Table I. Subtilisin-Catalyzed Hydrolysis of 2,2,2-Trifluoro-*N*-methyl-*p*-nitroacetanilide at pH 8.42[a]

10^4[S], *M*	10^4[Inh],[b] *M*	10^5[E], *M*	Rate constant, $\times 10^3$, sec^{-1}	k_2/K_s, M^{-1} sec^{-1}
1.33	0	4.82	8.48	153
1.42	0	0	1.10	
1.31	7.74	4.72	1.10	

[a] I = 0.1 *M* Tris buffer, 0.62% acetonitrile, 25°. Kinetics were observed at 390 nm. [b] The inhibitor is phenylmethanesulfonyl fluoride.

ical explanation for the absence of subtilisin activity toward *p*-nitroacetanilide while showing large activity (comparable to that of highly specific amide substrates[14,15]) toward the 2,2,2-trifluoro analog.

Finally, it should be mentioned that the difference between rate constants shown by the *N*-methylanilide (I) and its unmethylated analog (II) is not necessarily due to the presence of the methyl group alone but may be related to ionization of the unmethylated analog[16] with a resulting loss in nucleophilic susceptibility.

Table II. Subtilisin-Catalyzed Hydrolysis of 2,2,2-Trifluoro-*p*-nitroacetanilide at pH 8.42[a]

$10^4[S]_0$, *M*	10^2[Inh],[b] *M*	10^5[E], *M*	Rate constant, sec^{-1}	k_2/K_s, M^{-1} sec^{-1}	K_I(Inh), *M*
1.33	0	4.82	1.193×10^{-3}	17.7	
1.42	0	0	3.39×10^{-4}		
1.42	1.53	0	3.40×10^{-4}		
1.33	1.436	4.82	6.73×10^{-4}		5.34×10^{-2}

[a] I = 0.1 *M* Tris buffer, 0.62% acetonitrile, 25°. Kinetics were observed at 390 nm. [b] The inhibitor is *N*-benzoyl-L-arginine.

While these substrates are structurally analogous to the nonspecific subtilisin substrate *p*-nitrophenyl acetate[3] it seemed useful to determine whether catalysis of these highly activated compounds is dependent on the active site serine hydroxyl. Therefore, the rate of hydrolysis was also determined in the presence of phenylmethanesulfonyl fluoride (PMSF)[4] inhibited subtilisin (Table I). In another experiment the inhibitory effect of *N*-benzoyl-L-arginine was also determined (Table II).[5]

The results obtained are unexpected in light of the pretransition state protonation theory of chymotryptic anilide hydrolysis[6,7] but are consistent with the view that anilide hydrolysis proceeds through an initial tetrahedral intermediate[8-10] followed by rate-determining breakdown to give products. This latter view is strengthened by the observation that chymotryptic hydrolytic constants toward specific amides are apparently independent of any electronic effect.[8] Since highly specific subtilisin ester substrates also show no electronic effect on acylation[9] while less specific substrates show an electronic effect comparable to that in chymotrypsin,[11-13] it is evident that nonspecific amides may also show an electronic effect that is absent in specific amides.

In any case, such an electronic effect is the only log-

(3) L. Polgar and M. L. Bender, *Biochemistry*, **6**, 610 (1967).
(4) C. S. Wright, R. A. Alden, and J. Kraut, *Nature (London)*, **221**, 235 (1969), and references therein.
(5) Ethyl *N*-benzoyl-L-argininate is a subtilisin substrate with a K_m of 1×10^{-2} *M*: A. N. Glazer, *J. Biol. Chem.*, **241**, 635 (1966).
(6) T. Inagami, A. Patchornik, and S. S. York, *J. Biochem. (Tokyo)*, **65**, 809 (1969).
(7) J. H. Wang and L. Parker, *Proc. Nat. Acad. Sci. U. S.*, **58**, 2451 (1967).
(8) M. Philipp, R. M. Pollack, and M. L. Bender, *Proc. Nat. Acad. Sci. U. S.*, in press.
(9) A. R. Fersht, *J. Amer. Chem. Soc.*, **94**, 293 (1972).
(10) M. Caplow, *ibid.*, **91**, 3639 (1969).
(11) M. Philipp, Doctoral Dissertation, Northwestern University, Evanston, Ill., 1971.
(12) M. L. Bender and K. Nakamura, *J. Amer. Chem. Soc.*, **84**, 2577 (1962).
(13) Chymotrypsin also demonstrates ester hydrolysis Hammett ρ values that vary with substrate specificity, but the effect is not nearly so pronounced as in subtilisin; see (a) R. E. Williams and M. L. Bender, *Can. J. Biochem.*, **49**, 210 (1971); (b) A. Williams, *Biochemistry*, **9**, 3383 (1970); (c) C. D. Hubbard and J. F. Kirsch, *ibid.*, **11**, 2483 (1972).

Acknowledgment. This work was supported by a National Institutes of Health predoctoral fellowship to M. P. and Grant RO1-HLO-5726 of the NIH to M. L. B.

(14) K. Morihara, T. Oka, and H. Tsuzuki, *Arch. Biochem. Biophys.*, **138**, 515 (1970).
(15) It is interesting that this activity is comparable to that shown toward the nonspecific activated ester *p*-nitrophenyl acetate (2).
(16) P. M. Mader, *J. Amer. Chem. Soc.*, **87**, 3191 (1965).

M. L. Bender,* M. Philipp
Division of Biochemistry, Department of Chemistry
Northwestern University, Evanston, Illinois 60201

Received December 4, 1972

A New Rhodium(I)–Porphyrin Complex. II.[1] Synthesis and Oxidative Alkylation

Sir:

A new type of metalloporphyrin with two metal ions bonded to a porphyrin has been reported for Re and Rh complexes.[1,2] The crystallographic study of its rhenium(I) complex has shown that each Re ion is bonded to three nitrogen atoms and that two Re atoms are bonded to one porphyrin on opposite sides of the plane of the porphyrin.[3] Recently James and Stynes have reported on the synthesis of the rhodium(I)–porphyrin complex formulated as H[Rh(porphyrin)]·$2H_2O$ and the reactivity of lower valent metal porphyrins.[4] A rhodium porphyrin in various oxidation states may provide an interesting model to investigate the reaction behavior of vitamin B_{12} as well as the other models.[5,6]

We wish to report a new rhodium(I)–porphyrin

(1) For the previous paper in this series, see Z. Yoshida, H. Ogoshi, T. Omura, E. Watanabe, and T. Kurosaki, *Tetrahedron Lett.*, 1077 (1972).
(2) D. Ostfeld, M. Tsutsui, C. P. Hrung, and D. C. Conway, *J. Amer. Chem. Soc.*, **93**, 2548 (1971).
(3) D. Cullen, E. Meyer, T. S. Srivastava, and M. Tsutsui, *ibid.*, **94**, 7603 (1972).
(4) B. R. James and D. V. Stynes, *ibid.*, **94**, 6225 (1972).
(5) J. H. Weber and G. N. Schrauzer, *ibid.*, **92**, 726 (1970).
(6) J. Kwiatek, "Transition Metals in Homogeneous Catalysis," G. N. Schrauzer, Ed., Marcel Dekker, New York, N. Y., 1971, p 31.

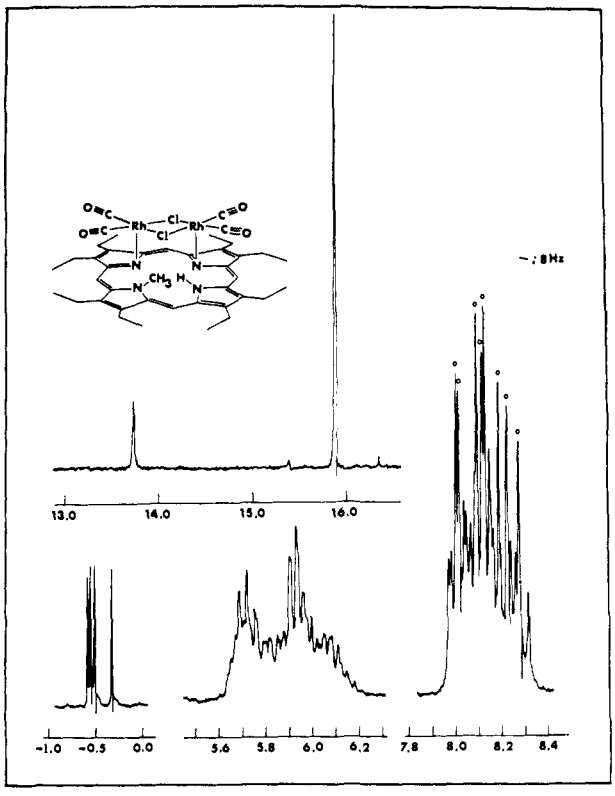


Figure 1. The 220-MHz nmr spectrum of **1** in $CDCl_3$ using TMS as internal standard. The symbol O indicates the central peak of each triplet.

complex and its novel chemical behavior. Equimolar amounts of *N*-methyloctaethylporphyrin[7] and $[Rh(CO)_2Cl]_2$ in benzene were stirred at room temperature for 1 hr under nitrogen atmosphere. After the solvent was removed under reduced pressure, the residue was crystallized from chloroform–benzene–pentane to afford dark violet crystals (**1**), mp 96° dec. *Anal.* Calcd for $C_{41}H_{48}N_4O_4Cl_2Rh_2$: C, 52.52; H, 5.16; N, 5.98; Cl, 7.56. Found: C, 52.45; H, 4.83; N, 5.46; Cl, 7.28. The visible spectrum of **1** in $CHCl_3$ shows the absorption maxima at 398 nm (log ϵ 4.56), 430 (4.48), 555 (3.70), 570 (3.78), 595 (3.75), and 617 (3.54). The ir spectrum in KBr has four strong absorptions at 2075, 2050, 2010, and 1980 cm^{-1} assigned to the terminal carbonyl stretching vibrations of the metal carbonyl groups and two medium absorptions at 605 and 490 cm^{-1} assignable to the Rh–CO stretching vibrations. The frequencies of these bands are quite similar of those of $[Rh(CO)_2Cl]_2 \cdot bipy$[8] and $[Rh(CO)_2Cl]_2$. The existence of a N–H bond is confirmed by the ir band at 3160 cm^{-1}. The 220-MHz nmr spectrum (see Figure 1) in $CDCl_3$ indicates four sharp, well-resolved proton signals at τ −0.35, −0.50, −0.55, and −0.58. If we assume the coupling constant $J_{CH_3-CH_2}$ as 8.0 Hz, the complicated signals in the region of τ 7.9–8.4 can be resolved into the eight different methyl proton signals of the peripheral ethyl groups at τ 8.28, 8.23, 8.20, 8.16, 8.13, 8.11, 8.04, and 8.02 (triplet, 3 H, respectively). Therefore the symmetry of the porphyrin ring is markedly reduced compared with metal free porphyrins and usual metalloporphyrins. The two singlets at τ 13.74 (1 H) and 15.90 (3 H) are assigned to the N–H and N–CH_3 protons, respectively. The striking upfield shifts of these signals are interpreted in terms of the anisotropic effect due to the porphyrin ring current. These spectroscopic results indicate that the structure of the $[Rh(CO)_2Cl]_2$ group is still maintained and the N–H and N–CH_3 bonds exist in **1**. The Rh–Rh distance in $[Rh(CO)_2Cl]_2$ has been reported to be 3.12 Å,[9] and the distance between the two adjacent nitrogen atoms of planar porphyrin is about 2.9 Å.[10] Thus it is presumably considered that two Rh ions of $[Rh(CO)_2Cl]_2$ moiety are bonded to the two adjacent nitrogen atoms of the porphyrinato core as is shown in Figure 2.

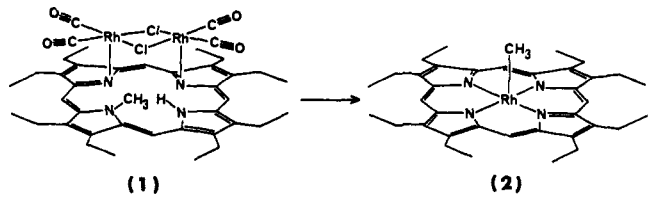


Figure 2. The reaction profile from the complex **1** to the methylrhodium(III) complex **2**.

Gentle heating of **1** in chloroform or chromatography of **1** on silica gel afforded the methylrhodium(III) complex of octaethylporphyrin, $(OEP)Rh^{III}CH_3$ (**2**), mp 260° dec. *Anal.* Calcd for $C_{37}H_{47}N_4Rh$: C, 68.29; H, 7.59; N, 8.61. Found: C, 68.02; H, 7.22; N, 8.71. The complex **2** was confirmed to be identical with the product obtained by the reaction of the aquochlororhodium(III) complex $(OEP)Rh^{III}Cl \cdot 2H_2O$ with methyllithium.[1] The visible spectrum of **2** in $CHCl_3$ shows four absorption maxima at 384 nm (log ϵ 4.98), 396 (5.11), 512 (4.30), and 544 (4.51). Its ir spectrum in KBr shows the absence of carbonyl stretching vibrations and the existence of one medium absorption at 560 cm^{-1} assigned to Rh–C stretching vibration which is shifted to 530 cm^{-1} by the deuteration of the methyl group. The nmr spectrum in $CDCl_3$ shows the proton chemical shifts at τ 8.10 (triplet, $-CH_2CH_3$, 24 H), 5.99 (quartet, $-CH_2CH_3$, 16 H), 0.04 (singlet, =CH—, 4 H), and 16.47 (doublet, Rh–CH_3, 3 H, J_{Rh-H} = 3.0 Hz).

In the related system to metalloporphyrins, Grigg and his coworkers have reported that the alkylation of nickel corrole ambident anions with alkyl halides occurs at the pyrrolic nitrogen atom and the moderate heating of *N*-alkyl nickel corrole causes alkyl migration from the pyrrolic nitrogen atom to the β-carbon atom of the pyrrolic ring.[11] A similar migration has been reported for the palladium corrole.[12] On the contrary, our result is the first case of alkyl migration from a nitrogen atom to a metal ion. The alkyl migration may proceed concertedly with oxidation of rhodium(I) to rhodium(III). The N–CH_3 bond fission seems to be facilitated by the aid of a low-valent rhodium ion. The reaction of *N*-ethyloctaethylporphyrin with $[Rh(CO)_2Cl]_2$ affords the similar rhodium(I) complex to **1**, which is easily oxidized to $(OEP)Rh^{III}CH_2CH_3$. The

(7) W. K. McEwen, *J. Amer. Chem. Soc.*, **68**, 711 (1946).
(8) D. N. Lawson and G. Wilkinson, *J. Chem. Soc.*, 1900 (1965).
(9) L. F. Dahl, C. Martell, and D. L. Wampler, *J. Amer. Chem. Soc.*, **83**, 1762 (1961).
(10) S. J. Silvers and T. Tulinsky, *ibid.*, **89**, 3331 (1967).
(11) R. Grigg, A. W. Johnson, and G. Shelton, *Justus Liebigs Ann. Chem.*, **746**, 32 (1971).
(12) M. J. Broadhurst, R. Grigg, G. Shelton, and A. W. Johnson, *Chem. Commun.*, 231 (1970).

mechanism of metal oxidation and alkyl migration is currently under investigation.

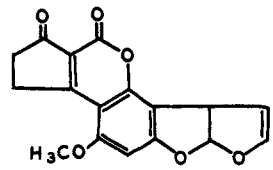
H. Ogoshi, T. Omura, Z. Yoshida*
Department of Synthetic Chemistry, Kyoto University Yoshida, Kyoto, 606, Japan

Received November 10, 1972

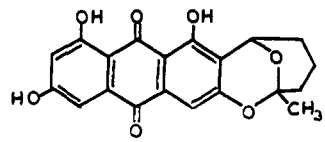
Averufin in the Biosynthesis of Aflatoxin B_1

Sir:

Chemical degradation[1] of aflatoxin B_1 (I) prepared from ^{14}C-labeled acetate by cultures of *Aspergillus parasiticus* ATCC 15517[2] has revealed that the basic skeleton of the aflatoxin molecule is totally derived from acetate units and that methionine contributes the methoxy methyl group. Based on the label distribution in the molecule, a number of fungal metabolites have been proposed as precursors of aflatoxin B_1.[3]



I



II

Recently, a mutant of *A. parasiticus* ATCC 15517 impaired in aflatoxin biosynthesis has been found to accumulate averufin (II),[4] a fungal pigment not being included in the above proposed intermediates for aflatoxin biosynthesis. ^{14}C-Labeled averufin was thus prepared from cultures of the mutant supplemented with [1-^{14}C]acetate, and was used to test if averufin cannot be incorporated into aflatoxin B_1 by the wild type cells.

Conidia of the deficient mutant, W49,[4] were cultivated in a synthetic medium[5] supplemented with 0.1% yeast extract as shaken cultures. Mycelial pellets at 48 hr were harvested to prepare resting cell cultures[6] of the mutant, which was then fed [1-^{14}C]acetate and incubated to accumulate [^{14}C]averufin. The labeled averufin was exhaustively extracted from the mycelial pellets with acetone and purified successively using three tlc systems: ChromAR 500 sheets (Mallinckrodt Chemical Works, St. Louis, Mo.) developed with chloroform–acetone–*n*-hexane (85:15:20, CAH), benzene–ethanol–water (46:35:19, BEW), and benzene–petroleum ether–acetone (90:8:2, BPeA). The purity of [^{14}C]averufin was verified by coincidence of spots in chromatograms and autoradiograms and by comparing the uv spectrum of the [^{14}C]averufin with that of pure averufin obtained from Donkersloot.[4]

The resting cell techniques used to incorporate [1-^{14}C]acetate into aflatoxin[7] were employed in the incorporation studies. [^{14}C]Averufin (1.5 μmol, 0.3 μCi/μmol) dissolved in 0.2 ml of acetone was placed in each 50-ml baffled flask to which was then added slowly 9.8 ml of the resting cell medium.[7] By this procedure 68% of the [^{14}C]averufin remained dissolved in the aqueous medium. One gram of wet cells was added to each flask and the flasks shaken (150 rpm) for 20 hr at 30°. Control flasks containing 30 μmol of [1-^{14}C]-acetate and 2% acetone were used to check the *de novo* synthetic activity of the cells. Acetone at this concentration (2%) reduced the incorporation efficiency of [1-^{14}C]acetate into aflatoxins by 37%. Another set of control flasks containing autoclaved cells was used to demonstrate the enzyme activities involved in the label incorporation.

In the first experiment, 150 μg of unlabeled aflatoxin B_1 was added to each flask as a carrier to facilitate extraction and purification. Aflatoxins were extracted from the fermented broths with chloroform and were separated from residual averufin and other compounds successively using two tlc systems: ChromAR-500 sheets developed with ethyl acetate–isopropyl alcohol–water (10:2:1, EaIpW) and BPeA. Aflatoxins eluted with chloroform from the ChromAR strips were further purified twice on Adsorbosil-1 plates developed with CAH and EaIpW. The purified aflatoxin B_1 retained 8.4 and 0.6% of the labels from [^{14}C]averufin and [1-^{14}C]-acetate, respectively. To ascertain that the radioactivity in aflatoxin B_1 was not due to the contamination of radioactive impurities, aflatoxin B_1 was subjected to further purification with two different tlc systems. The specific radioactivity of aflatoxin B_1 remained constant after each purification. The aflatoxin B_1 similarly isolated and purified from the control flasks containing deactivated cells did not contain any radioactivity.

In the second experiment, no carrier aflatoxin B_1 was added to the flasks and the labeled aflatoxin B_1 was isolated and purified by the same series of tlc systems. The relative specific activity or RIC[8] of the aflatoxin B_1 derived from [^{14}C]averufin was more than twice that of aflatoxin B_1 from [1-^{14}C]acetate, despite the fact that averufin concentration in the medium was much lower than acetate.

The above results as well as the accumulation of averufin by the deficient mutant suggested that averufin is a precursor of aflatoxin B_1. To confirm this, coexistence of averufin and aflatoxin in the parent strain culture was examined. Fermented broths and mycelia of *A. parasiticus* ATCC 15517 which had been grown for 48 hr in synthetic medium[5] were extracted with

(1) M. Biollaz, G. Büchi, and G. Milne, *J. Amer. Chem. Soc.*, **90**, 5017 (1968).
(2) J. A. Donkersloot, D. P. H. Hsieh, and R. I. Mateles, *ibid.*, **90**, 5020 (1968).
(3) M. Biollaz, G. Büchi, and G. Milne, *ibid.*, **90**, 5019 (1968).
(4) J. A. Donkersloot, R. I. Mateles, and S. S. Yang, *Biochem. Biophys. Res. Commun.*, 47, 1051 (1972).
(5) J. C. Adye and R. I. Mateles, *Biochem. Biophys. Acta*, **86**, 418 (1964).
(6) D. P. H. Hsieh and R. I. Mateles, *Appl. Microbiol.*, 22, 79 (1971).
(7) D. P. H. Hsieh and R. I. Mateles, *Biochem. Biophys. Acta*, **208**, 482 (1970).
(8) RIC, relative isotope content, shows the precursor–product relationship of a labeled compound with aflatoxin. The higher the RIC value the closer is the product to the precursor. See ref 7 for details.