Bioreduction of *N*-oxide moiety

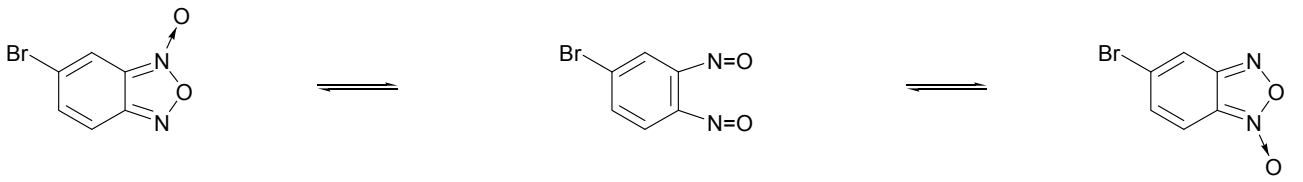
Supplementary Material

This experiment aims at the biotransformation of one *N*-oxide containing heterocycle, phenazine 5,10-dioxide, using two different reductor bio-systems. Since the monitoring of the biotransformation requires standards of product of the metabolisms, the first sessions of the experiment involve synthetic procedures to prepare them.

The first reaction, transformation of 4-bromo-2-nitroaniline to 5(6)-bromobenzofuroxan, involves an oxidation process promoted by a very economic reagent, sodium hypochlorite solution for domestic household. Due to this kind of sodium hypochlorite solutions varying in their active chlorine concentration (1-3 %) the student should check the progress of the reaction after adding portions of this reactive. For that, students should use thin layer chromatography (TLC). The oxidation process produces a less polar product, 5(6)-bromobenzofuroxan, with the highest R_f values. The spot from the TLCs could be visualized with or without UV-C light (254 nm) exposition (Figure SM 15.1.1).

The mechanism of this cyclization could be rationalized in different ways[a,b,c].

The generated benzofuroxan exhibit, at room temperature, the isomerization or, well known, ring chain tautomerism equilibrium[d] (Scheme SM 15.1.1).



6-bromobenzofuroxan *5-bromobenzofuroxan*

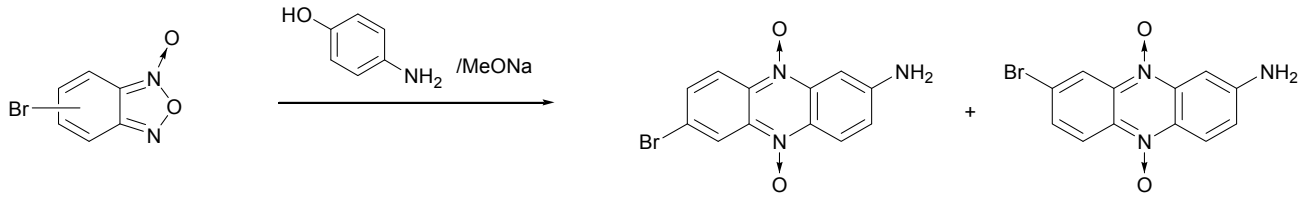
Scheme SM 15.1.**1** **–** Ring chain tautomerism of benzofuroxan exemplified with 5-bromobenzofuroxan.

This phenomenon can not be evidenced in the laboratory, so a unique spot on TLC and a defined melting point, if this were to be measured, are obtained.

The reproducibility of this reaction was assessed by its repetitive execution (Table SM 15.1.1), namely by 1st year Chemistry M.Sc. students from School of Sciences (Montevideo).

The second reaction, transformation of 5(6)-bromobenzofuroxan to 2-amino-7(8)-bromophenazine 5,10-dioxide, involves a heterocycles expansion process promoted by an anionic attack, from the phenolate of *p*-aminophenol, to the furoxan cycle (Beirut reaction). The sodium methoxide preparation should be done carefully due to it involves the use of metallic sodium. In this reaction, the mixture solvent ratio, methanol:tetrahydofuran (1:1), should be fulfilled in order to allow the correct precipitation of the product. Storing the mixture of reaction at low temperature for 24 hours could improve the amount of precipitated solid.

The Beirut reaction with a substituted-benzofuran produces a mixture of positional isomers (Scheme SM 15.1.2), non-separable by ordinary chromatographic techniques.



5(6)bromobenzofuroxan *7-bromo derivative* *8-bromo derivative*

Scheme SM 15.1.**2 –** Synthetic condition in the preparation of 2-amino-7(8)-bromophenazine 5,10-dioxide showing the mixture of generated products.

In this case the proportion of 7:8 isomers is near to 5:5 (in general 5.4:4.6). This proportion will be determined by ^{1}NMR (Figure SM 15.1.5).

The mechanism of this heterocycles expansion could be rationalized as indicated in reference [e].

The mixture of products should be characterized by ^{1}NMR (Figure SM 15.1.5).

The reproducibility of this reaction was assessed by its repetitive execution (Table SM 15.1.1), namely by 1st year Chemistry M.Sc. students from School of Sciences (Montevideo).

The third reaction, transformation of 2-amino-7(8)-bromophenazine 5,10-dioxide to 2-amino-7(8)-bromophenazine and 2-amino-7(8)-bromophenazine 5-oxide, involves the chemical *N*-oxide reduction. Since the starting material is brown-red, it is possible to follow the reaction by direct observation of the color changes, whilst the reaction mixture proceeds to a brown-yellow color. In this session students should use chromatographic isolation process. To check the advance of the isolation students should use TLC. The reduction process produces a less polar product, 2-amino-7(8)-bromophenazine, with the highest R_f values, and a intermediate polar product, 2-amino-7(8)-bromophenazine 5-oxide. The spot from the TLCs could be visualized with or without UV-C light (254 nm) exposition (Figure SM 15.1.2). The product 2-amino-7(8)-bromophenazine should be characterized by ^{1}NMR (Figure SM 15.1.6).

The reproducibility of this reaction was assessed by its repetitive execution (Table SM 15.1.1), namely by 1st year Chemistry M.Sc. students from School of Sciences (Montevideo).

The last session involves the *N*-oxide bioreduction where two different bio-systems could be used with the same results: in one case, the use of commercial product, S9-fraction[f], and in the other from a bio-system available in countries with cow production. S9-fraction isolation could be done in other course, related to biochemistry, and the same students could be the responsible of this process.[g] In the case of the use of ruminal bovine fluid, due to it is a reductive milieu, the atmosphere should be maintained free of oxygen. For that reason an equipment with connection to nitrogen gas should be installed (Figure SM 15.1.4). For the check of the evolution of these processes the students should use TLCs. In these cases, the students should include as standards the synthesized products, i.e. 2-amino-7(8)-bromophenazine 5,10-dioxide, 2-amino-7(8)-bromophenazine and 2-amino-7(8)-bromophenazine 5-oxide, and the mixture of the bio-reactions. As in the previous chromatographies, the spot from the TLCs could be visualized with or without UV-C light (254 nm) exposition (Figure SM 15.1.3). When the bio-reactions are quenched, after 60 minutes of incubation, it is possible that the bio-processes have not completed, due to the efficacy of enzymes and organism present in the milieu. In this case, the TLCs demonstrate an uncompleted process.

Table SM 15.1.**1 –** Experiments conducted using different amounts of starting materials.

	Synthesis of 5(6)-bromo benzofuroxan		**Synthesis of 2-amino-7(8)-bromophenazine 5,10-dioxide**		**Synthesis of 2-amino-7(8)-bromophenazine and 2-amino-7(8)-bromophenazine 5-oxide**	
Entry[a]	**Reactive**		**Reactive**		**Reactive**	
	4-Bromo-2-nitroaniline (mmol)	**Isolated Yield,%[b]**	**5(6)-Bromo benzofuroxan (mmol)**	**Isolated Yield,%[c]**	**2-amino-7(8)-bromophenazine 5,10-dioxide (mmol)**	**Isolated Yield,%[d]**
1	17	72	10	40	2.5	25 / 20[e]
2	25	80	15	48	3.8	27 / 22
3	34	85	20	52	5	30 / 25

a) Experiments executed by 1st year MSc students (the yields are the average of the results obtained by 3 to 5 students). **b)** Isolating the product by filtration on a Büchner funnel and drying *in vacuo* on SiO_2 for 24 hours. **c)** Isolating the product by filtration on a Büchner funnel and drying by successive washing the solid with increasing-volatility solvents. **d)** Isolating the products by column chromatography. **e)** 2-amino-7(8)-bromophenazine / 2-amino-7(8)-bromophenazine 5-oxide yields.

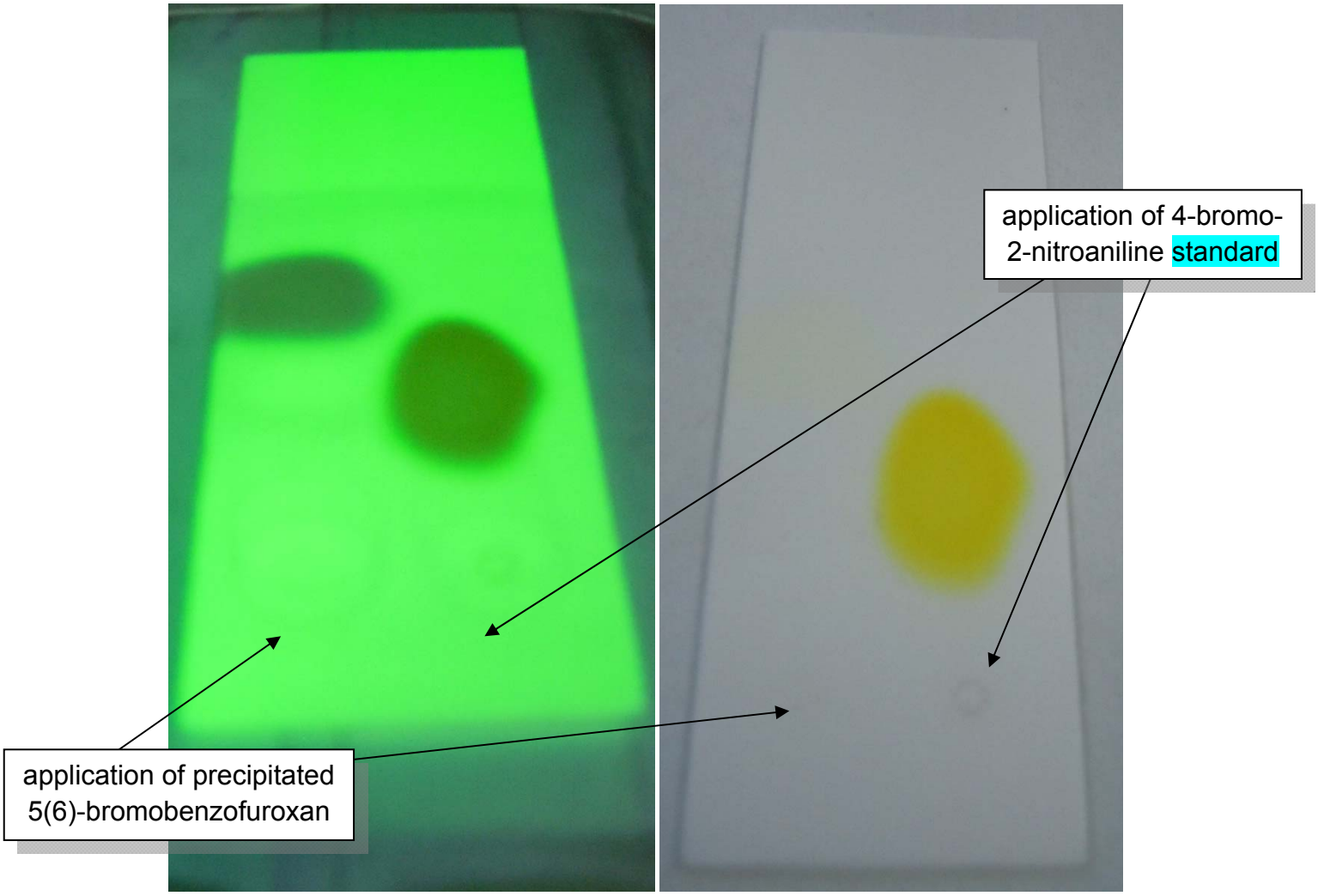


Figure SM 15.1.**1 –** TLC, from students, during the check of 5(6)-bromobenzofuroxan generation from 4-bromo-2-nitroaniline, with (left) and without (right) UV-C light (254 nm) exposition. Conditions: SiO_2, *n*-hexane:ethyl acetate (9:1).

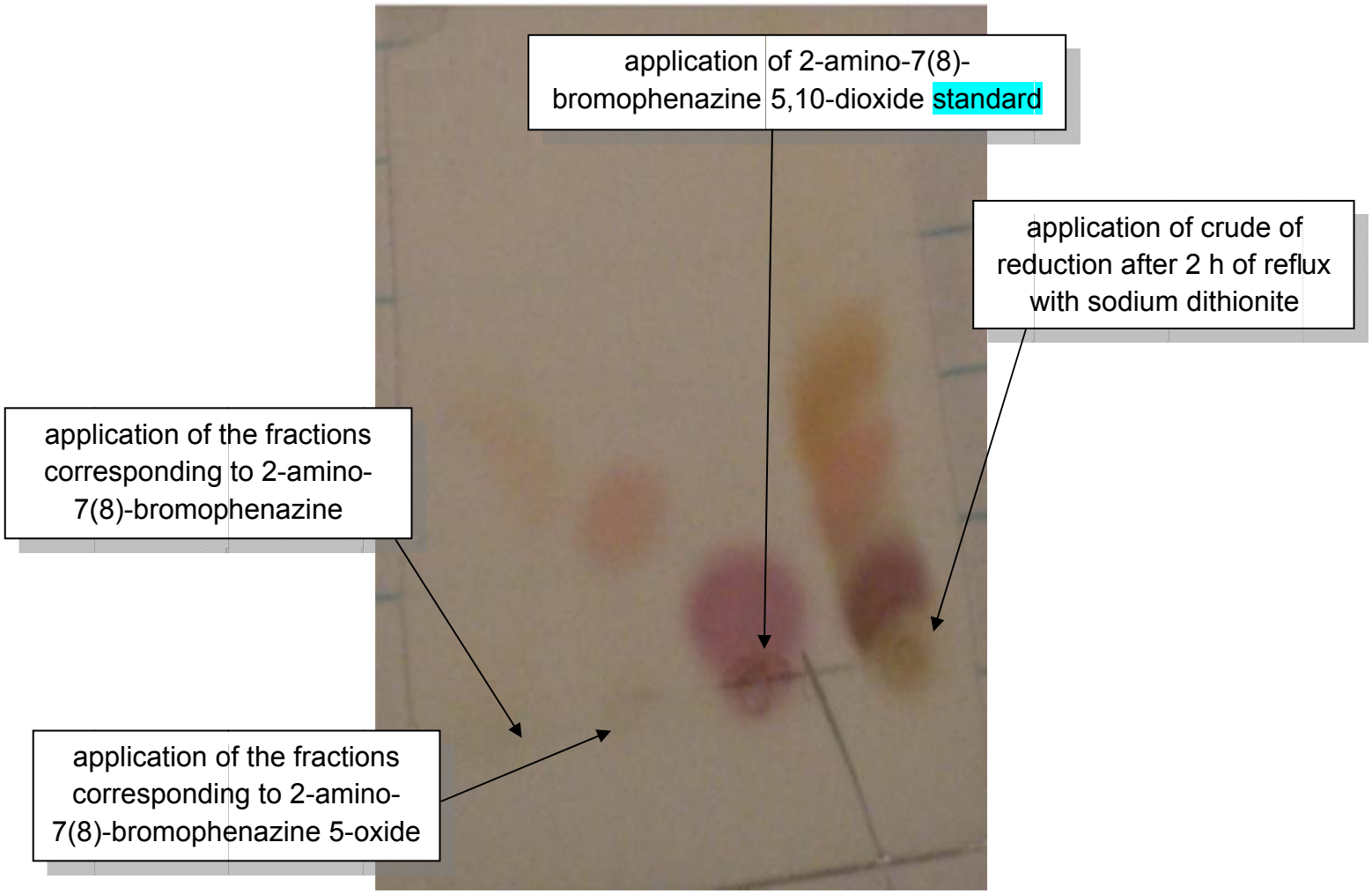


Figure SM 15.1.**2** – TLC, from students, during the check of 2-amino-7(8)-bromophenazine and 2-amino-7(8)-bromophenazine 5-oxide formation and isolation. Conditions: SiO_2, *n*-hexane:ethyl acetate (5:5).

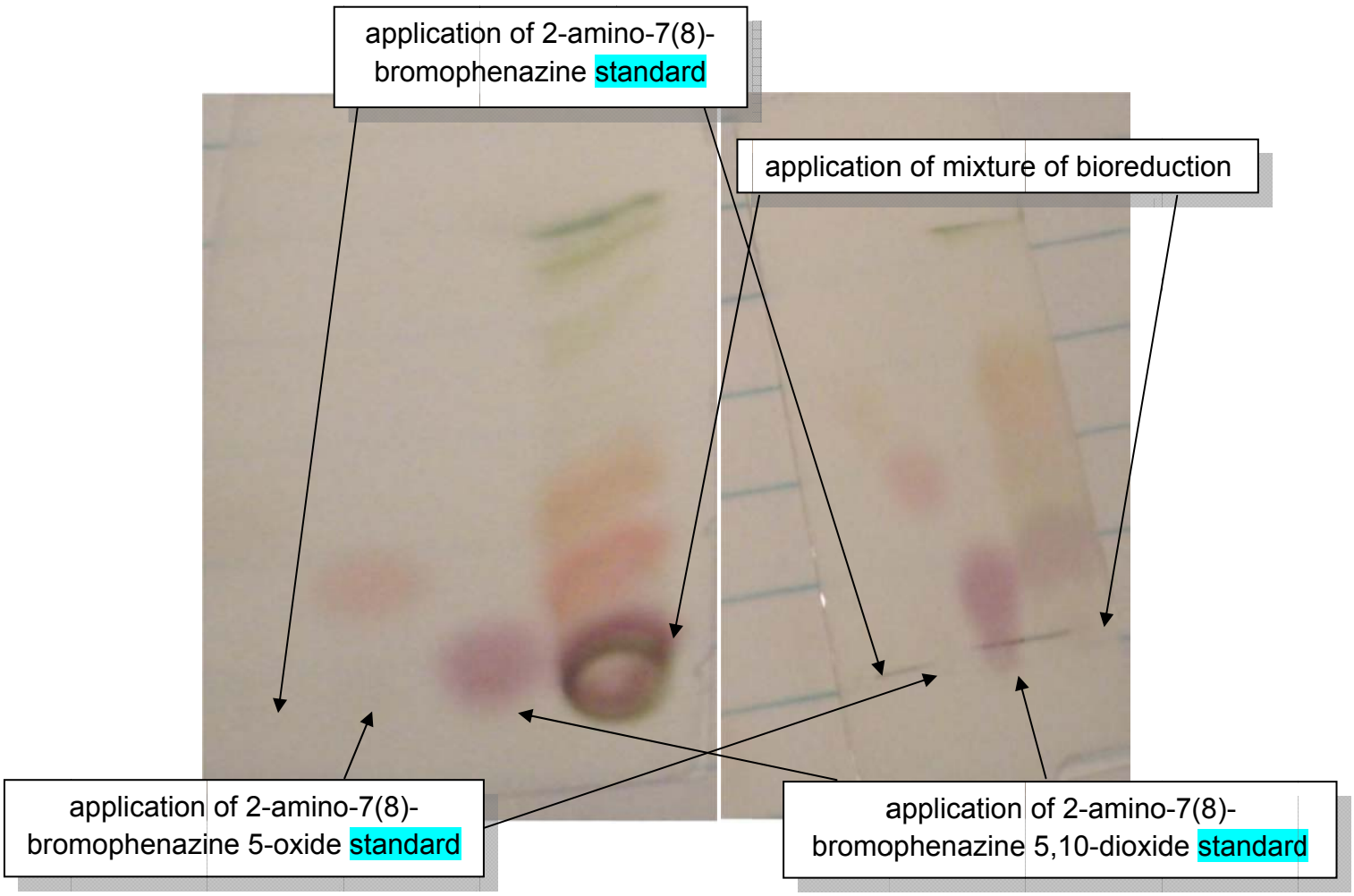


Figure SM 15.1.**3 –** TLC, from students, during the check of bio-reduction processes, with ruminal bovine fluid at different times. Conditions: SiO_2, *n*-hexane:ethyl acetate (5:5).

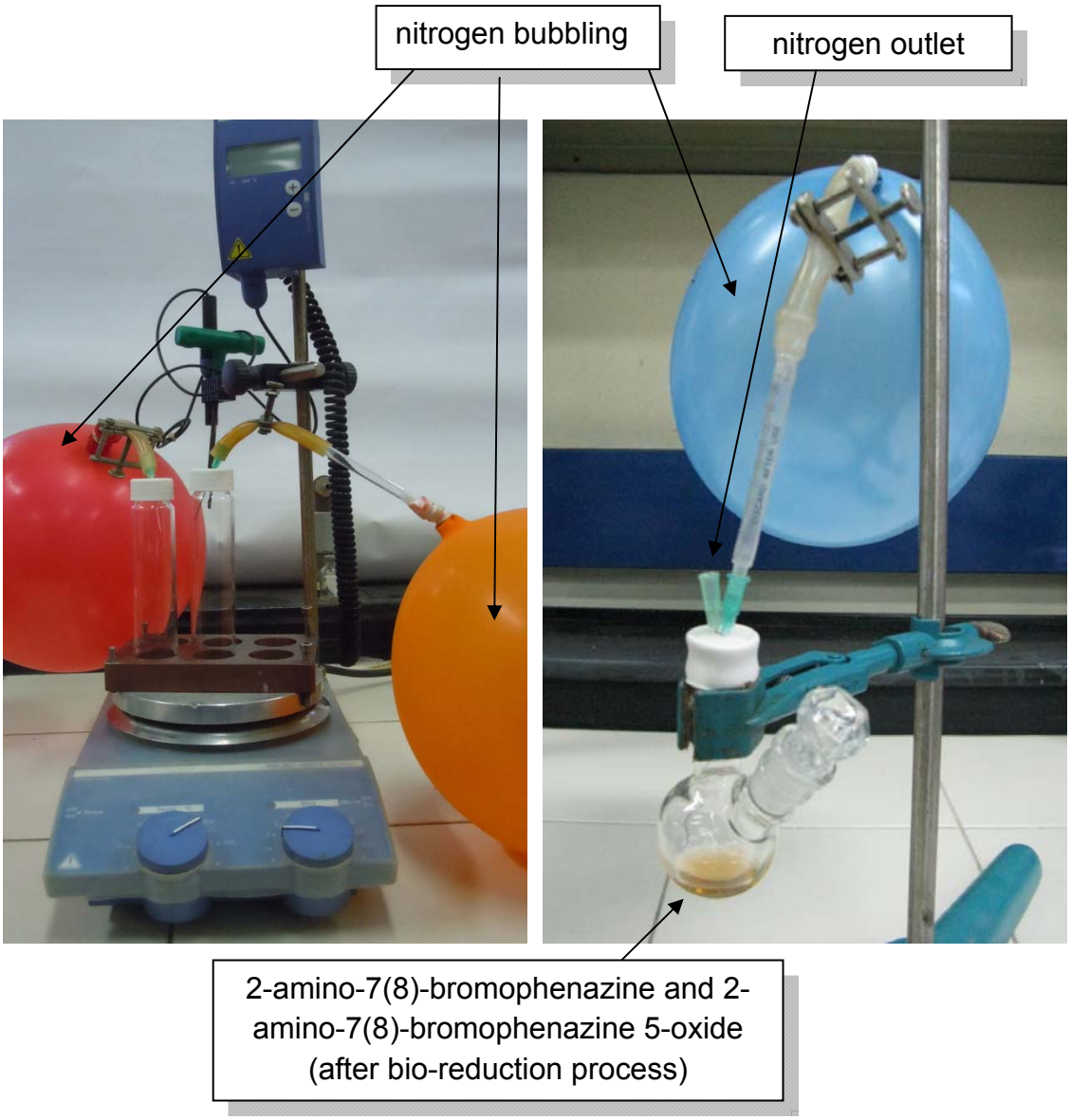


Figure SM 15.1.**4 –** Two possible apparatus used for the ruminal bovine fluid bioreduction. In the right photograph the endpoint of 2-amino-7(8)-bromophenazine 5,10-dioxide reduction.

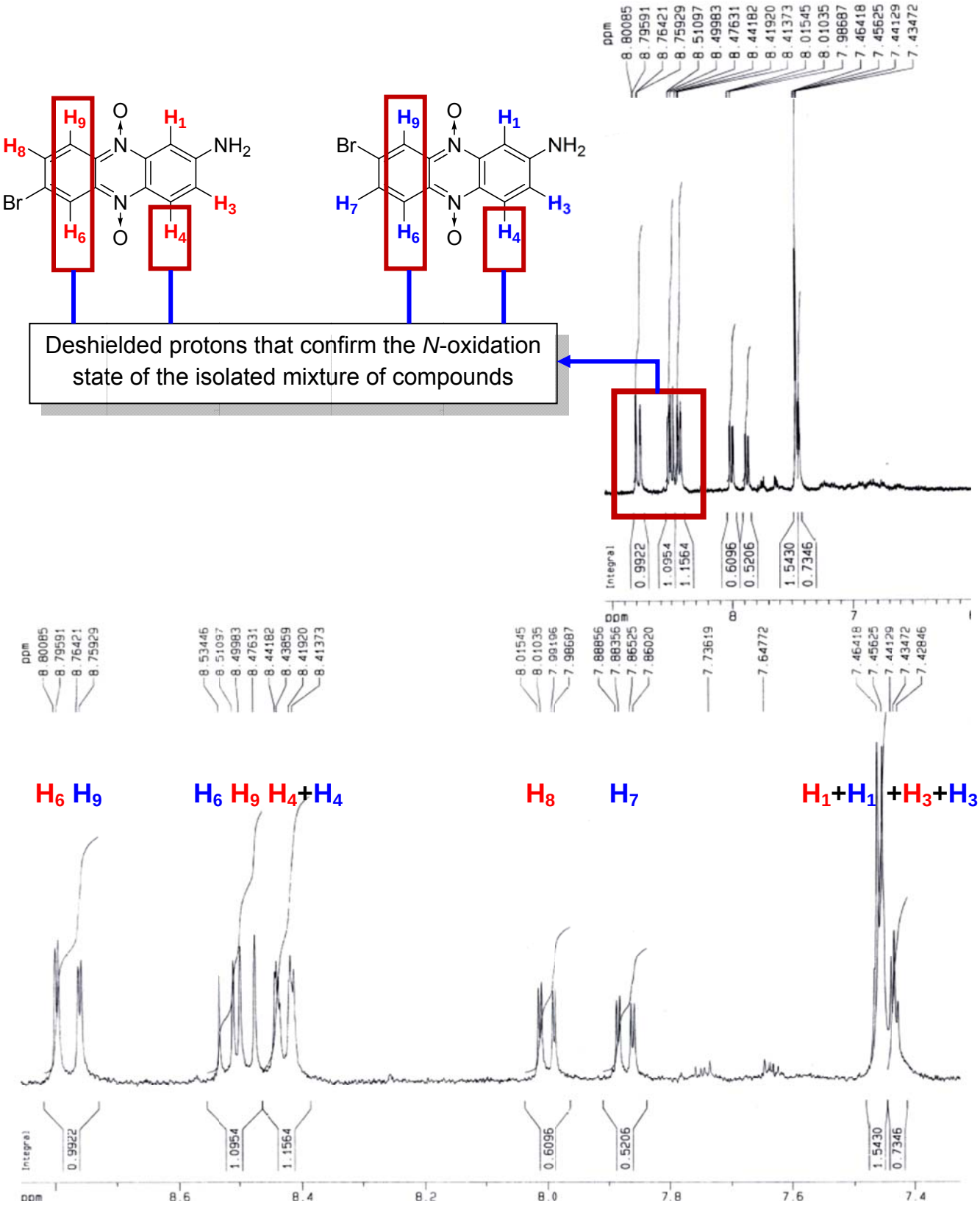
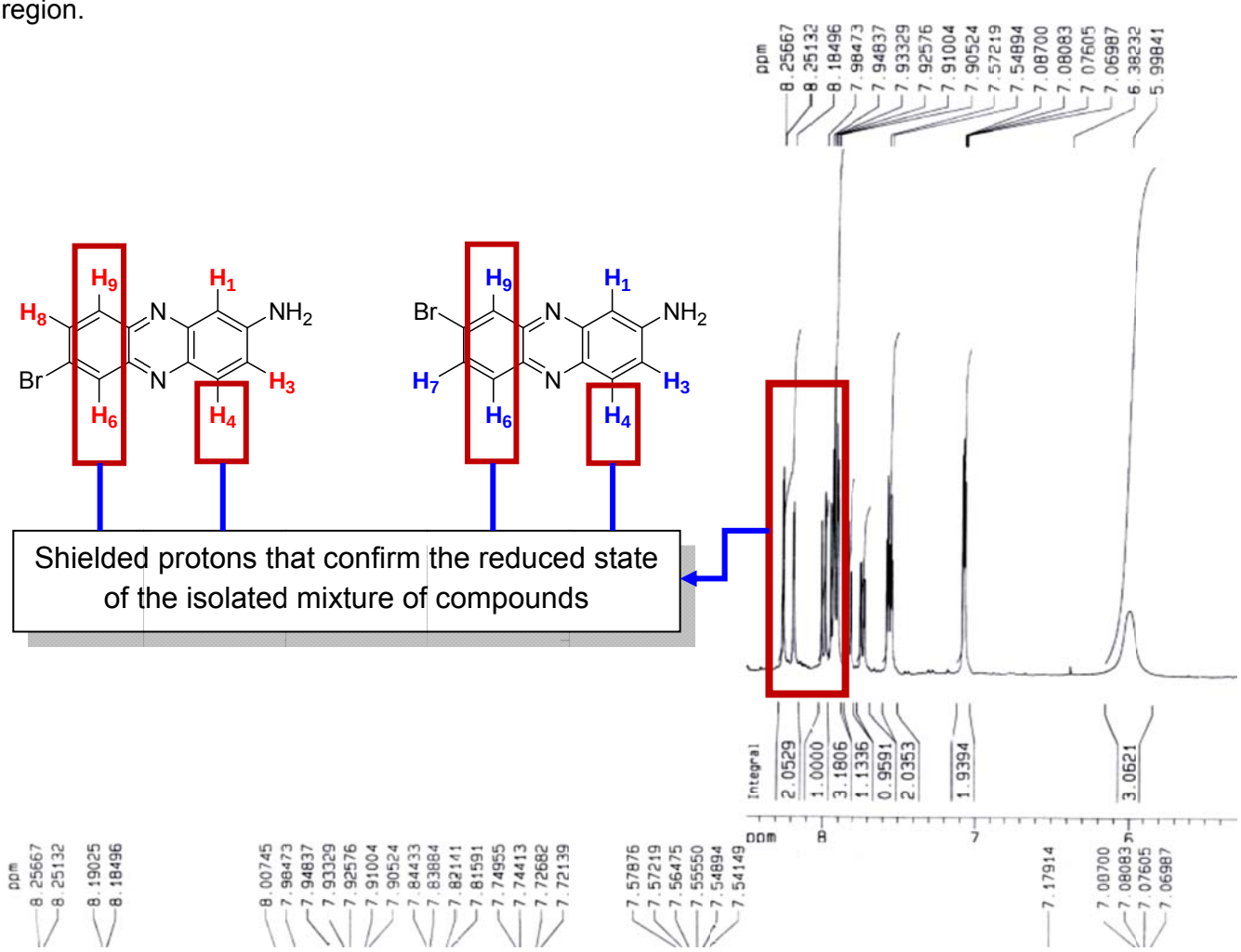


Figure SM 15.1.**5 –** Relevant region, aromatic, of the ^{1}H NMR spectrum (400MHz, 500 µL CD_3OD) of isolated 2-amino-7(8)-bromophenazine 5,10-dioxide at 303 K. Down: Enlarged region of the relevant region.



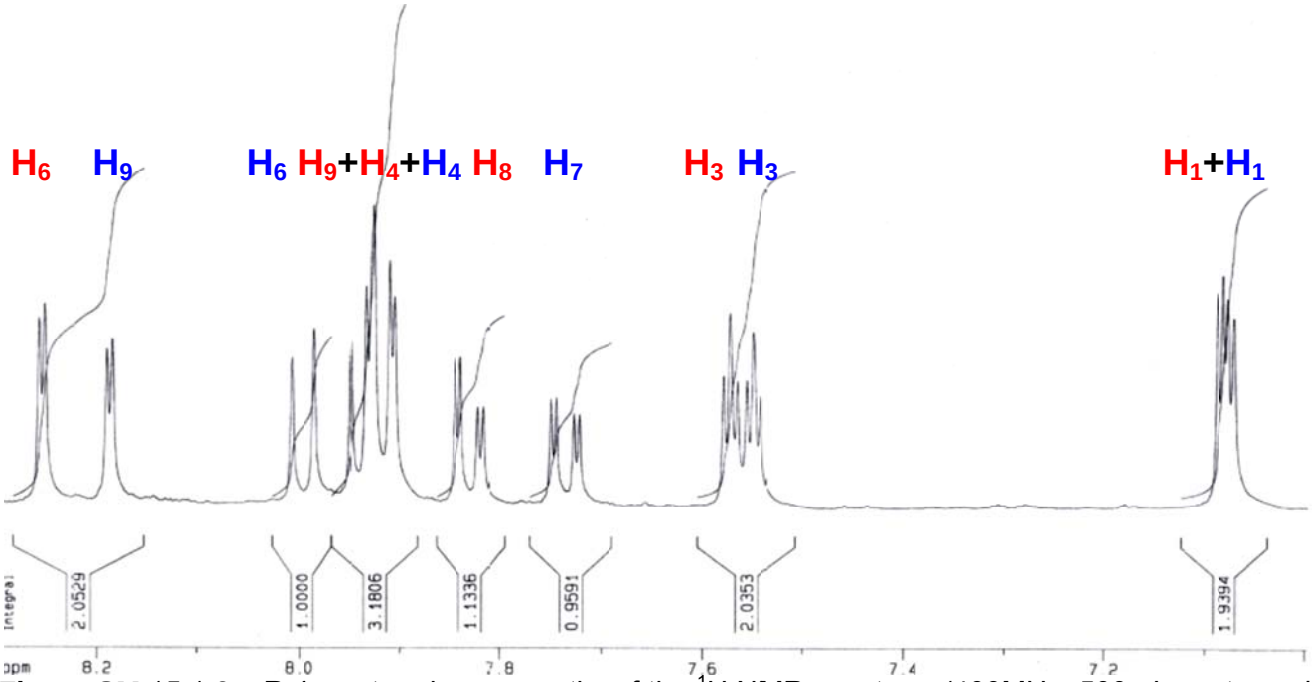


Figure SM 15.1.**6 –** Relevant region, aromativ, of the ^{1}H NMR spectrum (400MHz, 500 µL acetone-d_6) of isolated 2-amino-7(8)-bromo phenazine at 303 K. Down: Enlarged region of the relevant region.

References

[a] A. G. Green and F. M. Rowe, *J. Chem. Soc. Trans.*, 1912, **101**, 2443.
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[e] H. Cerecetto, M. González, M. L. Lavaggi and W. Porcal, *J. Braz. Chem. Soc.*, 2005, **16**, 1290.
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[g] M. L. Lavaggi, M. Cabrera, L. Celano, L. Thomson, H. Cerecetto and M. González, *J. Chem. Educ.*, 2013, **90**, 1388.