, and eluted at room temperature with this buffer. A flow rate of 4 mL/min was maintained; fractions of 7 mL were collected and monitored at 428 nm. The fractions eluting after number 17 contained the glutathione derivative, **7**. The earlier peak contains unreacted flavin.

pH 7.0 (neutral species) 427 nm (10 900), 348 (5550), 282 (32 300). Anal. Calcd for $C_{13}H_{10}N_4O_3$: C, 57.77; H, 3.73; N, 20.73. Found: C, 57.63; H, 3.66; N, 20.26.

7α-Bromoacetyl-10-methylisoalloxazine (5). To a warm solution (85–90 °C) of 292.6 mg (1.08 mmol) of **4** in 35 mL of glacial acetic acid was added 5 mL of 0.26 M Br_2 in glacial acetic acid (1.30 mmol) dropwise over a period of 10 min. The solution was then stirred for an additional 10 min and cooled to room temperature resulting in the precipitation of the brominated flavin derivative. The crystals were collected, washed with ether, and dried overnight under high vacuum to yield 333.6 mg (0.97 mmol) of material which contained less than 5% starting material and no detectable amount of flavin dibrominated in the acetyl group, as judged by NMR. The NMR spectrum of the product showed new absorption peaks at δ 4.78 ($-CH_2Br$), 8.17, 8.93, and 9.27 (aromatic H) when compared to the spectrum of **4**. In addition, the N-10 methyl resonance at δ 4.67 was somewhat broadened. The UV-vis spectrum of the product is virtually identical with that of **4** (λ_{max} 427, 350, and 282 nm). Several attempts to recrystallize the material resulted in the cocrystallization of compounds **4** and **5**. Since we were able to show[24] that the presence of a small amount of compound **4** should not have a significant effect on the modification of papain by the brominated flavin **5**, we used the latter compound as a modifying reagent without further purification.

7α-(*S*-Glutathionyl)acetyl-10-methylisoalloxazine (7). Compound **5** (1.7 mg, 5 mmol) was dissolved in 0.5 mL of Me_2SO, and 2.4 mg (8 mmol) of glutathione in 0.5 mL of 0.1 M Tris-HCl buffer, pH 7.5, was added in one portion. The resultant solution was stirred at room temperature for 3–4 h. Subsequently, the solution was applied to a DEAE Sephadex A-25 ion exchange column (14 × 300 mm) preequilibrated with 0.1 M Tris-HCl, pH 7.5. THe column was eluted using this buffer and fractions of 7 mL were collected (see Figure 1). The glutathione adduct was detected by its absorption at 428 nm.

Determination of ϵ_{428} for 7. The absorbance at 428 nm of a freshly prepared sample of the flavin-modified glutathione, **7**, in 0.1 M Tris-HCl, pH 7.5, was carefully measured. An aliquot (1 mL) of this solution was then transferred to a soft glass test tube containing 1 mL of concentrated HCl. The tube was evacuated, sealed, and heated at 140 °C for 18 h. The concentrations of glycine and glutamic acid were then determined by amino acid analysis. These concentrations, along with the original absorbance value, allowed the value of ϵ_{428} to be calculated. Two independent hydrolyses yielded four calculated values for ϵ_{428}. Averaging these values gave $(1.85 \pm 0.13) \times 10^4\ M^{-1}\ cm^{-1}$ as the value for ϵ_{428}.

Kinetics. The rate of aerobic oxidation of each dihydronicotinamide by the flavin under study was determined by observing the decrease in absorbance at the appropriate λ_{max} in the region 340–360 nm for each dihydronicotinamide. In a typical experiment, 2 mL of a buffered solution (0.1 M Tris-HCl, pH 7.5) containing the flavin derivative was pipetted into a 2.5-mL capacity stoppered cuvette with a 1-cm light path. After the system had equilibrated at 25.0 °C, the reaction was initiated by adding an aliquot of a solution of dihydronicotinamide in ethanol (NBzNH, NPrNH) or in buffer (NADH).

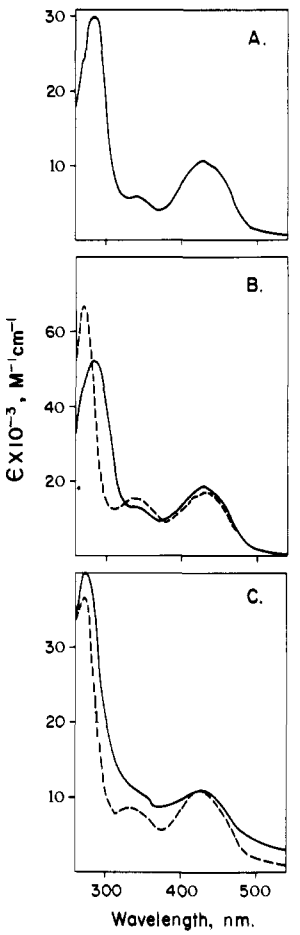


Figure 2. Absorption spectra of flavin derivatives **4**, **6**, and **7**: (A) 7-acetyl-10-methylisoalloxazine; (B) 7α-(*S*-glutathionyl)acetyl-10-methylisoalloxazine (**7**, solid line), 7-sulfone (dotted line); (C) flavopapain (**6**, solid line), **6** sulfone (dotted line). All spectra were recorded at 25.0 °C in 0.1 M Tris-HCl, pH 7.5.

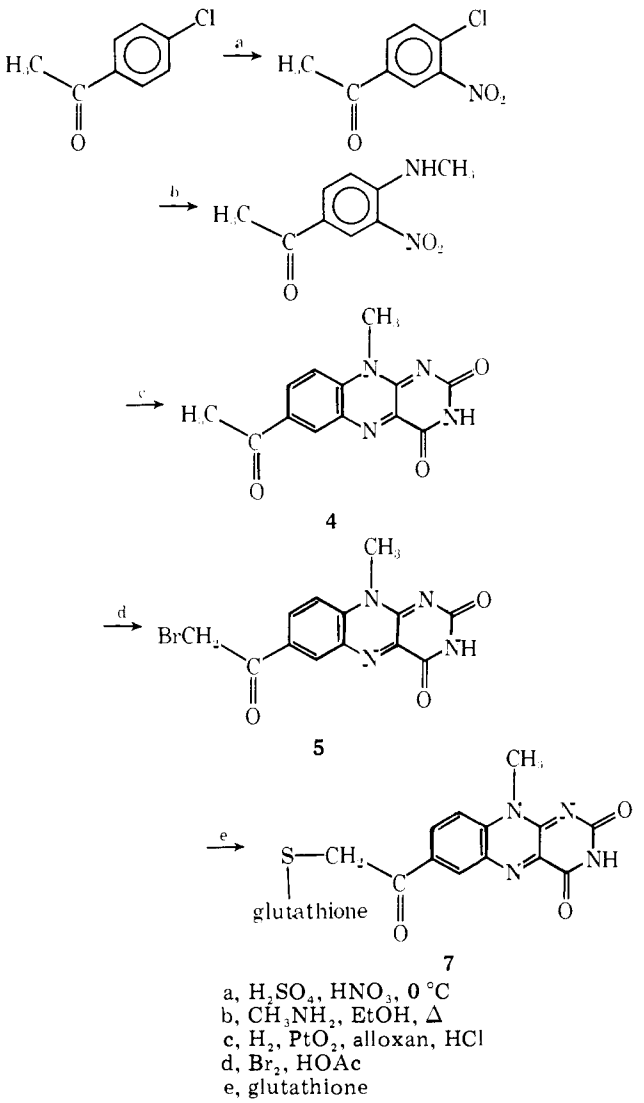
The kinetics of the anaerobic oxidation of NBzNH by **4** and **6** were measured by stopped-flow spectrophotometry. In experiments involving excess substrate (dihydronicotinamide), the decrease in absorbance at 427 nm with time was followed after mixing equal volumes of flavin and dihydronicotinamide solutions. In experiments involving excess flavopapain, the decrease in absorbance at 355 nm was monitored. In all cases, anaerobic conditions were maintained by adding 50 μL of a 5 mg/mL solution of glucose oxidase, 10 μL of a 25 mg/mL solution of catalase, and 50 μL of an aqueous saturated glucose solution to 10 mL of the reaction solutions.

Performic Acid Oxidation of 6 and 7.[25] To an ice-cold solution of either the flavin-glutathione derivative **7** or flavopapain **6** in 0.1 M Tris-HCl, pH 7.5, was added 0.002 mL of a performic acid mixture (9/1 v/v H_2O_2/HCOOH) per nmol of sulfur to be oxidized. After 2 h at 0 °C, the solution was diluted with five volumes of cold deionized water and lyophilized. The dried samples were then dissolved in sufficient deionized water to return the solution to a concentration of 0.1 M Tris, and the pH was carefully readjusted to 7.5. Fluorescence and absorbance measurements were then made using these solutions.

Results and Discussion

Synthesis and Properties of Flavins 4 and 7. The syntheses of 7-acetyl-10-methylisoalloxazine (**4**), 7α-bromoacetyl-10-methylisoalloxazine (**5**), and the flavin derivative of glutathione, **7**, are outlined in Scheme I. The flavins **4** and **7** exhibit UV-visible absorption spectra (Figure 2) similar to those of other flavins containing electron-withdrawing substituents at the 7 position.[26] In particular, at pH 7 the visible absorption

Scheme I



a, H_2SO_4, HNO_3, 0 °C
b, CH_3NH_2, EtOH, Δ
c, H_2, PtO_2, alloxan, HCl
d, Br_2, HOAc
e, glutathione

band for **4** occurs at 427 nm, blue shifted 16 nm when compared to that for lumiflavin (λ_{max} 443 nm). Furthermore, the near-UV absorbance at 340 nm in **4** (shifted from 360 nm in other flavins) is also less intense when compared to the corresponding band for the other flavins.

The presence of the 7-acetyl group in **4** also affects the redox potential of this flavin. The half-wave potential ($E_{1/2}$) for $2e^-$ reduction of **4** measured in 0.1 M Tris-HCl, pH 7.5, 25.0 °C, is −348 mV (vs. SCE). This is an increase of $E_{1/2}$ by almost 100 mV when compared to FMN ($E_{1/2}$ = −444 mV vs. SCE under the same conditions) and confirms that an electron-withdrawing group attached to the isoalloxazine ring system increases the oxidizing power of flavins, as measured thermodynamically.[26]

The attachment of glutathione to the acetyl group of **4** to give the species **7** does not drastically alter the UV-visible spectrum of the flavin moiety (Figure 2). However, there is a slight increase in the molar absorbtivity of **7** as compared to **4** (1.85×10^4 M^{-1} cm^{-1} for **7** vs. 1.09×10^4 M^{-1} cm^{-1} for **4**) and a slight shift in the lowest wavelength peak (289 nm for **7** vs. 282 nm for **4**).

Modification of Papain. In preliminary experiments it was found that treatment of papain with a tenfold excess of the brominated flavin **5** for 24 h resulted in a loss of more than 90% of the original enzymatic activity. However, the activity observed was always 3–5% higher using the BAPA assay than with the Cbz-Gly-PNP assay. This discrepancy is probably the result of the formation of a small amount of a Cys-25 thiol-C-4A flavin adduct similar to that reported by Loechler and Hollocher in the oxidation of dithiothreitol by flavins.[27] The cysteine present in the BAPA assay system should catalyze the decomposition of this adduct releasing active, unmodified papain. To overcome the competition between alkylation of the active site sulfhydryl group of papain by the bromoacetyl function of flavin **5** and the formation of the Cys-25 thiol-C-4A adduct, the procedure described in the Experimental Section involving repetitive treatment of the enzyme with the flavin derivative **5** and with cysteine was developed. From the treatment which was carried out we consistently obtained modified papain with less than 1% activity in either the BAPA or Cbz-Gly-PNP assay systems.

The site of modification in the inactivated enzyme was determined by amino acid analysis. The modified enzyme was treated with iodoacetate, hydrolyzed in 6 N HCl, and then subjected to amino acid analysis. Comparing this analysis to a similar analysis of native papain showed a loss of only one amino acid residue upon modification, Cys-25.

Properties of Flavopapain 6. The absorption spectrum of flavopapain **6** (Figure 2C) resembles those of **4** and **7**. However, there are several noticeable differences. In the vicinity of 427 nm the absorbance in the case of **6** is broader than that in either **4** or **7**. It is known that in model systems, the flavin visible absorption band is split in a nonpolar environment.[28] Also, some flavoproteins show shoulders in their visible absorption spectra at 430 and 480 nm, presumably owing to the nonpolar environment in their active sites.[29] The broadening of the visible absorption in the flavopapain **6** could be an indication that the flavin moiety in this enzyme lies in a relatively nonpolar environment. Indeed, Lowbridge and Fruton[30] have provided evidence that the active site of papain is nonpolar. The 342-nm absorbance found in **4** and **7** is shifted slightly in flavopapain **6** to ca. 340 nm as well as being reduced in intensity. These changes are again consistent with the flavin being in a nonpolar environment.[28] Free flavin molecules do not absorb significantly above 500 nm (see Figure 2, for example). However, in the flavopapain **6**, there is a significant long wavelength tailing absorbance which extends beyond 600 nm. The cause of this tailing is not known, but it could be the result of a charge-transfer interaction between a tyrosine or tryptophan residue in the active site with the flavin moiety.

Modification of papain with 8α-bromo-2′,3′,4′,5′-tetra-*O*-acetylriboflavin has been found to result in the formation of a flavopapain species in which the sulfur of Cys-25 linking the flavin moiety to the enzyme is in the sulfone oxidation state.[11] In view of this observation, a careful determination of the oxidation state of the sulfur at the flavin–enzyme linkage in flavopapain **6** was undertaken. Performic acid oxidation of the glutathione derivative **7** yields a sulfone species with λ_{max} 428, 338, and 270 nm (Figure 2B). The shift to shorter wavelength and increase in intensity of the 340-nm absorbance are consistent with the spectral changes observed in the oxidation of 8α-(*N*-acetylcysteinyl)-2′,3′,4′,5′-tetra-*O*-acetylriboflavin.[31] The increase in intensity of the shortest wavelength absorption maximum is also consistent with the postulate that sulfone formation occurred as a result of performic acid treatment.[31]

Treatment of flavopapain **6** with performic acid resulted in the spectral changes shown in Figure 2C. In addition to the appearance of an absorption peak at 335 nm consistent with sulfone formation,[31] the spectrum of performic acid oxidized flavopapain no longer exhibited the long-wavelength tailing seen in **6**. If this tailing in the spectrum of **6** is the result of a form of charge-transfer interaction as suggested above, then its disappearance in the flavopapain sulfone could indicate that this interaction is unimportant for the oxidized species.

The fluorescence properties of flavopapain **6** and its sulfone were also examined. The fluorescence of flavins **4** and **7** as well

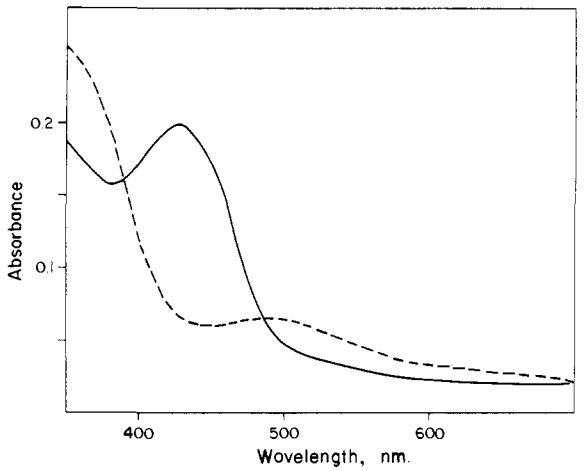


Figure 3. Reversible reduction of flavopapain. An anaerobic solution of flavopapain **6** (18 μM) in 0.1 M Tris-HCl, pH 7.5, was placed in the main compartment of a Thunberg cuvette and its spectrum recorded (solid line). NBzNH in ethanol was added from the side arm of the cuvette to give a final concentration of 30 μM (2% ethanol, v/v). The spectrum of the reduced enzyme was recorded when the decrease in A_{427} stopped (ca. 15 min, dotted line).

Table I. Relative Fluorescence Intensities of **4, 6,** and **7** and Their Respective Sulfones[a]

flavin	rel intensity, %	flavin	rel intensity, %
4	100	**7**	9
6	6	**7** sulfone	94
6 sulfone	61		

[a] Measured in 0.1 M Tris-HCl, pH 7.5, 25 °C. Excitation 430 nm, emission 510 nm.

as that of the flavopapain species was found to be independent of pH over the range pH 4.5-8.0. These results correspond to those reported by Falk and McCormick[32] and Kearney et al.[33] for other flavinyl peptides and flavins. The relative fluorescence intensities observed for the flavins we have studied at pH 7.5 are listed in Table I. The fluorescence emission peaks seen for flavopapain **6** and the flavinyl peptide **7** are markedly quenched when compared to **4**.[32] Oxidation of these species to their corresponding sulfones results in an increase in fluorescence intensity of 60 and 90%, respectively, for **6** and **7**. This return of the flavin fluorescence upon oxidation is a general property of sulfur-linked flavinyl peptides.

Reversible reduction-oxidation of flavopapain **6** can be accomplished by treatment first with either sodium dithionite or dihydronicotinamides under anaerobic conditions, followed by reaction with air. Figure 3 illustrates the results obtained when modified papain was mixed anaerobically with a 1.7-fold excess of NBzNH. The absorbance at 427 nm decreased and a broad absorption centered around 500 nm, but tailing well past 600 nm, was observed. Such long-wavelength tailing was not observed either with reduced or oxidized forms of the model flavins **4** and **7**. When the solution of reduced flavopapain **6** was exposed to air, the original spectrum of the oxidized form of the flavin reappeared within minutes.

Oxidation of Dihydronicotinamides by Model Flavin Compounds. The oxidation of dihydronicotinamide by flavins has been reported to proceed via preequilibrium charge transfer complexes.[34-36] The dissociation constant for the complexes formed by various flavins is on the order of 0.1 M.[36] Furthermore, it has been reported that the reoxidation of reduced flavin is not rate limiting in these reactions.[20,37] Therefore, under conditions of dihydronicotinamide in excess, if one maintains the concentration of dihydronicotinamide substantially less than 0.1 M, complex formation will be negligible,

Table II. Second-Order Rate Constants for the Oxidation of Dihydronicotinamides by **4** and **7**[a]

flavin	dihydronicotinamide	k, M^{-1} s^{-1}
4	NPrNH	798 ± 13
	NBzNH	121 ± 28
		(96.9 ± 16.5)[b]
	NADH	12.9 ± 2.9
7	NPrNH	1550 ± 230
	NBzNH	303 ± 32
	NADH	16.1 ± 1.0

[a] Measured at 25.0 °C in 0.1 M Tris-HCl, pH 7.5, 0-5% ethanol, v/v. [b] Obtained under anaerobic conditions.

Table III. Rate Parameters for the Oxidation of Dihydronicotinamide by Flavopapain **6**[a]

dihydronicotinamide	$K_m \times 10^5$, M	k_{cat}, s^{-1}	k_{cat}/K_m, M^{-1} s^{-1}
NPrNH	2.25 ± 0.37	0.0715 ± 0.0042	3180
NBzNH	3.00 ± 0.85	0.103 ± 0.024	3430
NADH	22.0 ± 6.0	0.015 ± 0.004	68.1

[a] Measured at 25 °C in 0.1 M Tris-HCl, pH 7.5, 0-5% ethanol, v/v.

and the observed kinetics will be first order with respect to the dihydronicotinamide. The observed rate constant, k_{obsd}, then will vary in direct proportion to the flavin concentration, permitting the calculation of the second-order rate constant, k, for each flavin-dihydronicotinamide couple. The secord-order rate constants which we have measured for the model flavins are listed in Table II. In all of our experiments, the concentration of both the flavin and the dihydronicotinamide never exceeded 1 mM, and no evidence for complex formation was observed.

Oxidation of Dihydronicotinamide by Flavopapain 6. In contrast to the oxidation of dihydronicotinamides by the model flavins **4** and **7**, using dihydronicotinamide in excess, the oxidation of the dihydronicotinamides by **6** exhibits saturation kinetics at low concentrations of this type of substrate. Under aerobic conditions the observed kinetics are readily explained by eq 1, where E_{ox} and EH_{2red} represent oxidized and reduced flavopapain, respectively, NRNH and NRN represent reduced and oxidized dihydronicotinamide, and ES represents a Michaelis complex formed between the substrate and the enzyme. Assuming that under the aerobic conditions used, the oxidation of NRNH is independent of oxygen, i.e., $k_0 (O)_2 \gg k_{cat}$, then the scheme of eq 1 leads to the expression of eq 2 for the rate of oxidation of the dihydronicotinamides where $(E)_0$ is the total enzyme concentration. Computer fitting of the data for the oxidation of the three dihydronicotinamides by flavopapain **6** to eq 2 gave the values of k_{cat} and K_m listed in Table III.

$$E_{ox} + NRNH \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} EH_{2red} + NRN \qquad (1a)$$

$$EH_{2red} + O_2 \xrightarrow{k_0} E_{ox} + H_2O_2 \qquad (1b)$$

$$v = \frac{k_{cat}(E)_0(NRNH)}{K_m + (NRNH)} \qquad (2)$$

When the second-order rate constants for the reactions of flavins **4** and **7** with the dihydronicotinamides examined in the present study are compared to the values measured for k_{cat}/K_m for the corresponding flavopapain-catalyzed reactions, it can be seen that the enzymatic reactions are more rapid, although the rate accelerations measured in this way are always less than a factor of 10^2. Though the rate accelerations are not especially large, it is significant that in the cases we have examined, saturation kinetics were observed indicating that flavopapain

Table IV. Kinetic Parameters for Several Naturally Occurring Flavoenzymes

enzyme	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, M^{-1} s^{-1}
NADH specific FMN oxidoreductase (B. Harveyi)[a]	15.5	47.5	3.26×10^5
NADPH specific FMN oxidoreductase (B. Harveyi)[a]	34.0	40.0	8.50×10^5
old yellow enzyme (Yeast)[b]	0.71	220	3.23×10^3
NADH dehydrogenase (bovine heart)[c]			$\sim 10^8$

[a] F. Jabolonski and M. DeLuca, *Biochemistry*, **16**, 2932 (1977). [b] T. Honma and Y. Ogura, *Biochem. Biophys. Acta*, **484**, 9 (1977). [c] Reference 38.

Table V. Rate Parameters for the Anaerobic Reduction of Flavopapain **6** by NBzNH[a]

$[E]_0$, μM	k_2/K_s, M^{-1} s^{-1}	k_3, s^{-1}
1.7	2.6×10^4	0.097
3.9	1.1×10^4	0.101
4.4	2.2×10^4	0.086
5.5	1.2×10^4	0.134
11	0.91×10^4	0.113
av	1.6×10^4	0.11

[a] Measured in 0.1 M Tris-HCl, pH 7.5, containing 0–5% ethanol, v/v, 25.0 °C.

binds the dihydronicotinamde substrates reasonably well. Since no binding of the dihydronicotinamides to the flavin-peptide derivative **7** was observed under comparable conditions, the binding of these substrates to flavopapain **6** appears to be the result of specific enzyme-substrate interactions and not simply a phenomenon associated with having a peptide substituent attached to the flavin moiety.

Table IV shows k_{cat}/K_m values for four naturally occurring flavoenzymes which utilize either NADH or NADPH as substrates. The oxidation of NBzNH or NPrNH by flavopapain **6** proceeds faster than the reaction illustrated for one of the enzymes, somewhat slower than those illustrated for two of the enzymes, and much slower than that observed for bovine heart NADH dehydrogenase, for which the turnover number is one of the highest known for any flavoenzyme.[38] The k_{cat} and K_m values we have measured for the reactions of NBzNH and NPrNH with flavopapain **6** are comparable to those found for NADH or NADPH reactions with naturally occurring flavoenzymes.

The effective binding of the dihydronicotinamides to flavopapain **6** can be rationalized by examining the properties of the active site of native papain. In the native enzyme, a hydrophobic binding pocket is formed near the active site by the side chains of Tyr-67, Pro-68, Trp-69, Phe-207, Ala-160, Val-133, and Val-157.[39] This site, designated S_2 by Schechter and Berger,[40] is responsible for papain's propensity to hydrolyze peptide bonds one residue removed from such hydrophobic amino acids as phenylalanine and tyrosine. If this binding site remains intact upon modification of papain with the brominated flavin **5**, then the alkyl groups of NPrNH and NBzNH can be fitted snugly into this pocket, giving rise to the tight binding observed for these substrates. With NADH, the large groups attached to the dihydronicotinamide ring sterically prevent this tight binding, resulting in a larger value of K_m for this substrate.

If one examines a three-dimensional model (Lab Quip) of papain in which the flavin group has been covalently attached to the sulfhydryl of Cys-25, the reactivity of flavopapain can be understood in structural terms. In particular, the structures of the papain derivatives produced by the reaction of three chloromethyl ketone substrate analogues with the sulfhydryl of Cys-25 have been recently examined by X-ray diffraction.[41] In each case the carbonyl oxygen of what was originally the chloromethyl ketone group was found to be near two potential hydrogen bond donating groups, the backbone NH of Cys-25 and the NH_2 of Gln-19. In our own model building, we oriented the flavin moiety of the flavopapain **6** in a fashion such that the carbonyl group of what had formerly been the bromomethyl ketone function in the modifying reagent **5** was brought to a position similar to that occupied by the carbonyl group mentioned above. When this was done, it was possible to bind the substrate dihydronicotinamide (NBzNH or NPrNH) in close proximity to the flavin, presumably facilitating hydrogen transfer.

The reaction of flavopapain **6** with NBzNH has also been studied anaerobically. Under conditions of substrate in excess, biphasic kinetic behavior was observed, and computer fitting of the experimental data to a scheme employing two consecutive first-order processes could be carried out to obtain rate constants for the two phases. We believe that these results imply the formation of a labile intermediate, as illustrated in eq 3, where E_{ox} and EH_{2red} again represent the oxidized and

$$E_{ox} + NBzNH \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} EH_{2red} + NBzN \quad (3)$$

reduced enzymes, ES the Michaelis complex between the enzyme and substrate, and ES′ represents the intermediate. The second, substrate independent, slower phase of the reaction corresponds to the breakdown of the intermediate ES′ (the k_3 step in eq 3). The initial, faster phase of the reaction corresponds to the formation of the intermediate from E_{ox} and NBzNH. The calculated rate constant for this phase, k_f, can be related to K_s and k_2 by eq 4. According to eq 4, k_2 and K_s

$$k_f = \frac{k_2(NBzNH)}{K_s + (NBzNH)} \quad (4)$$

can be determined from plots of $1/k_f$ vs. $1/(NBzNH)$. In practice, however, at substrate concentrations equal to and slightly lower than K_s (i.e., concentrations required for a good estimate of k_2 and K_s), the observed rate constants for the initial phase of the reaction approaches the value of k_3. Under these conditions, it became difficult to separate k_f and k_3 with a high degree of accuracy. Consequently, only the ratio k_2/K_s and not the individual parameters k_2 and K_s can be determined reasonably well. This ratio measured at several different enzyme concentrations is listed in Table V, along with the corresponding k_3 values. The results of our anaerobic kinetic experiments indicated that as a crude estimate k_2 is on the order of 5 s^{-1} while the value of K_s calculated was approximately 3×10^{-4} M. Using eq 5 and 6, these values of k_2 and K_s give estimates of k_{cat} and K_m of 0.1 s^{-1} and 7 μM, respectively, in fair agreement with the values obtained aerobically.

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \quad (5)$$

$$K_m = K_s \left(\frac{k_3}{k_2 + k_3}\right) \quad (6)$$

Several possible explanations exist for the observation of biphasic kinetics in the reduction of flavopapain **6** by NBzNH. One of these is that the dissociation of product (oxidized nicotinamide) from the enzyme could be rate determining as in

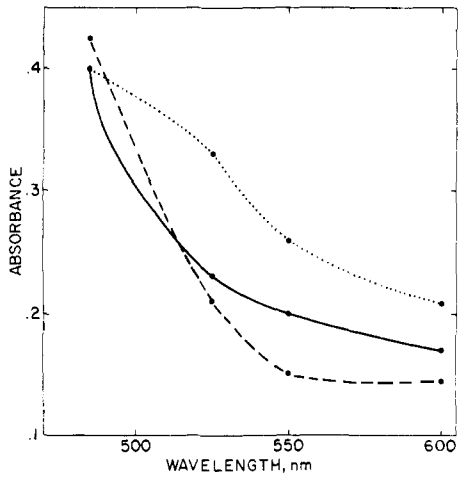


Figure 4. Absorbance change on mixing flavopapain **6** with NBzNH. Absorbance changes were recorded 5 s after mixing equal volumes of NBzNH and flavopapain anaerobically in the stopped flow instrument. Final concentrations were NBzNH, 0.148 mM; flavopapain, 7.4 μM (dashed line). The spectra of the oxidized (solid line) and reduced enzyme (dotted line) are shown for comparison.

the cases of the flavoprotein amino acid oxidases.[42] In this instance, the k_2 step of eq 3 would correspond to the actual redox reaction while the k_3 step would represent product dissociation. A test can be made for this possible situation by studying the kinetics of disappearance of NBzNH at 355 nm under conditions of enzyme in excess. If ϵ_{355} for the free and enzyme-bound forms of the product 1-benzylnicotinamide is the same, then the kinetics observed at 355 nm would be first order, and the intercept of a plot of $1/k_{obsd}$ vs. $1/[\mathbf{6}]$ should be $1/k_2$. If the ϵ_{355} values for the free and enzyme-bound forms of 1-benzylnicotinamide are different, then the kinetics seen at 355 nm under conditions of flavopapain in excess should be biphasic.

When the anaerobic oxidation of NBzNH was carried out in the presence of excess flavopapain **6**, the observed kinetics of disappearance of NBzNH measured at 355 nm were pseudo first order (see Table VI). A plot of $1/k_{obsd}$ vs. $1/[\mathbf{6}]$ yielded a straight line with an ordinate intercept yielding a k_{cat} value of 0.18 s^{-1}. From the slope/intercept ratio a value of $K_m = 32$ μM was calculated. The k_{cat} value measured under these conditions corresponds reasonably well with the k_3 value obtained under conditions of NBzNH in excess (Table V) and not to the k_2 value. This indicates that dissociation of the product 1-benzylnicotinamide from the reduced form of the flavopapain was not the cause of the biphasic kinetics seen under the latter conditions.

Since the k_3 step of eq 3 cannot be ascribed to slow product dissociation, a possibility to be considered is that the species ES′ may correspond to a chemically altered intermediate along the reaction pathway from oxidized flavin-reduced nicotinamide to reduced flavin-oxidized nicotinamide. Whatever the nature of the intermediate is, it appears unlikely that the species is one in which the double bonds or the ring nitrogen of the dihydronicotinamide moiety are structurally modified, since no change in the absorbance due to NBzNH is seen in the formation of ES′. This rules out the possibility that the intermediate might be a flavin-nicotinamide radical pair of some sort.

In an effort to observe the formation and breakdown of the intermediate ES′ without spectral contributions from the oxidized and reduced forms of the enzyme, some kinetic measurements were made at 485 nm, an isosbestic point in the spectra of oxidized and reduced flavopapain **6** (see Figure 3). The absorbance changes measured at this wavelength were too small to allow us to obtain reliable rate constants, but it was found that at a NBzNH substrate concentration of 0.15 mM the absorbance change at 485 nm was at a maximum after approximately 5–7 s. Consequently, the spectrum of the intermediate shown in Figure 4 was constructed by measuring the changes in absorbance at several wavelengths during the first 10 s of the reaction.

Table VI. Anaerobic Oxidation of NBzNH by Excess Flavopapain **6**[a]

$[E]_0$, μM	[S], μM	k_{obsd}, s^{-1}
8.0	3.7	0.033
8.0	4.1	0.034
8.0	1.4	0.040
13	1.2	0.053
6.5	1.2	0.031

[a] Measured in 0.1 M Tris-HCl, pH 7.5, 25.0 °C, 0–5% ethanol, v/v.

From the spectrum in Figure 4 it can be seen that the intermediate exhibits less long-wavelength tailing than either oxidized flavopapain **6** or the reduced flavopapain-NBzN mixture. This suggests that if a charge-transfer complex exists between the flavin moiety and an aromatic amino acid in the enzyme's active site, then prior to the redox reaction with NBzNH this complex is disrupted. The disruption of such a complex should result in a decrease in the long-wavelength absorption observed for the oxidized enzyme. We propose, therefore, that the transient intermediate ES′ (eq 3) observed kinetically in the reaction of flavopapain **6** with NBzNH corresponds to a species in which the flavin moiety has moved to a distinctly different environment than it occupies in the Michaelis complex ES. During the step producing ES′, the flavin remains in the oxidized state while the substrate NBzNH remains reduced. Once the realignment of the flavin moiety has taken place to give ES′, the redox reaction proceeds via the normal mechanism.[34-36]

In summary, the results presented here demonstrate that our semisynthetic enzyme, flavopapain **6**, is capable of binding reduced nicotinamide substrates with considerable specificity as well as catalyzing their oxidation with modest, though appreciable, rate accelerations. An investigation of the stereospecificity of flavopapain **6** as a redox catalyst is currently underway in our laboratory.[43]

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Minor and Trace Sterols in Marine Invertebrates. 8.[1] Isolation, Structure Elucidation, and Partial Synthesis of Two Novel Sterols—Stelliferasterol and Isostelliferasterol

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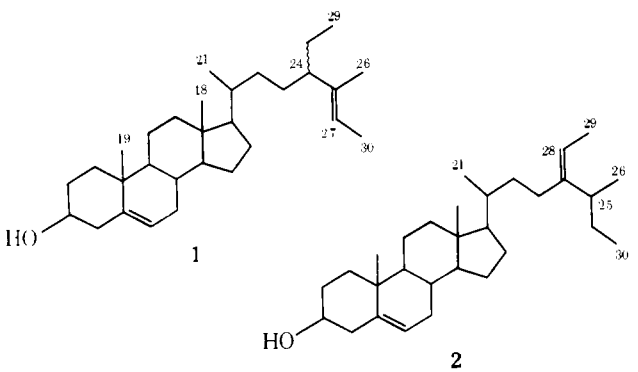
Abstract: The sterol constituents of the Australian sponge *Jaspis stellifera* consist of 12 3β-hydroxy-Δ^5-sterols and five stanols as shown by GC–MS and NMR spectroscopy. Among these were found two novel C_{30} sterols—stelliferasterol and isostelliferasterol—with unique side chains as depicted by structures **1** and **2**. Partial synthesis of these new sterols confirmed their structures and allowed the assignment of some stereochemical details of the sterol side chain. Attention is called to the significance of **1** and **2** in terms of sterol side chain biosynthesis.

The early predictions of Bergmann[2] in 1962 concerning the large diversity of marine sterols have been confirmed in the last 15 years by the discovery of more than 80 new sterols. Scheuer's review[3a] in 1973 and especially the most recent one by Schmitz[3b] showed again that marine organisms contain far more complex mixtures of sterols than terrestrial plants or animals. This amazing variety includes sterols with side chains that have no precedent in terrestrial sources such as cyclopropyl rings, alkylation at carbon atoms 22 and 23, a C_3 substituent at position 24, or an extra carbon at C-27. The diversity seems to be especially pronounced in primitive animals such as sponges and coelenterates. Therefore, our search for novel sterols has included a large number of sponges in an attempt to look for additional "missing links" which would shed some light on the interesting biochemical questions raised by the existence of these uniquely marine sterols.

From the Australian sponge *Jaspis stellifera* we obtained a sterol mixture with a fairly complex composition which forms the subject of this communication. By medium-pressure silver nitrate–silica gel chromatography of the sterol acetates and high-pressure liquid chromatography (LC) of the free sterols on reversed phase (C_{18}) columns we could identify 5 stanols and 12 3β-hydroxy-Δ^5-sterols (see Table I). Among these were found two novel C_{30} sterols which were named stelliferasterol and isostelliferasterol and whose structures were shown to be **1** and **2**.



The mass spectrum[4] of stelliferasterol showed the presence of an important peak at *m/e* 328 which has so far not been noted among marine sterols. By analogy to the well-known[5] McLafferty rearrangement of Δ^{24}-sterols to a mass 314 ion (Scheme I) we attribute the intense *m/e* 328 peak (a, Scheme I) to a similar rearrangement associated with a Δ^{25} double bond. The simultaneous occurrence of an *m/e* 314 peak (b) in the stelliferasterol spectrum can be rationalized by assuming a double bond migration (see Scheme I) in the mass spectrometer to the Δ^{24} position with subsequent McLafferty cleavage. A similar electron impact induced migration of a trisubstituted double bond to a tetrasubstituted position was

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