Stylocheilamide, an Unusual Constituent of the Sea Hare *Stylocheilus longicauda*[1]

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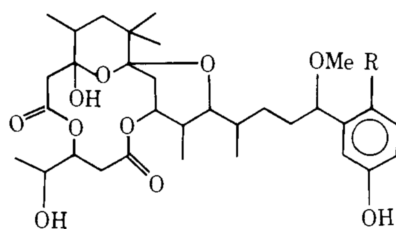
Abstract: From the sea hare *Stylocheilus longicauda* we have isolated, in addition to the previously reported aplysiatoxins (**1**, **2**), a novel nontoxic amide of composition $C_{28}H_{44}ClNO_6$. Stylocheilamide (**3**) is a tertiary amide constructed of a methoxyolefinic C_{14} acid and a methyl(2-chloro-3-cyclohexyl-2-propenyl)amine. The highly functionalized carbocyclic ring gives rise to a related compound **4**, differing only by the elements of HOAc. The structures have been elucidated by spectral analysis and chemical degradation. A key degradation product (**8**) was crystalline and its structure was determined by X-ray diffraction.

Sea hares are herbivorous marine mollusks that lack an external shell. Yet these soft-bodied, slow-moving animals appear to have few predators.[2] A rational explanation of this apparent dichotomy is provided by the toxic nature of these invertebrate animals, a reputation that they have enjoyed since Roman times.[3] However, the chemical nature of sea hare toxins



1, R = Br
2, R = H

had remained obscure until recently, when we reported the isolation and structural elucidation of two toxic constituents of the sea hare *Stylocheilus longicauda*, aplysiatoxin (**1**) and debromoaplysiatoxin (**2**).[4] The structure of these toxins is unprecedented: the principal moiety is an ω-phenylpentadecanoic acid which bears five methyl substituents, one a *gem*-dimethyl, at seemingly random positions. This unusual structural feature raises the question of the biogenesis and, more immediately, of the biological origin of the toxins. An early surmise that most if not all of the sea hares' secondary metabolites originate in their algal diet has now been conclusively demonstrated.[5] In our work, we observed *Stylocheilus longicauda* feeding on the red alga *Acanthophera spicifera* and, less commonly, on the blue-green alga *Lyngbya majuscula.* We could not trace the aplysiatoxins (**1**, **2**) to the macroscopic alga and we did not investigate *L. majuscula,* which was less abundant at our principal collection site in Kaneohe Bay, Oahu. Since then, however, Moore and co-workers[6] did isolate debromoaplysiatoxin (**2**) from the related blue-green alga *L. gracilis* and, interestingly, revealed its antileukemia activity.

Early indications of the unconventional constitution of the aplysiatoxins (**1**, **2**)[7] prompted us to screen ether extracts of *Stylocheilus* for, hopefully, simple toxin precursors. We took advantage of two prominent structural features of the toxins, the ^1H NMR signal of the methoxyl at δ 3.2 and a positive Beilstein test for halogen. Although our results shed no light on the biogenesis or structure of the aplysiatoxins, the screening procedure led us to a new, interesting nontoxic constituent, stylocheilamide (**3**), and its olefinic analogue **4**, which is for-

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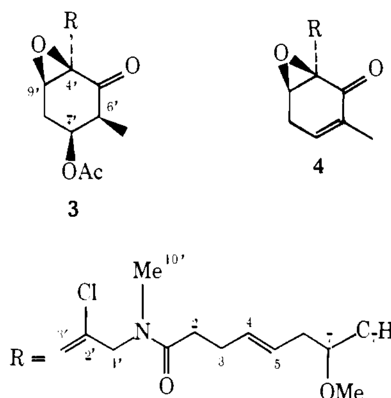

mally related to **3** by loss of acetic acid. The compounds are amides of a methoxy monoolefinic myristic (C_{14}) acid and a highly functionalized 3-cyclohexyl-2-chloro-2-propenylmethylamine. While the unfunctionalized C_{14} acid is a common constituent of terrestrial plants, the amine appears to be a unique natural product. Structure determination of the stylocheilamides is the subject of this report.

Stylocheilus is abundant only during parts of May and June when the animals appear inside the coral reef to spawn. We froze our collections and extracted the frozen whole animals in a blender with acetone as needed. The aqueous organic residue after removal of most of the acetone was partitioned between water and ether. The ether phase residue when directly applied to a silicic acid column yielded the aplysiatoxins.[4a] Further solvent partitioning followed by extensive chromatography on BioSil, Florisil, and Sephadex led to the stylocheilamides **3** and **4** as an oily mixture in about 3×10^{-3}% yield from the frozen animals. Preparative TLC on silica gel achieved separation of **3** from **4** in a ratio of 2:1. Most of our structural work was carried out with the more abundant **3**.

Stylocheilamide (**3**) is an optically active, colorless oil, stable in refluxing ethanol and to silicic adsorbents, but unstable to mineral acid, to base, and to alumina adsorbents. All conventional criteria of homogeneity (one- and two-dimensional TLC in several systems, MS, combustion analysis) prove stylocheilamide to be a single substance, $C_{28}H_{44}ClNO_6$. NMR spectra, however, clearly indicate that the chloropropenylmethylamine part of the molecule lacks unambiguous steric definition. Two *N*-methyl singlets at δ 2.90 and 2.98 integrate for a total of 3 protons and are assigned to the C-10′ protons. In Me_2SO-d_6 at 100 °C these two signals coalesce to a 3 H singlet at δ 2.95. The olefinic proton at C-3′ also gives rise to two signals at δ 6.14 and 6.26 which integrate for one proton. These signals are allylically coupled to a slightly broadened singlet at δ 4.02 (H-1′) and remain unchanged at elevated temperature. Existence in stylocheilamide of unresolved geo-

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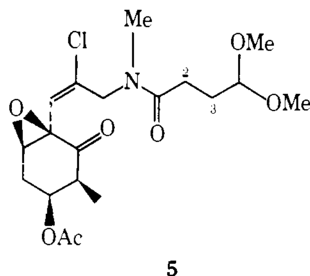
metrical isomers is also seen in the olefinic ^{13}C NMR signals, which appear as doublets at 131.6 (cis with respect to H and Cl) and 132.1 (trans) (C-2′) and at 120.2 (cis) and 121.5 (trans) ppm (C-3′). The dual signals for C-1′ at 48.3 and 50.8 ppm result from amide stereochemistry. In saturated aliphatic amides this effect is felt even by the δ carbon.[8] Additional doublets in the carbon spectrum, at 202.3 and 202.8 (C-5′), 172.5 and 172.8 (acetate CO), and 60.6 and 61.2 (C-7′) ppm, may arise from different conformers of the cyclohexane ring.

Spectral data (see below and Experimental Section) of stylocheilamide unambiguously defined all functional groups and characterized **3** as a bicyclic, diolefinic molecule. Hydrogenation over platinum in ethyl acetate led to a dechlorotetrahydro derivative corresponding to $C_{28}H_{49}NO_6$. Quantitative hydrogenation over palladium on carbon in isopropyl alcohol confirmed the presence of two double bonds and revealed that one olefin was reduced much more slowly than the other. The less hindered C-4,5 olefin was successfully cleaved by neutral Lemieux oxidation, which led to a single isolable acidic product characterized as 3-methoxydecanoic acid and further purified as its methyl ester. Synthesis of racemic methoxy ester proved its identity and secured ten skeletal carbons of the myristic acid moiety. Ozonolysis of stylocheilamide at −75 °C in dry methanol with a reductive workup also cleaved the C-4,5 double bond selectively but, in addition, permitted isolation and characterization of an oily aldehyde as its dimethyl acetal **5**. Spectral data of this key degradation



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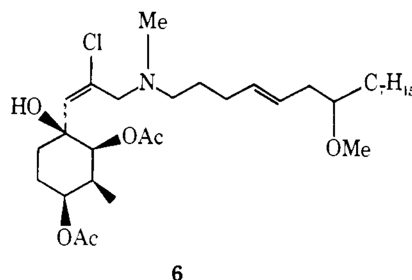
product showed the amine portion of stylocheilamide unchanged from the natural product, thereby allowing interpretation of the C-2, C-3 methylene NMR signals. In the ^{1}H NMR spectrum of **5** the acetal methine proton is a triplet at δ 4.40 that becomes a singlet when a multiplet at δ 1.85 is irradiated. Likewise, irradiation of the δ 4.40 triplet collapses the multiplet at δ 1.85, which can now be assigned to the C-3 methylene. The remaining methylene (C-2) of the myristic acid moiety is deduced from an abundant mass spectral fragment at *m/e* 131, $C_6H_{11}O_3$, which can arise by α-cleavage of the amide carbonyl bond and be represented by $(MeO)_2CHCH_2CH_2CO^+$, which in turn loses methanol to form an abundant ion at *m/e* 99, depicted as $MeOHCH{=}CHCH_2CO^+$.

The IR spectrum of **3** exhibits three distinct bands in the carbonyl region, at 1745, 1720, and 1655 cm^{-1}, which we assigned to acetate (supported by a 1230-cm^{-1} band), ketone, and tertiary amide functions, thereby accounting for four of the six oxygen atoms of stylocheilamide (**3**). The fifth oxygen embodied in the methoxy with its telltale ^{1}H NMR signal at δ 3.3 was secured by the degradation to 3-methoxydecanoic acid. We assigned the sixth oxygen atom to an epoxide, principally defined by two ^{13}C NMR resonances, a singlet at 61.2 and a doublet at 64.0 ppm. Single frequency on resonance decoupling showed that the sole proton associated with this function appeared as a multiplet at δ 3.62. Metal hydride reduction of **3**, which we had hoped would lead to an amine triol, produced in fact complex mixtures. Rigorous exclusion of moisture and a large excess of $LiAlH_4$ yielded manageable mixtures, which upon acetylation produced some tri- but largely diacetates. Spectral data of the principal diacetate, assigned structure **6**, showed that both olefinic linkages and



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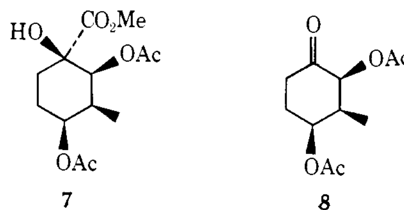
the chlorine atom had survived the reduction. Further degradation of **6** by ozonation in methanol with a reductive workup led, in addition to 11 minor products, to two major compounds, **7** and **8**, which appeared to be closely related by the similarity



7 **8**

of their ^{1}H NMR spectra. 2,4-Diacetoxy-3-methylcyclohexanone (**8**) could be crystallized from cyclohexane, mp 89–90 °C. Its structure was secured by X-ray diffraction. The corresponding α-hydroxymethyl carboxylate (**7**) was homogeneous by GLC and TLC. Distinguishing spectroscopic features of **7**, $C_{13}H_{20}O_7$, included hydroxyl bands at 3600, 3500, and 1160 cm^{-1} and a 3 H singlet at δ 3.74 assigned to a carbomethoxy group. Generation of **7** and **8** by ozonation of **6**, while at first surprising, has a precedent in the ozonolysis of 1-vinylcyclohexanol, which is transformed to cyclohexanone, formaldehyde, and formic acid.[9]

Under reductive workup conditions of the ozonolysis an α-hydroxy aldehyde, rather than an α-hydroxy acid, would have been expected.

Ester **7** argues a priori for a stylocheilamide (**3**) structure that places the chlorine atom at C-3′ rather than C-2′. Such a reaction course was in fact shown by Johnson and co-workers[10] in the ozonolysis of a chlorocyclohexene. In our case, however, the ^{1}H NMR evidence is conclusive for the assigned structure of the 2-chloro-2-propenylmethylamine portion of the molecule.

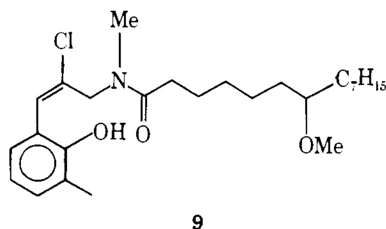
This moiety remained, in our hands, inaccessible to simple degradative or crystallographic analysis. Structure proof of this moiety by spectral analysis was impeded by the observed dual NMR signals of the *N*-methyl and olefinic protons (vide supra). There is ample precedent for temperature-dependent amide conformers.[11] We showed their existence in stylocheilamide by recording ^{1}H NMR spectra at 100 °C, which eliminated the corresponding twin signals. The slightly broadened 2 H singlet at δ 4.02, which is allylically coupled to the one-proton olefin (doublets at δ 6.14, 6.26), can only be assigned to the allylic methylene (C-1′) of 2-propenylamine. The chlorine atom is inert. It fails to react with refluxing ethanolic silver nitrate and must therefore be linked to an olefinic carbon. Allylic coupling between the olefinic and the C-1′ protons implies that the chlorine is at C-2′, rather than C-3′. By analogy the C-1 methylene of *N*,*N*-3-trimethyl-2-butenylamine is a doublet at δ 2.9.[12] The same author[12] also reports that in *N*,*N*-2-trimethyl-2-butenylamine the C-1 methylene is a singlet at δ 2.9. By comparison our observed proton chemical shifts appear reasonable. The olefinic ^{13}C NMR signals of stylocheilamide (**3**) are observed at 132.3 and 131.6 (singlets) and at 121.5 and 120.2 ppm (doublets). Multiplicities and chemical shifts argue for the assigned

structure. By comparison, C-1 of *cis*-1-chloro-1-propene resonates at 126.2 ppm, trans C-1 at 128.9 ppm, while the respective C-2 resonances occur at 119.6 and 117.2 ppm.[13] For additional evidence we synthesized *trans*-5-chloro-5-decene and observed the C-5 resonance at 134.5 ppm and the C-6 signal at 125.1 ppm. Our assignments appear therefore secure.

The only remaining structural feature without direct degradative evidence is the epoxide. We have assigned a carbon resonance at 64.0 ppm (doublet in off-resonance) to C-9′ and resonances at 61.2 and 60.6 (singlets) to C-4′. The observed doubleting of this resonance is probably caused by geometrical isomerism about the C-2′,3′ double bond. By analogy, Magnusson and Thorén[14] in a series of α,β-epoxycyclohexanones report values of 56.3–61.6 ppm for the α and 61.2–64.4 ppm for the β carbon. None of the three compounds in their study was α-alkylated, but the values nevertheless correspond well. By single frequency on resonance decoupling the ^{1}H NMR resonance at δ 3.62 was related to the carbon resonance at 64.0 ppm and placed at C-9′. It is somewhat surprising that C-9′, which is β to the carbonyl and β to the chlorovinyl, should resonate at lower field than C-4′, which is α to these electronegative groups. The clue seems to lie in the influence of the C-7′ acetoxy group. When this group is replaced by a C-6′,7′ olefin as in **4**, the C-9′ resonance moves upfield to 60.3 ppm. Incidentally, the C-4′ dual resonances in compound **4** are virtually unchanged at 61.2 and 59.4 ppm from 61.2 and 60.6 ppm in **3**.

Relative stereochemistry of the epoxide is implied from the conformation of its hydride reduction product, alcohol **7**, which bears an axial hydroxyl, and which probably arose from hydride attack on the less hindered side. Further stereochemical definition was not possible as the pertinent ^{1}H NMR signal at C-9′ is neither a clean triplet nor a doublet of doublets, but a multiplet that was not amenable to first-order analysis.

The olefinic companion product **4**, which is related to stylocheilamide (**3**) by the elements of acetic acid and which trivially may be referred to as deacetoxystylocheilamide, may well be an artifact. It was recognized as an α,β-unsaturated ketone by its UV maximum at 241 nm, its single broad IR band in the carbonyl region at 1660 cm^{-1} replacing the acetoxy (1745 cm^{-1}), ketone (1720 cm^{-1}), and amide (1655 cm^{-1}) in **3**. The additional double bond is recognized by a new ^{1}H NMR signal at δ 6.35 (C-7′), allylically coupled (J = 1.5 Hz) to a new methyl signal at δ 1.80, and two new olefinic ^{13}C NMR signals at 137.6 (d, C-7′) and 133.4 (s, C-6′) ppm. Except for these data, all other spectral data, diagnostic tests, and oxidative degradations showed the deacetoxy compound to differ from stylocheilamide only in this respect. Attempted demonstration of the third double bond by hydrogenation over Pd/C led to saturation of the unhindered C-4,5 olefin and to aromatization of the carbocyclic ring, thus yielding the phenol **9** as the major



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product. The phenol was readily characterized by its UV maximum at 283 nm (ϵ 2300), shifted to 290 nm (ϵ 4100) in base, and by its strong IR band at 3250 cm^{-1}. The phenolic proton signal at δ 8.1, rapidly exchangeable, the one-proton doublet of doublets at δ 7.10, and the two-proton multiplet at δ 6.6–6.9 are consistent with the assigned structure. We generated by computer a close fitting 1,2,3-trisubstituted benzene with chemical shifts of δ 7.103 (H$_a$), 6.836 (H$_b$), and 6.741

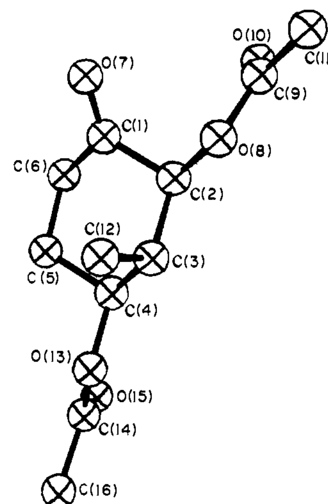
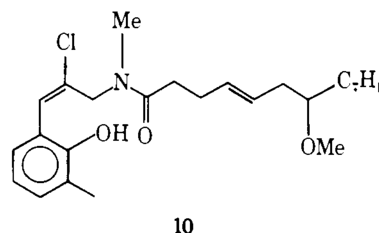


Figure 1. A computer-generated perspective drawing of the stylocheilamide degradation product (**8**). Hydrogens are omitted for clarity and no absolute configuration is implied.

(H$_c$) for the three aromatic protons, and coupling constants of J_{ab} = 1.3, J_{ac} = 8.1, and J_{bc} = 7.8 Hz. By using Jackman and Sternhell's[15] substituent constants and replacing the chlorovinyl with a phenyl, we arrive at a close match only for the substitution pattern in **9**: δ 7.13 (H$_a$), 6.88 (H$_b$), and 6.74 (H$_c$). Elucidation of the phenol structure not only confirmed the substitution pattern of the carbocyclic ring, but cleared up the structure of an unexpected phenol that we had obtained upon chromatography of stylocheilamide on neutral alumina, which accordingly must have structure **10**. Loss of acetic acid,



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opening of the epoxide followed by dehydration, and enolization of the ketone resulted in aromatization. Related A-ring alumina-catalyzed aromatizations of 2-acetoxy-3-keto-4,5-oxido-19-norsteroids to the corresponding 2,3-catechols have recently been observed.[16] It is worth noting that in a related transformation of stylocheilamide (**3**) with sodium acetate in refluxing ethanol, one of the products is deacetoxy compound **4**. The major product, isomeric with **4**, has not aromatized, but instead has rearranged to a γ-lactone of as yet undetermined structure.

The X-ray diffraction study of the stylocheilamide degradation product (**8**)[17] is summarized in Figure 1, which is a computer-generated prespective drawing. The absolute configuration was not determined in the X-ray study. The six-membered cyclohexanone ring adopts a chair conformation with equatorial acetates at C(2) and at C(4). The methyl group at C(3) is axial. The relative configuration is (2R*), (3S*), (4S*). Bond distances and angles agree with generally accepted values.

Experimental Section

Isolation. Specimens of *Stylocheilus longicauda* collected on a reef in Kaneohe Bay, Oahu, Hawaii, were either processed immediately or stored intact in a freezer (−10 °C). Whole animals (8.5 kg) were mildly mascerated with acetone in a Waring Blendor and the tissue was removed by filtration through glass wool. The filtrate was concentrated in vacuo and the resulting oil partitioned between ether and water (1/1). The ethereal layer was dried ($MgSO_4$) and concentrated

to a dark green oil (fraction A, 80 g). Fraction A was distributed between 10% aqueous MeOH (1 L) and petroleum ether (3 × 1 L). The separated layers were concentrated to oils to give fractions B (40 g) and C (39 g), respectively. Fraction B was then distributed between 20% aqueous MeOH (1 L) and CCl_4 (3 × 0.5 L) to afford, after concentration, fractions D (6.0 g) and E (35 g, 0.41%), respectively.

Fraction E was subjected to column chromatography (BioSil A, 1200 g) with hexane followed by hexane containing ether, then ether containing increasing amounts of MeOH. Column fractions eluted with 60–80% ether/hexane gave fraction F, which showed a positive Beilstein flame test for halogens and Dragendorff's test for nitrogenous compounds. Chlorophyll and extraneous coloration were removed from fraction F by short path length column chromatography (Florisil, 50 g) eluting with 33% ether in hexane (200 mL). Concentration of the eluant gave fraction G (5.50 g, 0.065%). Column chromatography (BioSil A, 550 g) of fraction G eluting with 5% MeOH in benzene afforded, after concentration, fraction H (1.256 g, 0.015%). Fraction H was further purified by gel filtration (Sephadex LH-20) and elution with MeOH. Column fractions were monitored and combined by similar TLC profiles, positive Dragendorff's test, and positive Beilstein halogen test to yield, after concentration, fraction I (0.971 g, 0.011%). TLC analysis (silica gel HF 254 + 366, 25% ether in *n*-hexane; 5% MeOH in benzene; 33% ether in EtOAc; toluene/2-butanone, 4/3; 5% MeOH in CH_2Cl_2) and LC (Spherisorb, 5% EtOAc in hexanes; μ-Porisil, 4% EtOAc in hexanes; C-18-reverse phase, 75% MeOH in water) of fraction I indicated that it was approximately a 4:1 mixture of stylocheilamide (**3**) and deacetoxystylocheilamide (**4**).

Fraction I was purified by preparative TLC (silica gel HF 254 + 366) by multiple development using ether/hexane (3/1). Elution of the minor component as a UV-active band afforded pure **4** (0.191 g, 0.002%). Elution of a strip immediately below the UV-visible band afforded pure **3** (0.603 g, 0.007%). All attempts to crystallize **3** or **4** were unsuccessful.

Stylocheilamide (**3**): $[\alpha]^{28}$ +10.6 (*c* 28.2, MeOH); UV λ max (MeOH) end absorption; IR (neat) 3040, 2920, 2860, 2820, 1745 (acetate), 1720 (ketone), 1655 (amide), 1450, 1390, 1360, 1230, 1100, 1040, 1000, 960, 880 cm^{-1}; 220-MHz ^1H NMR ($CDCl_3$) δ 6.26, 6.14 (each s, 1 H), 5.45 (bt, 2 H), 5.23 (bs, 1 H), 4.02 (bs, 1 H), 3.62 (m, 1 H), 3.30 (s, 3 H), 3.14 (m, 2 H), 2.98, 2.90 (each s, 3 H), 2.57–2.23 (m, 6 H), 2.18 (t, J = 5 Hz, 2 H), 2.00, 1.99 (each s, 3 H), 1.55–1.14 (bs, 12 H), 1.03 (overlapping d, J = 6 Hz, 3 H), 0.86 (bt, J = 6 Hz, 3 H); 25.2-MHz ^{13}C NMR ($CDCl_3$) (off-resonance mult) ppm 202.8, 202.3 (s, ketone), 172.8, 172.5 (s, acetate), 168.9 (s, amide), 132.3, 131.6 (s), 131.0 (d), 126.8 (d), 121.5 (d), 121.5, 120.2 (d), 80.5 (d), 76.3 (d), 64.0 (d), 61.2, 60.6 (s), 56.3 (q), 50.8, 48.3 (t), 41.2, 36.2, 35.0, 33.8, 33.2, 32.3, 31.8, 29.7, 29.7, 28.0, 25.2, 22.6, 20.8, 14.1, 10.8; mass spectrum *m/e* 526 (chemical ionization, no additional MH^+), *m/e* 525.2856 (M^+, calcd for $C_{28}H_{44}ClNO_6$, 525.2856), 21 eV, *m/e* (rel intensity) 527 (2), 525 (M^+, 5), 512 (2), 510 (5), 490 (10), 385 (33), 383 (100), 325 (15), 323 (40), 294 (50), 143 (50), 111 (15), 69 (25).

Deacetoxystylocheilamide (**4**): $[\alpha]^{25}$ −11.3 (*c* 15.7, MeOH); UV max (MeOH) 241 nm (ϵ 6800); IR (neat) 3040, 2920, 2860, 2820, 1665 (α,β-unsaturated keteone), 1660 (tertiary amide), 1450, 1400, 1120, 1090, 1060, 960, 880, 830 cm^{-1}; 100-MHz ^1H NMR ($CDCl_3$) δ 6.35 (bs, 1 H), 6.22, 6.06 (each t, J = 2 Hz, 1 H), 5.48 (m, 2 H), 4.35 (bd, J = 15 Hz), 3.87 (bd, J = 15 Hz), 4.27 (bd, J = 16 Hz), 3.94 (bd, J = 16 Hz) (total 2 H), 3.72 (t, J = 2 Hz, 1 H), 3.30 (s, 3 H), 3.2–2.8 (m, 4 H), 3.03, 2.96 (each s, 3 H), 2.5–2.3 (m, 3 H), 2.3–2.1 (m, 2 H), 1.80 (overlapping d, J = 1.5 Hz, 3 H), 1.6–1.2 (bs, 12 H), 0.88 (bt, J = 6 Hz, 3 H); 25.2-MHz ^{13}C NMR ($CDCl_3$) (off-resonance mult) ppm 191.7, 191.5 (s, ketone), 172.6, 172.2 (s, amide), 137.8, 137.6 (d), 133.3 (s), 132.5 (s), 132.3 (s), 131.3 (d), 127.0, 126.8 (d), 120.8, 118.8 (d), 80.6 (d), 61.2 (d), 59.4, 59.0 (s), 55.9 (q), 50.4, 48.0 (t), 36.3, 35.2, 33.9, 33.3, 32.3, 31.7, 29.6, 27.9, 25.2, 22.5, 16.1, 14.0; mass spectrum *m/e* 466 (chemical ionization, no additional MH^+), *m/e* 465.2621 (M^+, calcd for $C_{26}H_{40}ClNO_4$, 465.2641), 20 eV *m/e* (rel intensity) 467 (3), 465 (8), 452 (2), 450 (5), 430 (8), 325 (35), 323 (100), 271 (7), 269 (25), 234 (13), 143 (20), 69 (10).

Anal. ($C_{26}H_{40}ClNO_4$) C, H, N.

Hydrogenation of Stylocheilamide (3). A. Over Platinum. To a round-bottomed flask containing prereduced platinum oxide (3 mg) in EtOAc (2 mL) in a H_2 atmosphere (1 atm) was added stylocheilamide (15 mg, 0.067 mmol) in EtOAc (1 mL). Hydrogenation was continued for 1.5 h when TLC analysis indicated the absence of starting material. The reaction flask was flushed with N_2, the catalyst removed by filtration through a Celite mat, and the filtrate concentrated to an oil. Preparative TLC (silica gel HF 254 + 366) eluted with MeOH/benzene (1/20) gave dechlorotetrahydrostylocheilamide: IR (CCl_4) 2920, 2860, 2820, 1745 (acetate), 1725 (ketone), 1650 (amide), 1455, 1400, 1370, 1225, 1090, 1000, 900 cm^{-1}; 100-MHz ^1H NMR (CCl_4) δ 5.50 (m, 1 H), 3.8 (m, 1 H), 3.22 (s, 3 H), 3.0 (bs, 3 H), 2.3 (bt, 2 H), 1.97 (s, 3 H); mass spectrum (20 eV) *m/e* (rel intensity) 495 (M^+, 5), 470 (23), 463 (15), 435 (10), 396 (38), 336 (20), 297 (40), 268 (30), 252 (25), 241 (45), 164 (85), 44 (100).

B. Over Palladium. A Brown microhydrogenation apparatus[18] was charged with 10% Pd/C (5 mg) and 2.5 mL of 2-propanol, then flushed with N_2. Hydrogen was introduced via syringe and the catalyst was stirred until no further hydrogen uptake occurred. Stylocheilamide (20.58 mg, 0.0563 mmol) in 0.15 mL of 2-propanol was added. Hydrogen uptake (0.077 mmol) was complete in 6 h. Ratio: 1.4 mol hydrogen per mol stylocheilamide.

Lemieux Oxidation of Stylocheilamide (3). To stylocheilamide (0.500 g, 0.95 mmol) in acetone (15 mL) was added 5.5 mL of $NaIO_4$ (10.70 g in 50 mL of water) and 0.2 mL of $KMnO_4$ (15.8 g in 100 mL of water). After refluxing for 4 h, additional potassium permanganate solution (1.0 mL) was added and the reflux continued for 4 h. Acetone was removed by distillation, and the pot residue was cooled to room temperature and extracted with ether (2 × 60 mL). The separated ethereal phase was extracted successively with 15% aqueous Na_2CO_3 (2 × 30 mL) and saturated aqueous NaCl, then dried ($MgSO_4$) and concentrated to an intractable mixture.

The aqueous basic phase was acidified (pH 2) with concentrated H_3PO_4, saturated with NaCl, and extracted with ether (3 × 50 mL); the combined ether extracts were washed with saturated aqueous NaCl, dried ($MgSO_4$), and concentrated to 3-methoxydecanoic acid (0.067 g, 35% from **3**): IR (CCl_4) 3400–2400 (acid), 1715, 1270, 1070 cm^{-1}; 100-MHz ^1H NMR ($CDCl_3$) δ 10.7 (bs, 1 H, D_2O exchangeable), 3.65 (m, 1 H), 3.38 (s, 3 H), 2.51 (dd, J = 6, 3 Hz, 2 H), 1.7–1.2 (12 H), 0.9 (bt, J = 6 Hz, 3 H); mass spectrum (20 eV) *m/e* (rel intensity) 187 (M^+ − 15, 12), 172 (4), 170 (7), 133 (19), 103 (100), 69 (17), 61 (21).

Esterification of 3-Methoxydecanoic Acid. Diazomethane in ether was added dropwise to an ice-cooled solution of 3-methoxydecanoic acid (60 mg, 0.298 mmol) in ether (20 mL) until the solution was bright yellow. The solution was stirred at 0 °C for 30 min. The reaction mixture was concentrated to a yellowish oil. Pure methyl 3-methoxydecanoate (59 mg, 0.273 mmol, 92%) was obtained by column chromatography (SilicAR CC-7) eluting with dichloromethane/MeOH (20/1): IR (CCl_4) 2940, 2860, 1740 (ester), 1260, 1090 cm^{-1}; 100-MHz ^1H NMR ($CDCl_3$) δ 3.68 (s, 3 H), 3.7 (m, 1 H), 3.35 (s, 3 H), 2.47 (dd, J = 6, 3 Hz, 2 H), 1.6–1.3 (12 H), 0.9 (bt, J = 6 Hz, 3 H); mass spectrum (20 eV) *m/e* (rel intensity) 201 (M^+ − 15, 15), 186 (12), 184 (8), 143 (33), 127 (15), 117 (100), 65 (80).

3-Hydroxydecanoic Acid. Octanal (Aldrich, 12.0 g, 0.094 mol) and ethyl bromoacetate (Aldrich, 43.5 g, 0.284 mol) were dissolved in 70 mL of dry benzene and added dropwise to activated Zn (16.2 g, 0.25 mol, acid washed, MeOH rinse) and covered with dry benzene. I_2 was required to catalyze the reaction mixture, which slowly turned gray. When nearly all the Zn had reacted, the reaction mixture was refluxed for 2.5 h. The cooled reaction mixture was poured into cold, dilute H_2SO_2 and extracted with ether. The orange organic layer was separated, washed with water (twice), dried ($MgSO_4$), and concentrated to an oil. This oil was dissolved in MeOH and 6 N KOH was added to about pH 10. The solution was stirred at room temperature for 1 h, then refluxed for 1 h. The solvent was removed at reduced pressure and the resulting basic aqueous solution was extracted with ether. The separated basic solution was acidified with concentrated H_3PO_4 to about pH 2 and extracted with CH_2Cl_2. The organic phase was separated, washed with water, dried ($MgSO_4$), and evaporated to a semisolid. Recrystallization from cyclohexane gave 3-hydroxydecanoic acid, white solid (10.2 g, 58%): mp 55.0–55.5 °C; IR (CCl_4) 3600–2400 (acid tailing), 1715 (C=O), 1420, 1290, 1220 cm^{-1}; 100-MHz ^1H NMR ($CDCl_3$) δ 7.35 (bs, 2 H, D_2O exchangeable), 4.02 (m, 1 H), 2.52 (s, 1 H), 2.45 (d, J = 5 Hz, 1 H), 1.6–1.2 (12 H), 0.88 (bt, J = 6 Hz, 3 H); ^{13}C NMR ($CDCl_3$), ppm, sp^2, 177.0 (C=O); sp^3, 68.2 (d), 41.2, 36.4, 31.8, 29.6, 29.5, 25.5, 22.6, 14.0.

3-Hydroxydecanoic acid (1.50 g, 8.0 mmol) was dissolved in 25 mL of ether and cooled to 0 °C and ethereal diazomethane (freshly prepared from Diazald) was added dropwise until the bright yellow color remained. After stirring at 0 °C for 1.5 h, the solution was extracted

with dilute $NaHCO_3$ and water and dried (Na_2SO_4). Evaporation of the solvent gave the methyl ester as a clear oil (1.62 g, 100%): IR (neat) 3550 (OH), 1740 (C=O), 1370, 1250, 1170 cm^{-1} (C-O); 100-MHz ^1H NMR ($CDCl_3$) δ 4.00 (m, 1 H), 3.68 (s, 3 H), 3.25 (bs, 1 H, D_2O exchangeable), 2.48 (br s, 1 H), 2.42 (d, J = 3 Hz, 1 H) (2.48 and 2.42 signals collapse to a bs at 2.46 upon irradiation at δ 4.00), 1.7-1.2 (12 H), 0.89 (bt, J = 6 Hz, 3 H); ^{13}C NMR ($CDCl_3$) sp^2, 173.0 (C=O); sp^3, 68.1 (d), 51.5 (q), 41.9, 37.1, 29.9, 29.7, 25.8, 22.9, 14.2 ppm.

Methyl 3-hydroxydecanoate (0.420 g, 0.210 mmol) was dissolved in 10 mL of $CHCl_3$, dried over $MgSO_4$, and filtered into a dry, round-bottomed flask. The solution was flushed with N_2 and Ag_2O (0.35 g) catalyst added. Three portions of CH_3I (0.5 mL each, 1.5 mL, 24.2 mmol) were added at 15-min intervals. The reaction mixture was stirred at room temperature for 2 days and the catalyst removed by filtration through Celite. Evaporation of the solvent gave a greenish oil (0.428 g, 95%). The oil was chromatographed on BioSil A (200-325 mesh) eluted with ether-hexanes (5:1) to give methyl 3-methoxydecanoate as an oil (0.401 g, 89%): IR (CCl_4) 1740 (ester), 1270, 1160 cm^{-1} (C-O); ^{13}C NMR ($CDCl_3$), ppm, sp^2, 173.0 (C=O); sp^3, 77.7, 56.8, 51.4, 41.4, 36.5, 31.7, 29.2, 29.1, 25.4, 22.5, 13.9; 100-MHz ^1H NMR ($CDCl_3$) δ 3.7 (s, 3 H), 3.52 (m, 1 H), 3.34 (s, 3 H), 2.44 (d, J = 6 Hz, 2 H), 1.6-1.3 (12 H), 0.95 (bt, J = 6 Hz, 2 H).

Ozonolysis of Stylocheilamide. Stylocheilamide (0.556 g, 1.06 mmol) in anhydrous MeOH (50 mL) was placed into a dry, N_2-flushed 125-mL side-arm filter flask fitted with gas inlet, drying ($CaCl_2$), and exit tubes. The solution was cooled to −78 °C (dry ice in acetone). A stream of O_3 was passed into the reaction flask until the exit tube indicated the presence of ozone. The cold reaction mixture was immediately flushed with nitrogen, palladium on carbon (10%, 200 mg) was added, and the ozonide was reductively cleaved by the addition of hydrogen (1 atm). After 40 min at −78 °C, the reaction mixture was allowed to warm to 0 °C. After 1 h the reaction mixture was thoroughly flushed with N_2 and the catalyst removed by filtration through a pad of Celite. Approximately 10 mL of the filtrate was distilled into a flask containing an aqueous methanolic dimedone solution. No reaction was observed. The pot residue was concentrated to an oil. Trituration of the oil with petroleum ether (bp 30-60 °C) gave a nonpolar, soluble fraction M (0.2291 g) and a polar, insoluble fraction N (0.3107 g). TLC examination of fraction M showed it to contain unreacted **3** and an air-oxidizable aldehyde. Preparative TLC (silica gel HF 254 + 366) gave unreacted stylocheilamide (0.1852 g) and an acid (0.086 g) identical with 3-methoxydecanoic acid.

Fraction N was subjected to gel filtration (Sephadex LH20) and eluted with MeOH. Six fractions were combined by identical TLC profiles to give fraction O (0.2204 g) as an oil. Preparative TLC (silica gel HF 254 + 366) of fraction O developed with CH_2Cl_2/MeOH (20/1) showed a faintly short-wavelength UV-visible band (R_f 0.45-0.55). Extraction of the band with acetone gave fraction P (0.1294 g) as an oil. Fraction P was purified by preparative TLC (silica gel HF 254 + 366) using multiple development with ether as solvent to give **5**, homogeneous by TLC: IR (CCl_4) 2980, 2940, 2880, 2740, 1730 (ketone), 1745 (acetate), 1650 (amide), 1230, 1115, 1070, 1050, 900 cm^{-1}; 100-MHz ^1H NMR ($CDCl_3$) δ 6.26 (t, 1 H, J = 1.5 Hz), 5.1 (m, 1 H), 4.40 (t, 1 H, J = 6 Hz), 4.04 (bs, 2 H), 3.64 (m, 1 H), 3.37 (s, 6 H), 0.9 (d, 3 H, J = 7 Hz); 25.2-MHz ^{13}C NMR ($CDCl_3$) ppm (off-resonance mult) 202.5 (s, ketone), 172.4 (s, acetate), 169.9 (s, amide), 131.9 (s), 121.8 (d), 103.7 (d), 75.7 (d), 63.9 (d), 60.9 (s), 53.1 (q), 53.1 (q), 48.3 (t), 41.2, 29.6, 27.8, 27.8, 27.8, 10.7; mass spectrum m/e 417.1549 (M^+, calcd for $C_{19}H_{28}ClNO_7$, 417.1554), 70 eV, m/e (rel intensity) 419 (2), 417 (6), 388 (6), 386 (19), 382 (70), 350 (22), 252 (10), 174 (20), 148 (15), 131 (60), 99 (30), 71 (100).

Lithium Aluminum Hydride Reduction of 3. To a suspension of $LiAlH_4$ (0.600 g, 65 mmol) in dry ether (50 mL) in N_2 atmosphere was added stylocheilamide (1.218 g, 2.31 mmol) in dry ether (20 mL). The reaction mixture was stirred at room temperature (1 h), then heated to reflux (3 h). Excess hydride was destroyed by dropwise addition of 2-propanol to the ice-cooled reaction. Saturated aqueous NaCl was slowly added until a white, granular precipitate formed. The reaction mixture was filtered and the filtrate extracted with 5% HCl (3 × 15 mL). The aqueous acidic phase was basified (pH 10) with solid Na_2CO_3 and extracted with diethyl ether (3 × 15 mL). The ethereal solution was washed with saturated aqueous NaCl (3 × 10 mL), dried ($MgSO_4$), and concentrated to a yellow oil (0.976 g). Preparative TLC (silica gel HF 254 + 366) by multiple development with ether/hexane (3/1) yielded one major band, fraction J (0.5163 g), as an oil: IR (CCl_4) 3400, 1190, 1160, 1125, 960 cm^{-1}.

Acetylation of Fraction J. To fraction J (0.5163 g) in dry (KOH) pyridine (5 mL) was added acetic anhydride (0.80 mL, 7.28 mmol) and the reaction mixture heated to 55 °C for 3 h. Excess acetic anhydride was hydrolyzed by the addition of water and the solvents removed under vacuum. The resulting oil was partitioned between water and CH_2Cl_2. The organic layer was washed sequentially with 10% aqueous Na_2CO_3 and saturated aqueous NaCl, then dried ($MgSO_4$) and concentrated to give fraction K as a colorless oil. Fraction K was purified by column chromatography (BioSil A) eluted with ether/cyclohexane (3:1) to give **6** (0.1988 g, 15% from **3**) as an oil, homogeneous by TLC: IR (CCl_4) 3100 (OH), 1735 (acetate), 1600, 1215, 1085, 1020, 1000, 955 cm^{-1}; 100-MHz ^1H NMR ($CDCl_3$) δ 6.04 (bs, 1 H), 5.44 (bt, 2 H, J = 3.5 Hz), 4.96 (m, 2 H), 3.65 (bd, 1 H, J = 14 Hz), 3.30 (s, 3 H), 2.40 (bs, 3 H), 2.05 (s, 3 H), 2.03 (s, 3 H); mass spectrum (20 eV) m/e (rel intensity) 559 (18), 557 (M^+, 50), 544 (15), 542 (40), 522 (80), 502 (6), 498 (20), 462 (22), 416 (7), 414 (22), 348 (23), 246 (60), 330 (33), 328 (100), 268 (23), 143 (45), 84 (25); 25.2-MHz ^{13}C NMR ($CDCl_3$) (off-resonance mult) ppm 170.1 (s, 2 C), 137.5 (s), 131.3 (d), 126.9 (d), 118.4 (d), 80.6 (d), 75.6, 75.1, 71.1, 63.1, 56.2, 56.1, 40.4, 36.3, 33.2, 32.8, 31.8, 29.8, 29.2, 26.9, 26.3, 25.2, 25.2, 20.8, 20.6, 13.9, 13.7.

Ozonolysis of 6. Compound **6** (0.1988 g, 0.357 mmol) in anhydrous MeOH (20 mL) at −78 °C (dry ice in acetone) was reacted with ozone for 15 min. Excess ozone was displaced in a stream of N_2. The solution was warmed to 0 °C (ice water), palladium on carbon (10%, 20 mg) added, and the reaction vessel flushed with H_2. The hydrogenation (1 atm) was continued for 3.5 h, H_2 displaced by N_2, and the catalyst removed by filtration. Concentration of the reaction mixture gave fraction Q, which showed two major spots on TLC. Preparative TLC (silica gel HF 254 + 366) of fraction Q developed with ether/hexane (3/1) gave two major bands, fraction R (R_f 0.25-0.35) and fraction S (R_f 0.35-0.45).

Fraction R was subjected to gel filtration (Sephadex LH-20) and eluted with MeOH. Column fractions were combined by TLC profiles to give fraction T, which was column chromatographed (BioSil A) eluting with ether to give methyl 1-hydroxy-2,4-diacetoxy-3-methylcyclohexanecarboxylate (**7**), homogeneous by TLC and GLC: UV max (MeOH) 217 nm (ε 158); IR (CCl_4) 3500 (OH), 2980, 1730 (acetate, ester), 1370, 1230, 1030 cm^{-1}; 100-MHz ^1H NMR ($CDCl_3$) δ 5.00 (q, 1 H, J = 3 Hz), 4.82 (d, 1 H, J = 2.5 Hz), 3.74 (s, 3 H), 2.5-1.8 (6 H), 2.10 (s, 3 H), 2.08 (s, 3 H), 0.95 (d, 3 H, J = 7.5 Hz); mass spectrum m/e 288.1232 (M^+, $C_{13}H_{20}O_7$, calcd 288.1209), 20 eV, m/e (rel intensity) 288 (M^+, 4), 261 (2), 246 (68), 228 (20), 186 (100), 169 (30), 170 (32), 109 (30), 84 (30).

Fraction S was recrystallized from cyclohexane to yield 2β,4β-diacetoxy-3β-methylcyclohexanone (**8**): mp 89.0-90.0 °C; IR (CCl_4) 2980 (w), 1760 (acetate), 1740 (ketone and acetate), 1370, 1210, 1080, 1030 cm^{-1}; 100-MHz ^1H NMR ($CDCl_3$) δ 5.35 (p, 1 H, J = 5 Hz), 5.3 (d, 1 H, J = 5.5 Hz), 2.8-1.8 (5 H), 2.15 (s, 3 H), 2.08 (s, 3 H), 0.95 (d, 3 H, J = 7 Hz); mass spectrum (20 eV) m/e (rel intensity) 186 ($M^+ - CH_2{=}C{=}O$), (29), 168 (18), 143 (45), 126 (100), 98 (50), 80 (40), 69 (18), 43 (40).

Hydrogenation of 4. Deacetylstylocheilamide (**4**, 0.0857 g, 0.184 mmol) in EtOAc (10 mL) was hydrogenated (1 atm) over Pd/C (10%, 8 mg). After 1 h TLC analysis indicated the absence of **4**. The reaction vessel was flushed with nitrogen and the catalyst removed by filtration. Concentration of the filtrate on a rotary evaporator yielded fraction U. Gel filtration (Sephadex LH-20) of fraction U eluting with MeOH gave strongly UV-absorbing material in six fractions. Combination of the column fractions and concentration in vacuo gave fraction V, which showed one major spot on TLC. Preparative TLC (silica gel HF 254 + 366) with ether/hexanes (3/1) yielded, after concentration, **9** (0.0292 g), homogeneous by TLC: UV max (MeOH) 283 nm (ε 2100); IR (CCl_4) 3250 (OH), 1640 (amide), 1460, 1420, 1340, 1280, 1240, 1200, 1090 cm^{-1}; 100-MHz ^1H NMR ($CDCl_3$) δ 8.1 (bs, 1 H, D_2O exchangeable), 7.11 (m, 1 H, J = 8.1, 1.3 Hz), 6.84 (m, J = 7.8, 1.3 Hz), 6.74 (m, 1 H, J = 7.8, 8.1 Hz), 6.20 (t, J = 1.5 Hz), 4.05 (bs, 2 H), 3.32 (s, 3 H), 3.1 (bs overlapping m, 4 H), 2.26 (s overlapping t, 5 H), 1.7-1.0 (23 H), 0.85 (bt, 3 H, J = 5 Hz); mass spectrum m/e 451.2835 (M^+, calcd for $C_{26}H_{42}ClNO_3$, 451.2854), 70 eV, m/e (rel intensity) 453 (1), 451 (3), 416 (60), 384 (32), 353 (8), 351 (25), 176 (38), 175 (70), 174 (40), 147 (35), 145 (100), 69 (50), 55 (35), 44 (70).

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.

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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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