1-Methyl-β-carboline-3-carboxylic Acid, an Unusual Metabolite from Cows Fed with Corn Silage

The blue-fluorescent substance which appears in urine and milk of cows fed with corn silage has been isolated and identified with 1-methyl-β-carboline-3-carboxylic acid. The formation and the possible biological activity of this compound are discussed.

Feeding cattle with corn silage has become a common practice in northern Italy, especially in winter. During a series of investigations aimed to establish the influence of such a diet on properties of milk and cheese (Battistotti and Bertoni, 1974), it was found that milk and urine of animals fed with corn silage contain a blue-fluorescent substance. The amount of this substance shows a clear-cut dependence on the amount and time of feeding with corn silage, reaching a maximum in milk approximately 2 months after the beginning of use of this kind of fodder. Comparison with control animals fed only with fresh fodder or hay indicated that the fluorescent substance is also present in their milk or urine, but in much lower amount. This paper reports the isolation and identification of the blue-fluorescent metabolite.

MATERIALS AND METHODS

Ultraviolet spectra for solution in 95% ethanol were measured with a Beckman DK-2 apparatus. The high-resolution mass spectrum was performed with a Hitachi RMU-6-D apparatus at 70 eV.

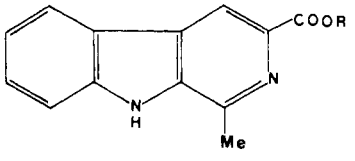
Isolation of the Metabolite (Ia). Ten liters of urine from a cow fed with corn silage was mixed with the same volume of ethanol/acetone (1:1) to precipitate proteins, the mixture was centrifuged, and the supernatant was concentrated in vacuo to a 50-mL volume and absorbed on the top of a chromatographic column (3 × 50 cm) of MN polyamid SC6 (Macherey and Nagel, Düren, West Germany). The column was eluted with water until the brown color of the eluate disappeared. The elution was monitored by thin-layer chromatography (TLC, Merck HF_{254} silica gel plates, 0.25 mm, eluant $CHCl_3$/MeOH/CH_3COOH 75:20:5, R_f of the fluorescent spot 0.34). Water fractions containing the fluorescent metabolite were recycled on the column, which was again eluted with methanol. Concentration of the methanol fractions gave a crude product, which was subjected to preparative (2 mm) TLC on silica gel Merck plates, with $CHCl_3$/MeOH/CH_3COOH 90:20:2, R_f 0.28. The fluorescent band in UV light (254 nm) was extracted with methanol, the solution was evaporated, and the residue was taken up with 3 mL of methanol and treated with excess diazomethane in ether. Preparative TLC of the product on silica gel Merck plates (2 mm) with $CHCl_3$/MeOH (30:1) afforded 1–2 mg of the methyl ester Ib (R_f 0.43): high-resolution mass spectra m/e 240.0898 (calcd for $C_{14}H_{12}N_2O_2$: 240.0890 ± 0.002, and 182.0844 (calcd for $C_{12}H_{10}N_2$: 182.0872 ± 0.003); UV (EtOH 95%): λ_{max} 235, 273, 304, 310 nm. Authentic samples of Ia and Ib were synthesized according to Snyder et al. (1948), and the identity with the natural compounds established by comparison of UV and mass spectra and direct TLC comparison.

Quantitation of Ia. The amount of Ia in biological fluids was measured with a Turner 430 spectrofluorimeter. Samples were mixed with equal volumes of 10% aqueous CCl_3COOH, centrifuged at 5000 rpm for 10 min, and fluorescence intensity read at 452 nm (excitation 380 nm). A solution of Ia in 10% aqueous CCl_3COOH was used as a standard.

RESULTS AND DISCUSSION

The isolation of the fluorescent metabolite Ia was performed only on urine due to the higher concentration and less cumbersome separation from other constituents. The identity with the compound present in milk was established by TLC comparison.

Although it was difficult to obtain Ia in pure state, the reaction with diazomethane allowed the easy isolation and purification of the ester Ib. This result, together with the loss of 58 m/e units in the mass spectrum of Ib, confirmed that Ia is a carboxylic acid. The formula $C_{14}H_{12}N_2O_2$ for Ib and the only main fragmentation peak at m/e 182 ($C_{12}H_{10}N_2$) indicated a stable polycyclic heteroaromatic structure, most probably a methylcarboline (Budzikiewicz et al., 1964). The highly characteristic UV spectrum (Scott, 1964), typical of a β-carboline nucleus, suggested the structure of 1-methyl-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid (Ia) for the metabolite. This structure corresponds



Ia, R = H
b, R = Me

to a known alkaloid, isolated for the first time from the plant *Aspidosperma polyneuron* (Hesse, 1964), which does not seem to have ever been found in animals. The great similarity of the properties of the fluorescent metabolite with this alkaloid prompted the preparation of a synthetic sample of Ia and Ib (Snyder, 1948). Spectral and chromatographic comparison confirmed unambiguously the identity with the natural material and therefore the structure Ia.

The concentration of Ia in milk and urine was measured fluorimetrically using synthetic Ia as a standard. These measures may obviously be affected by considerable error due to the possible presence of other fluorescent substances in the biological fluids. Average values of Ia in rumen, milk, and urine of control animals are respectively 0.2, 0.08, and 0.54 μg/mL. The concentration of Ia increased steadily in fluids of animals fed with corn silage, reaching an average value of 30 times the control value in rumen and 100 times the control value in urine after 20–25 days from the beginning of the diet. The concentration in milk (ca. five times the control value) reached the maximum after 2 months of feeding with corn silage.

TLC analysis (sensitivity ca. 1–2 ppm) of extracts of corn silage showed that no Ia is present in the feeding ration or at least that it is not enough to justify even the amount found in rumen of control animals. Therefore it must be a product of metabolism, clearly derived from tryptophan and acetaldehyde or an equivalent unit. TLC of rumen samples showed the presence of Ia. It is thus possible that the silage feeding conditions either induce different fermentation processes in the rumen, with a higher production of Ia, or hinder the normal catabolic activity, with an

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increasing absorption of the compound, which is then excreted in milk and urine.

We have also observed a correlation between the concentration of Ia (measured fluorimetrically) and delay in the acidification of milk due to lactic bacteria (Bertoni, 1978). However, no specific bacteriostatic activity was found in vitro for Ia against lactic bacteria. The hypothesis that Ia could be decarboxylated to 1-methyl-β-carboline (harman), which is known to inhibit the growth of molds, and which has been detected in fermentation products such as sake, beer, and wine (Takase et al., 1967), was ruled out as no trace of harman could be found in urine and milk of animals (TLC in comparison with a synthetic sample). Therefore it might be possible that other active substance(s), present in small amounts, and produced by processes correlated with those leading to an unusual amount of Ia, could be responsible for this effect.

LITERATURE CITED

Battistotti, B., Bertoni, G., *Scienza Tec. Latt. Casearia*, **25**, 7 (1974).
Bertoni G., unpublished results, 1978.
Budzikiewicz, H., Djerassi, C., Williams, D. H., "Structure Elucidation of Natural Products by Mass Spectroscopy", Vol. 1, Alkaloids, Holden-Day, San Francisco, 1964, p 85.
Hesse, M., "Indolalkaloide in Tabellen", Springer Verlag, Heidelberg, 1964, p 95.
Scott, A. I., "Interpretation of the Ultraviolet Spectra of Natural Products", Pergamon, Oxford, 1964.
Snyder, H. R., Hansch, C. H., Katz, L., Parmeter, S. M., Spaeth E. C., *J. Am. Chem. Soc.* **70**, 219 (1948).
Takase, S., Sakai, T., Sato, N., Murakami, H., *Nippon Jozo Kyokai Zasshi* **62**, 1415 (1967); *Chem. Abstr.* **68**, 67724.

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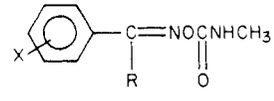
Inhibition of Acetylcholinesterase by *O*-(Methylcarbamoyl)oximes. Structure–Activity Relationships

Since the anticholinesterase activity and the mechanism of alkaline hydrolysis of *O*-(methylcarbamoyl)benzaldoximes and -acetophenoximes are analogous to those of phenyl *N*-methylcarbamates, these two groups of derivatives were compared by means of structure–activity relationships. The correlations with the electronic substituent parameter σ showed that the mechanism of inhibition of acetylcholinesterase by *O*-(methylcarbamoyl)oximes is the same as that observed for phenyl *N*-methylcarbamates bearing strongly electron-withdrawing substituents. The correlations with the bimolecular rate constant k_{OH} suggest that the mechanism of the alkaline hydrolysis of oxime carbamates may closely parallel their mechanism of interaction with acetylcholinesterase at the serine hydroxyl.

Fukuto et al. (1969), as well as Jones et al. (1972), showed that *O*-(methylcarbamoyl)oximes of substituted acetophenones and benzaldehydes have a poor insecticidal activity against species such as the housefly (*Musca domestica* L.) in spite of their good anticholinesterase activity as measured on fly heads by the I_{50} parameter (i.e., the molar concentration of carbamate necessary to cause 50% inhibition of cholinesterase activity). In contrast to phenyl *N*-methylcarbamates (Hansch and Deutsch, 1966; Jones et al., 1969), lipophilic bonding plays almost no role in the inhibition of cholinesterase by *O*-(methylcarbamoyl)-oximes. The fact that anticholinesterase activity is dependent only on the field (*F*) and resonance (*R*) constants of Swain and Lupton suggests that cholinesterase inhibition by *O*-(methylcarbamoyl) oximes is determined by the reactivity of the carbamoyl moiety, analogous to the hydrolysis of these esters.

The kinetic data reported earlier for the alkaline hydrolysis of *O*-(methylcarbamoyl)oximes (Mrlina and Calmon, 1980) can be discussed in terms of substituent effects on biological activity and in relation to the mechanism of inhibition of acetylcholinesterase. Two kinds of correlations, calling upon the electronic substituent constant σ or the bimolecular rate constant k_{OH}, were considered so as to check whether the mechanism of hydrolysis can account for the mechanism of inhibition of acetylcholinesterase. Since the bimolecular rate constant k_{OH} is closely related to σ and can be measured easily, it could be used in structure–activity relationships when σ values are unknown or when the transmission of the substituent effect is complex. The k_{OH} parameter is, as a matter of fact, liable to reflect the overall substituent effects.

Table I. Kinetic (log k_{OH}), Electronic (σ), and Biological (log $1/I_{50}$) Parameters Used in Structure–Activity Relationships for Oxime Carbamates $XC_6H_4C(R)$:NOCONHCH$_3$



R	X	log (1/I_{50})[a]	σ	log k_{OH}
H	H	4.34	0	−1.054
H	*p*-iPr	4.43	−0.150	−1.258
H	*p*-Br	4.42	0.232	−0.896
H	*m*-NO_2	5.08	0.710	−0.462
H	*p*-NO_2	4.85	0.780	−0.108
Me	H	4.15	0	−2.096
Me	*p*-Me	4.05	−0.170	−2.194
Me	*m*-MeO	3.82	0.115	−1.939
Me	*p*-Br	4.41	0.232	−1.730
Me	*m*-NO_2	5.35	0.710	−1.424
Me	*p*-NO_2	5.09	0.780	−1.250

[a] Fukuto et al. (1969); Jones et al. (1972).

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