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Artigo

Anaerobic Biotransformation of *N*-oxide Containing Aromatic Heterocycles by Bovine Ruminal Fluid

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Biotransformação Anaeróbia de Heterociclos Aromáticos Contendo *N*-óxido por Líquido Ruminal de Bovinos

Resumo: Micro-organismos do fluido ruminal de bovinos foram estudados como biocatalizadores para a redução de diferentes heterociclos aromáticos contendo *N*-óxido, isto é, quinoxalina N^1,N^4-dióxido, fenazina N^5,N^{10}-dióxido, indazol N^1-óxido, benzofuroxano e furoxano. Em anaerobiose, os micro-organismos biocatalisam a redução de alguns *N*-óxidos, dióxidos de quinoxalina e fenazina para se obter os heterociclos correspondentes, enquanto no caso do heterociclo benzofuroxano, ocorre abertura do anel, gerando a *o*-nitroanilina como único produto. Ao contrário, o furoxano não foi biotransformado nas condições estudadas. O indazol N^1-óxido foi biotransformado lenta e incompletamente ao indazol correspondente. Exceção foi observada apenas para um dos N^1-óxidos de indazol, que foi completamente convertido a um composto novo, diferente do indazol reduzido esperado. A biotransformação em escala semi-preparativa foi realizada e o novo produto obtido e identificado por métodos espectroscópicos.

***Palavras-chave*:** Biotransformação; *N*-óxidos; rúmen; líquido ruminal; bovino.

Abstract

Microorganisms of bovine rumen fluid have been studied as biocatalysts for the reduction of different *N*-oxide containing aromatic heterocycles, i.e. quinoxaline N^1,N^4-dioxide, phenazine N^5,N^{10}-dioxide, indazole N^1-oxide, benzofuroxan and furoxan. In anaerobiosis, the microorganisms biocatalyzed the reduction of some *N*-oxides, quinoxaline and phenazine dioxides, to yield the corresponding heterocycle while in the case of benzofuroxans the heterocycle-opening, *o*-nitroaniline was generated as the unique product. Contrarily, the furoxan was not biotransformed in the studied conditions. The indazole N^1-oxide was biotransformed very slowly and incompletely in the corresponding indazole. Only one of the studied indazole N^1-oxide derivative was completely converted to a new compound, different to the expected reduced indazole. For that, a semi-preparative scale biotransformation was performed and the new product was spectroscopically identified.

Keywords: Biotransformation; *N*-oxides; rumen; ruminal fluid; bovine.

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Anaerobic biotransformation of *N*-oxide containing aromatic heterocycles by bovine ruminal fluid

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1. Introduction

The digestive anatomy and physiology of cow and other ruminants is markedly different to that of monogastric animals such as human. The ruminants have three additional compartments (rumen, reticulum and omasum) before the true stomach (abomasum). These compartments allow the

microbial population to extract and the host to absorb energy from fibrous plant material not otherwise available to mammalian enzymes. Digestion of food in the rumen occurs by a combination of microbial fermentation and physical breakdown during rumination being the rumen a highly reductive anaerobic environment.[1] The products of fermentation are mainly acetate, propionate, butyrate, formate, ethanol, lactate, succinate, ammonia, carbon dioxide and hydrogen gas.[2] The total pool of hydrogen gas is produced during microbial fermentation of feed, and is used as an energy source by methanogenic archaea producing methane. This pool is small and the dissolved H_2 concentration is usually about 0.1-50 µM, which is 0.014 to 6.8% of its maximal solubility at 39 ^{o}C and one atmosphere pressure. Efficient H_2 removal is postulated to increase the rate of fermentation by eliminating the inhibitory effect of H_2 on the microbial degradation of plant material. Anaerobic microbial community in the rumen which consists of many bacterial, archaeal, protozoal and fungal species, could be able to biotransform xenobiotics to new compounds. Examples of xenobiotic transformations by rumen microbes have been reported for toxicants in forages such as pyrrolizine alkaloids, fumonisin, alflatoxin,[3-6] and nitropropionic acid, and TNT (2,4,6-trinitrotoluene) that were converted to the corresponding amine and hydroxylamine derivatives.[7,8] Additionally, we have recently described the ability of bovine ruminal fluid, in anaerobic conditions, to transform nitro-substrates into amines.[9]

The stereo-electronic structural similarity between the nitro moiety and the heterocyclic *N*-oxide group (Figure 1) has led us to ask which behavior of the latter would be under the cow rumen biotransformer. The deoxygenation of *N*-oxide-containing aromatic heterocycles to the corresponding heterocycles is an important step in the preparation of new compounds, for example in the field of medicinal chemistry, toxicology, environmental sciences, and new materials. A number of methods have been developed to perform these reductions, however, they often suffer from serious disadvantages, such as incompatibility with other functional groups, low yields, harsh reaction conditions, difficult work-up procedures and environmental unfriendly methodologies.[10,11] Hence, the development of alternative methods is awaited.

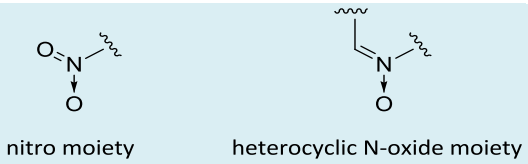
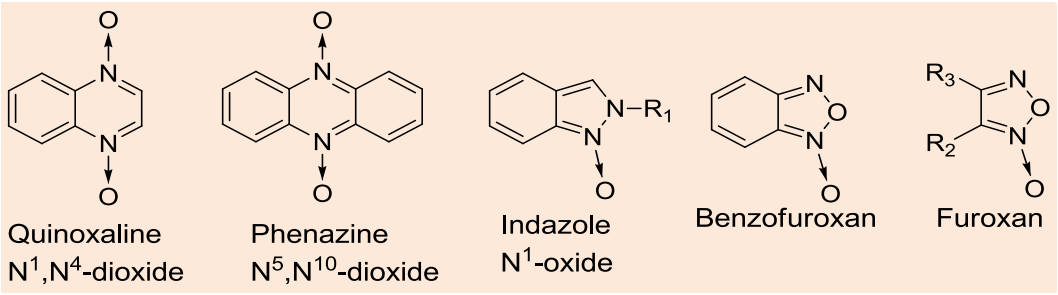


Figure 1. Nitro and heterocyclic *N*-oxide moieties

The present work describes the reductive potential of bovine ruminal microorganisms, with respect to five different *N*-oxide containing diaza-heterocycles (quinoxaline N^1,N^4-dioxide, phenazine N^5,N^{10}-dioxide, indazole N^1-oxide, benzofuroxan and furoxan, Scheme 1).



Scheme 1. *N*-oxide chemotypes employed herein as substrates in biotransformation studies involving anaerobic bovine rumen fluid

2. Experimental

2.1. General methods

Products of biotransformations were purified by column chromatography (SiO_2, 230–400 mesh; eluted with mixtures of EtOAC: petroleum ether, 9:1; 8:2, and 1:1). The purification and progresses of the biotransformations were checked by TLC (silica gel 60 F254 layers, EtOAc: petroleum ether, 1:1), visualized under UV light (λ = 254 nm), by exposing to iodine vapor, or by spraying with *p*-anisaldehyde/sulphuric acid reagent and heating at ca. 120 °C. The identities of products were determined using their chromatographic characteristics and ^{1}H NMR spectra by comparison with those of reference standards. ^{1}H NMR spectra were acquired at 400MHz using a Bruker DPX400 instrument with the analyte dissolved in $CDCl_3$.

2.2. Chemicals

The reagents, the *N*-oxide derivatives **1-8**, and the potential reduced products **9-20** and **22-24** were prepared as previously described.[12-16]

2.3. Standard procedure for small scale biotransformation reactions[9]

Microbial sources and collection: Rumen fluid was collected from a cannulated dairy cow (550 kg body weight, Figure 2), fed a 70 % forage (fresh pasture, mixture of *Lolium multiflorum, Trifolium repens* and *Trifolium pratense*) and 30 % concentrate (corn grain and sunflower meal, 16% crude protein) diet at an intake level of 3 % of the body weight. The procedure was approved by the Bioethics Committee of Veterinary Faculty (UdelaR). The collection of rumen fluid was performed approximately 1-2 h after the beginning of the main meal and filtered through two layers of cheesecloth into 1 L plastic prewarmed (39 °C) vessel with no remaining air space and under CO_2 atmosphere. The vessels were sealed and transported to the laboratory within 1 h. The ruminal fluid was maintained no more than a week at 4 °C.

Culture conditions and sampling protocols: Transformation experiments were conducted under anaerobic conditions in 10 mL vials containing 2 mL of the rumen fluid. The *N*-oxide derivatives dissolved in DMSO were added to a final concentration of 0.4 mM. The vials were purged with N_2 prior to being sealed with white rubber serum stoppers and plastic crimp closures. The vials were incubated at 39 °C, protected from light, without agitation. Samples were withdrawn from duplicate vials at the appropriate time intervals (0.5, 1, 4, 6, and 24 h) and analyzed immediately for *N*-oxide derivatives and metabolic products. Samples were treated with methanol (500 µL/1 mL of rumen) and stirred during 30 min. in order to destroy the biological matrix. Then EtOAc (3

mL) and saturated aqueous $NaHCO_3$ solution (3 mL) were added and the organic layer was evaporated *in vacuo* to be analyzed by chromatography. Metabolite identification was based on comparison of chromatographic behaviors. Samples were analyzed immediately following collection to minimize abiotic degradation of intermediates. Different controls, at 39 °C, were used to confirm the origin of the biotransformation: (a) autoclaved opened-rumen fluid and *N*-oxide derivatives; (b) aqueous solution and *N*-oxide derivatives; and (c) rumen fluid.

2.4. Procedure for the scale-up biotransformation

Semi-preparative protocol was performed with *N*-oxide derivative **6** (15 mg in 480 mL of ruminal fluid, and 20 mL of DMSO) incubated during 10 days in the same conditions that it is indicated in Section 2.3. After the incubation time the bio-material was treated as it is indicated in Section 2.3. The residue from *in vacuo* evaporation was purified by chromatographic column as it is indicated in Section 2.1., the identity of the main product, **21**, was confirmed by ^{1}H NMR, COSY, ^{13}C NMR, HETCOR experiments, IR and MS.

3. Results and discussion

3.1. Biotransformation of quinoxaline N^1,N^4-dioxide and phenazine N^5,N^{10}-dioxide derivatives

Biotransformations of the *N*-oxide derivatives **1-3** (Figure 3) were carried out using bovine rumen fluid obtained from dairy cows (Figure 2). The processes were conducted on a small scale (near to 0.1 mg of substrate solubilized in DMSO per mL of ruminal fluid) incubating the mixtures without agitation for 24 h at 39 °C. The biotransformations were checked at different times (0.5, 1, 4, 6, and 24 h) being quinoxaline dioxide **1** biotransformed by ruminal fluid to the corresponding monoxide **9** (Figure 3) after 2 h carrying out a complete reduction at 24 h of incubation. Interestingly, the **1** bioreduction took place regioselectively following the electronic effect of neighboring methyl group which increases the negative density of the adjacent *N*-oxide moiety increasing the redox potential of it. Monoxide **10** and quinoxaline **11** were not detected.

Figure 2. Cannulated dairy cow (the procedure was approved by the Bioethics Committee of Veterinary Faculty-UdelaR)

Similar behavior was observed for the phenazine dioxide **2**. It was bioconverted to the monoxide **12**, result of the reduction of the most reactive *N*-oxide, and the phenazine **14** from the first 0.5 h. Similarly, *N*-oxide **3** produced by action of the bovine ruminal fluid in anaerobic conditions the phenazine **14** after 0.5 h of incubation. For this phenazine, there was no presence of any of the mono-bioreduction products, **15** and **16** (Figure 3). Both *N*-oxides were completely biotransformed after 24 h of incubation.

The results for phenazine dioxides **2** and **3** were similar to that previously observed using liver-mammal systems, cytosolic and microsomal fractions, in simulated-hypoxia.[17] Once more, the electronic effect of substituents affected the redox status of the end products.

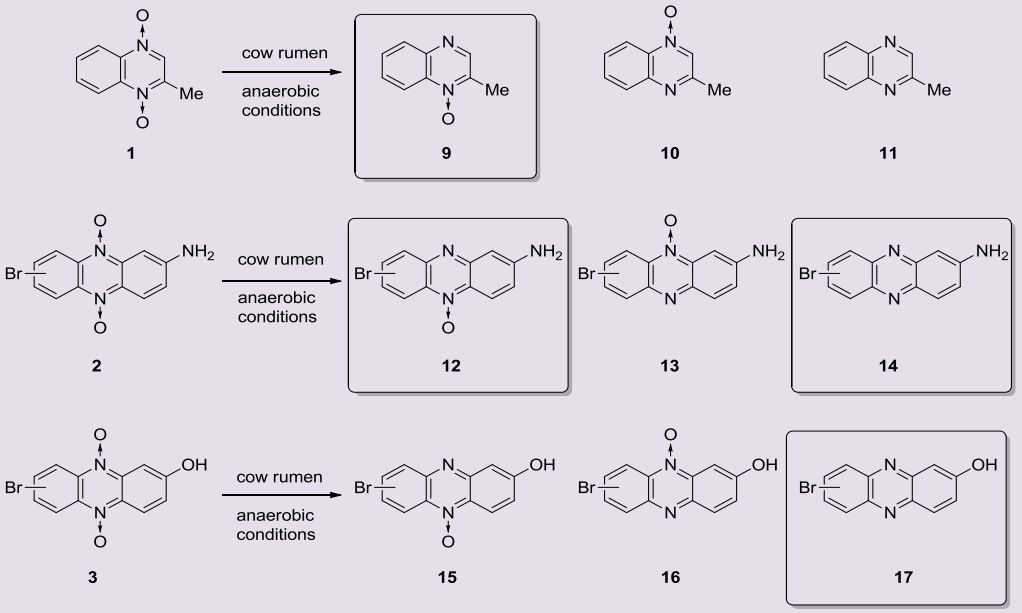


Figure 3. Quinoxaline N^1,N^4-dioxide and phenazine N^5,N^{10}-dioxide derivatives used herein and identified products of biotransformations (highlighted compounds)

3.2. Biotransformation of indazole N^1-oxide derivatives

Three different indazole N^1-oxides were studied in their capabilities as substrates of anaerobic rumen biotransformations (**4-6**, Figure 4). According to the results with quinoxaline and phenazine dioxides, we looked for the presence, in the incubation milieu, of the corresponding indazole (**18-20**, Figure 4). In these cases the bioreduction is slower than those of compounds **1-3**, i.e. reduction products of *N*-oxides **4** and **5** were observed since the first 4 h of incubation and after 24 h the starting materials still remained. Particularly, indazole **6** which ports two bioreduced moieties, i.e. *N*-oxide and nitro groups, did not generate the corresponding expected product, **20** (Figure 4). In this case it was observed a different product with lower retention time in thin layer chromatography. In order to identify the new generated compound a semi-preparative scale biotransformation was performed and the new product was spectroscopically identified (see 3.4. section).

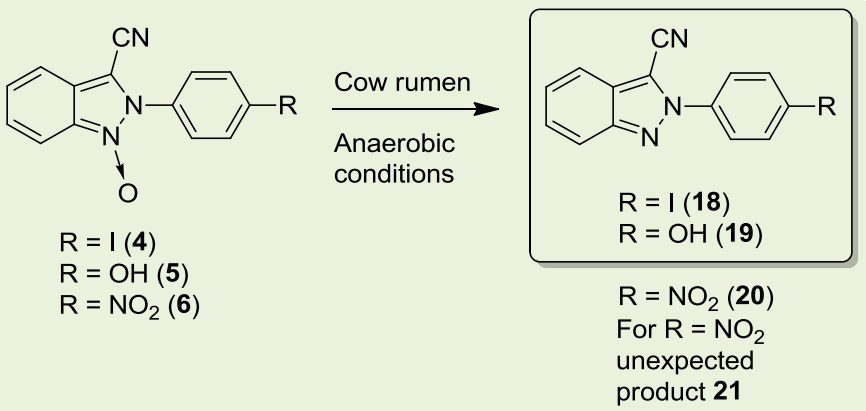


Figure 4. Indazole N^1-oxide derivatives used herein and identified products of biotransformations (highlighted compounds)

3.3. Biotransformation of benzofuroxan and furoxan derivatives

The 1,2,5-oxadiazole *N*-oxides **7** and **8** (Figure 5) were incubated in anaerobic conditions with ruminal fluid. Similarly to the rest of *N*-oxides, the reduced heterocycles (**22** and **24**, Figure 5) were sought as products of biotransformations. For benzofuroxan, the unique generated product was the corresponding aniline **23** (Figure 5) after 0.5 h of incubation. This kind of product of bioreduction has been previously described when benzofuroxans were confronted in different biological conditions.[18,19] On the other hand, the furoxan **8** was inert to the bioreductive conditions of the ruminal fluid for at least 24 h of study. Once again, the chemical behavior is associated to the biological behavior since furoxans are harder to reduce than their benzo-analogs, benzofuroxans.[20]

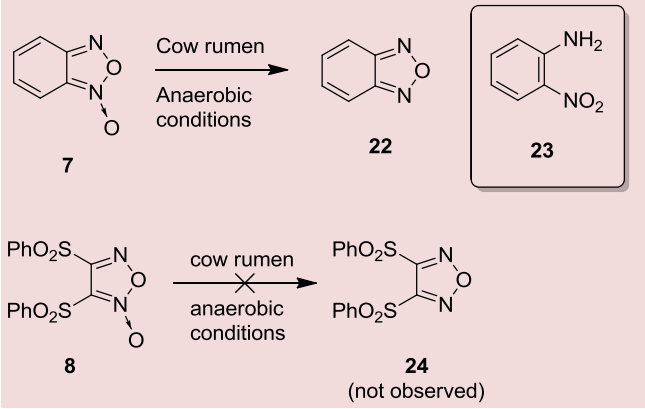


Figure 5. 1,2,5-Oxadiazole N^1-oxide derivatives used herein and identified product of biotransformation (highlighted compound)

3.4. Scale-up of indazole N^1-oxide 6 biotransformation

In order to determine the chemical structure of the main metabolite of indazole N^1-oxide **6** biotransformation by ruminal fluid in anaerobic conditions, a semi-preparative (15 mg of **6**) scale process was performed isolating, by column chromatography, 9 mg of product **21**. According to the results obtained with the different spectroscopic and

spectrometric tools (see Experimental section) (Table 1) one of the possible chemical structure of the generated product, **21**, is herein proposed (Figure 6a). The following most relevant signals allowed us to propose this chemical structure: 1) ^{1}H NMR: i) absence of protons in the region of 8.10-8.60 ppm, confirming reduction of the nitro group; ii) presence of new protons in the region of 3.30-4.20 ppm, and 1.29-2.90 ppm, confirming the incorporation of aliphatic moieties; iii) presence of one proton at 7.88 ppm assigned to a formamide proton; iv) broad singlet at 6.19 ppm assigned to a heteroatom-bonded proton, NH or OH. 2) ^{13}C NMR: i) absence of carbons in the region of 90.0-112.0 ppm, showing the absence of nitrile moiety;[21] ii) presence of two carbonyl-carbons, near to 169 and 173 ppm, indicating the possible presence of amide and hydroxamic acid moieties; iii) presence of carbon at 155.0 ppm that could be assigned to aromatic carbon that ports oxygen (C_{Ar}-O-R) confirming the substitution of nitrile group by an -OR moiety; iv) presence of carbon in the region of 129.0 ppm, and absence in the region of 149.0 ppm, confirming the *N*-oxidation state of indazole heterocycle.[14] 3) COSY and HETCOR experiments: i) coupling between protons at 4.14 and 3.34 ppm, indicating the presence of –OCH_2CH_2N- fragment; ii) couplings between protons at 2.27 and 1.58 ppm, protons at 1.58 and 1.29 ppm, and protons at 1.29 and 2.90 ppm, indicating the presence of -(O=C) $CH_2CH_2CH_2CH_2N$- fragment; iii) correlation, in HMBC experiment, between proton at 4.14 ppm and carbon at 155.0 ppm confirming C_{Ar}-O-R fragment; iv) correlation, in HMBC experiment, between proton at 2.90 ppm and carbon at 168.7 ppm confirming -CH_2NH(C=O)- fragment. 4) IR: i) absence of signal in the 2200 cm^{-1} region confirming lack of nitrile moiety; ii) presence of two signals in the 1630-1720 cm^{-1} region confirming presence of carbonyl moieties, hydroxamic acid[22] and amide groups; iii) apparition of a broad signal in 3342 cm^{-1} confirming presence of OH, from hydroxamic acid;[22] iv) presence of signals in 1277 and 1037 cm^{-1} that could indicate the presence of an unsymmetrical ether. 5) MS: presence of ions that could be assigned to the molecular ion of **6** and to the different logical fragmentations from this (Figure 6b).

Consequently, we could indicate that compound **6** suffered reduction via the nitro moiety to the corresponding hydroxylamine,[23] without modification on the *N*-oxide group, together with a series of reactions (i.e. substitution and condensation) participating the ruminal fluid metabolites ethanolamine,[24,25] γ-aminovaleic acid,[26,27] formate, and hydrogen gas.[2]

From this data we could demonstrate that this bioprocess is regioselective. Additionally, the result was in concordance with our previous results where the nitro moiety was susceptible to be reduced in this biological conditions.[9] Probably, the slower kinetics of reduction of this *N*-oxide, indazole N^1-oxide, and the ability of ruminal fluid to reduce nitro-aromatic systems[9] contribute to the generation of the isolated product **21**.

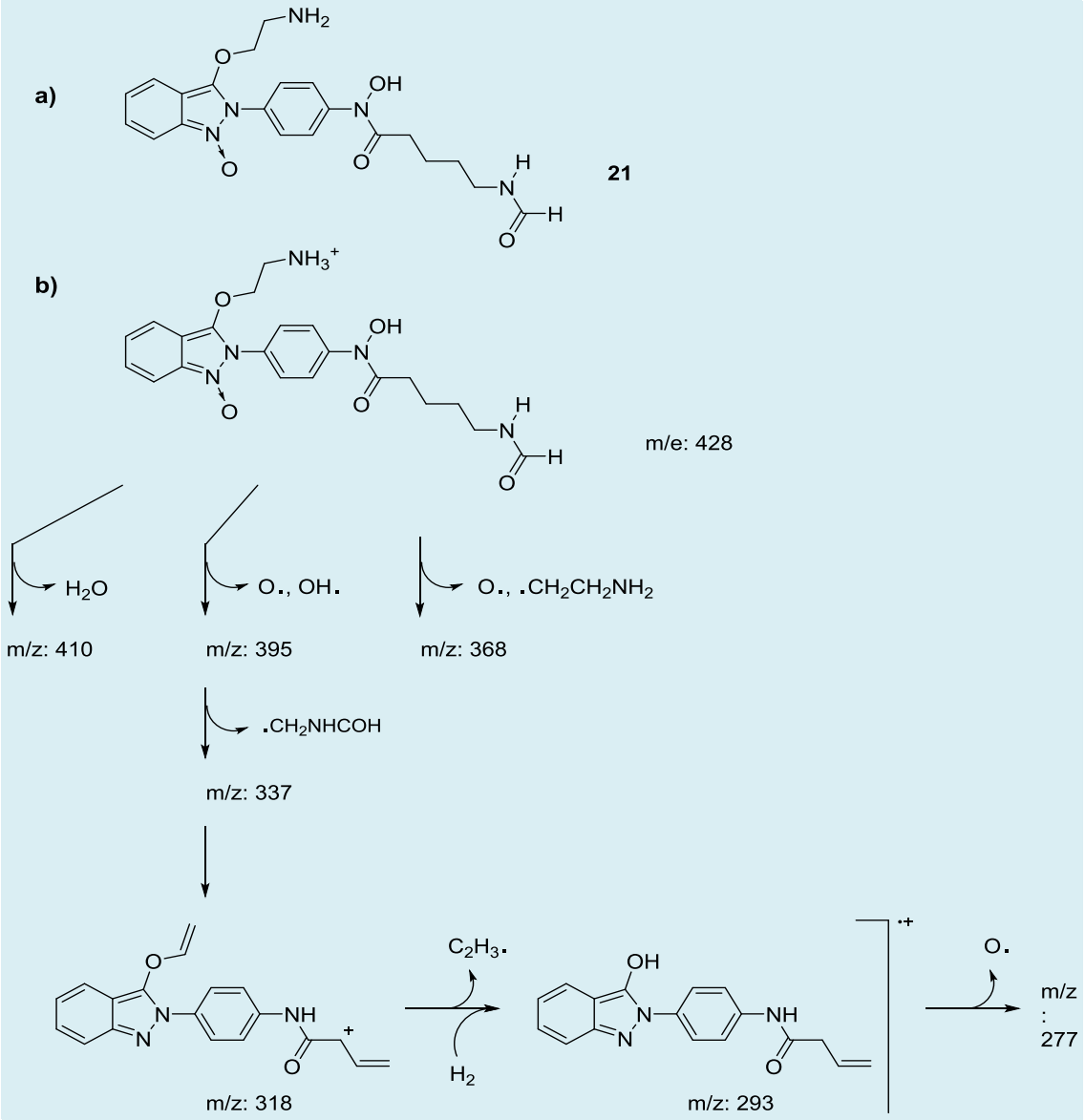


Figure 6. **a)** Proposed chemical structure for metabolite **21**. **b)** Interpretation of the main fragments obtained in the EI-MS of **21**.

Table 1. Selected information, from the spectroscopies and spectrometry, that allows to propose chemical structure of metabolite **21** (Figure 6)

compound	^{1}H NMR	^{13}C NMR	IR[a]	MS[b]
6[c,d]	7.52 (1H), 7.57 (1H), 7.86 (1H), 7.90 (1H), 8.17 (2H), 8.56 (2H)	92.6, 111.6, 115.1, 119.9, 123.5, 125.7, 128.9, 129.3, 130.1, 130.2, 136.8, 149.7	2205, 1612, 1536, 1438, 1353, 1299, 1239, 1035, 845, 749	280 ($M^{+\cdot}$, 88)
20[c,d]	7.52 (1H), 7.60 (1H), 7.97 (1H), 8.03 (1H), 8.27 (2H), 8.56 (2H)	107.9, 111.5, 119.6, 119.9, 126.1, 126.2, 126.3, 127.8,	2220, 1597, 1528, 1354, 857, 750	264 ($M^{+\cdot}$, 35)

		127.9, 129.8, 143.8, 149.3		
21[e]	1.29 (2H), 1.58 (2H), 2.27 (2H), 2.90 (2H), 3.34 (2H), 4.14 (2H), 6.19 (bs, 1H), 7.23 (2H), 7.55 (3H), 7.61 (1H), 7.68 (1H), 7.71 (1H), 7.88 (1H)	26.0, 26.9, 33.0, 34.5, 40.5, 67.7, 124.0-135.0 (ten carbons), 155.0, 168.7, 173.5	3342 (broad), 1715, 1707, 1637, 1277, 1174, 1037	428 ($[M+H]^{+\cdot}$, 0.2)

[a] KBr (cm^{-1}). [b] Electronic impact, 70 eV. In parentheses are indicated percentages. [c] NMR spectrum were acquired in DMSO-d_6. [d] Data from reference [21]. [e] NMR spectrum were acquired in acetone-d_6:D_2O (9:1).

4. Conclusions

Bovine rumen fluid has been shown to be efficient in the biotransformation of a number of *N*-oxide containing heterocycle-substrates, producing in some cases the corresponding heterocycles in good yields. The results suggest that ruminal fluid may offer some application in the synthesis of heterocycles, under very mild conditions, and maybe in the bioremediation of toxic *N*-oxide derivatives involving safety, scalability and environmentally friendly conditions.

Acknowledgments

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References

[1] Kamra, D. N. *Curr. Sci.* **2005**, *89*, 124. [Link]

[2] Janssen, P. H. *Anim. Feed Sci. Technol.* **2010**, *160*, 1. [CrossRef]

[3] Hovermale, J. T.; Craig, A. M. *Biophys. Chem.* **2002**, *101-102*, 387. [CrossRef] [PubMed]

[4] Gurung, N. K.; Rankins, D. L.; Shelby, R. A. *Vet. Hum. Toxicol.* **1999**, *41*, 196. [PubMed]

[5] Hedman, R.; Pettersson, H. *Arch. Tierernahr.* **1997**, *50*, 321. [CrossRef] [PubMed]

[6] Kiessling, K. H.; Pettersson, H.; Sandholm, K.; Olsen, M. *Appl. Environ. Microbiol*, **1984**, *47*, 1070. [PubMed]

[7] Fleischmann, T. J.; Walker, K. C.; Spain, J. C.; Hughes, J. B.; Craig, A. M. *Biochem. Biophys. Res. Commun*. **2004**, *314*, 957. [CrossRef] [PubMed]

[8] Anderson, R. C.; Rasmussen, M. A.; Allison, M. J. *Appl. Environ. Microbiol.* **1993**, *59*, 3056. [PubMed]

[9] Rodríguez, A.; Irazoqui, I.; Álvarez, G.; Cajarville, C.; Repetto, J.; González M.; Cerecetto, H. *Green Sustainable Chem.* **2011**, *1*, 47. [CrossRef]

[10] Reis, P.M.; Royo, B. *Tetrahedron Lett.* **2009**, *50*, 949. [CrossRef]

[11] Toganoh, M.; Fujino, K.; Ikeda, S.; Furuta, H. *Tetrahedron Lett.* **2008**, *49*, 1488. [CrossRef]

[12] Cerecetto, H.; Dias, E.; Di Maio, R.; González, M.; Pacce, S.; Saenz, P.; Seoane, G.; Suescun, L.; Mombrú, A.; Fernández, G.; Lema, M.; Villalba, J. *J. Agric. Food Chem.* **2000**, *48*, 2995. [CrossRef] [PubMed]

[13] Cerecetto, H.; González, M.; Lavaggi, M. L.; Azqueta, A.; López de Cerain, A.; Monge, A. *J. Med. Chem.* **2005**, *48*, 21. [CrossRef] [PubMed]

[14] Gerpe, A.; Piro, O. E.; Cerecetto, H.; González, M. *J. Mol. Struct.* **2007**, *871*, 98. [CrossRef]

[15] Cerecetto, H.; Di Maio, R.; González, M.; Risso, M.; Saenz, P.; Seoane, G.; Denicola, A.; Peluffo, G.; Quijano, C.; Olea-Azar, C. *J. Med. Chem.* **1999**, *42*, 1941. [CrossRef] [PubMed]

[16] Cabrera, M.; López, G. V.; Gómez, L. E.; Breijo, M.; Pintos, C.; Botti, H.; Raymondo, S.; Vettorazzi, A.; López de Ceráin, A.; Monge, A.; Rubbo, H.; González, M.; Cerecetto, H. *Drug Chem. Toxicol.* **2011**, *34*, 285. [CrossRef] [PubMed]

[17] Lavaggi, M. L.; Cabrera, M.; González, M.; Cerecetto, H. *Chem. Res. Toxicol.* **2008**, *21*, 1900. [CrossRef] [PubMed]

[18] Medana, C.; Visentin, S.; Grosa, G.; Fruttero, R.; Gasco, A. *Farmaco* **2001**, *56*, 799. [CrossRef]

[19] Boiani, M.; Merlino, A.; Gerpe, A.; Porcal, W.; Croce, F.; DePaula, S.; Rodríguez, M.A.; Cerecetto, H.; González, M. *Xenobiotica* **2009**, *39*, 236. [CrossRef]

[20] Cerecetto, H.; González, M. *Top. Heterocycl. Chem.* **2007**, *10*, 265. [CrossRef]

[21] Gerpe, A.; Aguirre, G.; Boiani, L.; Cerecetto, H.; González, M.; Olea-Azar, C.; Rigol, C.; Maya, J. D.; Morello, A.; Piro, O. E.; Arán, V. J.; Azqueta, A.; López de Ceráin, A.; Monge, A.; Rojas, M. A.; Yaluff, G. *Bioorg. Med. Chem.* **2006**, *14*, 3467. [CrossRef] [PubMed]

[22] Agarwal, H.; Agarwal, O. P.; Karnawat, R.; Sharma, I. K.; Verma, P. S. *Int. J. Appl. Biol. Pharmac. Technol.* **2010**, *I*, 1293. [Link]

[23] Hughes, J. B.; Wang, C. Y.; Zhang, C. *Environ. Sci. Technol.* **1999**, *33*, 1065. [CrossRef]

[24] Buccioni, A.; Decandia, M.; Minieri, S.; Molle, G.; Cabiddu, A. *Anim. Feed Sci. Technol.* **2012**, *174*, 1. [CrossRef]

[25] Klein, M. S.; Almstetter, M. F.; Schlamberger, G.; Nürnberger, N.; Dettmer, K.; Oefner, P. J.; Meyer, H. H. D.; Wiedemann, S.; Gronwald, W. *J. Dairy Sci.* **2010**, *93*, 1539. [CrossRef] [PubMed]

[26] Van Den Hende, C.; Oyaert, W.; Bouckaert, J. H. *Res. Vet. Sci.* **1964**, *5*, 491.

[27] Tsutsumi, W.; Onodera, R.; Kandatsu, M. *Agr. Biol. Chem.* **1975**, *39*, 711. [CrossRef]