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# NMR and DFT Calculation Study on Structures of 2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC) and Its Two Metabolites Isolated from Urine of Patients Suffering from Tyrosinemia Type I.

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**NMR and DFT Calculation Study on Structures of 2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC) and Its Two Metabolites Isolated from Urine of Patients Suffering from Tyrosinemia Type I.**

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# NMR and DFT Calculation Study on Structures of 2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC) and Its Two Metabolites Isolated from Urine of Patients Suffering from Tyrosinemia Type I.

Adam Gryff-Keller<sup>\*[a]</sup>, Przemysław Szczeciński<sup>[a]</sup> and Anna Kraska-Dziadecka<sup>[a]</sup>

2-[2-Nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC) is an active component of nitisinone, a medicine against tyrosinemia type I. Using <sup>1</sup>H, <sup>13</sup>C and <sup>19</sup>F NMR spectroscopy it has been found that in urine of patients treated with nitisinone two compounds possessing CF<sub>3</sub> group are always present. They have been isolated by TLC technique and identified as 4-hydroxy-2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione and 5-hydroxy-2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione, the latter being previously unknown. The constitution, tautomerism and stereochemistry of these compounds have been thoroughly investigated using <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy supported by theoretical calculations. Molecular structures have been optimized using density functional theory (DFT) with PBE1PBE functional and 6-31G\* basis set. In NMR parameter calculations the larger 6-311++G(2d,p) basis set has been used. At both calculation stages the polarizable continuum model of the solvent has been employed.

**Key words:** NTBC, metabolism, tautomerism, NMR spectroscopy, DFT calculation

## Introduction

Several natural and synthetic compounds possessing branched  $\beta,\beta'$ -triketonic structure are inhibitors of 4-hydroxyphenylpyruvate dioxygenase (HPPD), an important enzyme on tyrosine biochemical metabolism path.<sup>[1-6]</sup> The first part of this path is common for plants and

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3 animals. In plants blocking this enzyme prevents production of some vitally important  
4 compounds, whereas in animals blocking tyrosine catabolism does not have such dramatic  
5 consequences, so HPPD inhibitors can be widely exploited as herbicides in crops protection.  
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7 Search for new and more effective triketonic herbicides has led to invention of 2-[2-nitro-4-  
8 (trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC), which appeared to be so selective  
9 and effective that it could be used in therapy of patients suffering from tyrosinemia type I, a  
10 fatal metabolic disease.<sup>[2,4,6-9]</sup> Children with this genetic error lack an enzyme needed at the  
11 end of tyrosine catabolism path, which results in production of succinylacetone, a  
12 pathognomonic metabolite damaging liver and kidney. Application of an NTBC-based drug  
13 interrupts the biochemical path at the earlier stage which prevents the production of that  
14 harmful metabolite.<sup>[7-9]</sup>

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23 The mechanism of HPPD action and its inhibition has been widely discussed.<sup>[10-16]</sup> The  
24 details of molecular structures of the natural substrate of the enzyme and its inhibitors are  
25 obviously crucial in such considerations. It seems that the same concerns the inhibitor  
26 metabolites, occurrence of which has occasionally been reported.<sup>[17,18]</sup> Recently, it has been  
27 found that one such metabolite, 4-hydroxy-2-[2-nitro-4-  
28 (trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC-6-OH) appears in the urine of  
29 patients treated with nitisinone.<sup>[18]</sup> In this work, the separation and chemical identification of  
30 the second NTBC metabolite, 5-hydroxy-2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-  
31 1,3-dione (NTBC-5-OH), is reported. Apart from the spectroscopic characterisation of NTBC  
32 and its metabolites the detailed discussion on their molecular structures and conformational  
33 equilibria, in the light of <sup>1</sup>H and <sup>13</sup>C NMR spectra and the results of DFT calculations, is  
34 undertaken.

## 35 36 37 38 39 40 41 42 43 44 45 46 **Results and Discussion**

### 47 48 49 50 **Separation and chemical identification of NTBC metabolites.**

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53 In the previous paper we reported the results of our investigations of organic extracts of urine  
54 samples from children treated with nitisinone.<sup>[18]</sup> In <sup>19</sup>F NMR spectra of these samples two  
55 major signals of CF<sub>3</sub> groups were always present. At the same time, in <sup>1</sup>H NMR spectra, in  
56 the region  $\delta \approx 8.5$  ppm, two characteristic signals were observed for all investigated samples.  
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58 These signals were assigned to the H3' protons strongly deshielded because of three electron  
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3 withdrawing groups present in the aromatic ring of NTBC and its derivatives. One compound  
4 containing  $\text{CF}_3$  group could be separated from the extracts by TLC technique in a small  
5 amount. The NMR spectra showed that it was NTBC-6-OH (Fig. 1). Its identification was  
6 confirmed by comparison with the sample of the same compound synthesised *in vitro*.<sup>[18]</sup>  
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10 Following the literature suggestions<sup>[19,20]</sup> it seemed natural to suppose that the second  
11 of the compounds containing  $\text{CF}_3$  group present in urine extracts was the unchanged NTBC.  
12 Moreover, the comparison of the  $^1\text{H}$  NMR spectrum of a urine extract with the spectrum of  
13 the same sample recorded after addition of a portion of NTBC seemed to verify positively  
14 such a conjecture. In view of presently available data, however, it is clear that we  
15 underestimated the possibility that our identification method might be error prone. Namely,  
16  $\text{H}_3'$  proton and  $\text{CF}_3$  fluorine signals of NTBC derivatives are actually broadened, partially  
17 resolved multiplets and, simultaneously, the chemical shift differences involved are very  
18 small and concentration-dependent. Unfortunately, organic urine extracts contain, apart from  
19 NTBC metabolites, many different compounds whose concentrations overwhelmingly exceed  
20 the concentration of the compounds of interest. Therefore, most proton NMR signals of  
21 NTBC and its metabolites are covered by intense signals of other mixture components.  
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25 In order to check if NMR technique can be applied for medical assays during  
26 nitisinone treatment the fluorine NMR spectra of several samples of unprepared urine,  
27 originating from four different patients, have been measured. It has been found that also for  
28 these much more dilute samples two main  $\text{CF}_3$  signals are easily detectable. We have also  
29 repeated the measurements of the original urine sample with the addition of NTBC and  
30 NTBC-6-OH. The experiments have confirmed the presence of NTBC-6-OH, but, to our  
31 surprise, excluded the presence of NTBC, at least in an amount comparable with NTBC-6-  
32 OH. Addition of a portion of NTBC to the urine sample caused the new signal to appear  
33 downfield with respect to two original signals (Fig. 2). TLC examination of the organic urine  
34 extracts has yielded similar results. It was found that when silica gel plates and benzene-  
35 acetone mixture (1/1, v/v) as an eluent were used, the experiments performed for all organic  
36 urine extracts and their mixtures with the reference compounds revealed the presence of two  
37 main spots: one originating from NTBC-6-OH ( $R_f = 0.25$ ) and the second from an unknown  
38 compound ( $R_f = 0.15$ ) unequivocally different from NTBC ( $R_f = 0.5$ ). Separation of the  
39 compounds giving those two spots was done with the aid of the preparative TLC technique.  
40 The NMR and HRMS spectra confirmed that one of them was NTBC-6-OH, whereas the  
41 second one was a new metabolite NTBC-5-OH (Fig. 1). Analogous 6- and 5-hydroxylated  
42 derivatives were found in urine of rats and mice treated with 2-(4-methylsulfonyl-2-  
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3 nitrobenzoyl)cyclohexane-1,3-dione (mesotrione), a popular herbicide.<sup>[17]</sup> In this case,  
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5 however, unchanged mesotrione was also detected in huge amounts.  
6

7 Having a relatively pure sample of NTBC-5-OH we could compare the <sup>1</sup>H NMR  
8 signals of H3' protons for this compound and for NTBC. It appeared that their multiplet  
9 patterns were identical and that at comparable concentration the chemical shift difference  $\Delta\delta$   
10 was of the order of 2 Hz only, at  $B_0 = 11.7$  T. Apparently, this was the reason of the erroneous  
11 interpretation of this signal made previously.  
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## 16 17 18 **Tautomerism**

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21 So-called triketonic herbicides (leptospermon, mesotrione, sulcotrione, chalcotrione,  
22 etc.) as well as three compounds investigated in this work belong to the class of the branched  
23  $\beta,\beta'$ -diketones. Actually, probably all these compounds exist in organic solutions exclusively  
24 as enols stabilised by the formation of strong intramolecular hydrogen bonds.<sup>[10,14,21,22]</sup> In 2-  
25 acylcyclohexane-1,3-diones (Fig. 3) the acyl carbonyl and enol group originating from one of  
26 the two ring carbonyls form a ring *via* such a hydrogen bond. In the case of symmetrically  
27 substituted cyclohexane ring (NTBC or NTBC-5-OH), this leads to two chemically  
28 indistinguishable topomeric structures, while for the unsymmetrical substitution (NTBC-6-  
29 OH) the structures involving different oxygen atoms are different. To be specific, such a  
30 classification holds only if cyclohexane ring conformation is neglected; otherwise we deal  
31 with enantiomeric structures for symmetrical substitution and with diastereomers in the  
32 opposite case.  
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42 There is firm evidence coming from both <sup>1</sup>H and <sup>13</sup>C NMR that tautomeric  
43 transformation leading from one topomer to another is completely hindered in NMR time  
44 scale in CDCl<sub>3</sub> solution at room temperature. Already in our previous paper we noticed that  
45 several 2-acylcyclohexane-1,3-diones, including NTBC, show in <sup>13</sup>C NMR spectrum six sharp  
46 lines for the cyclohexane ring carbons.<sup>[14,22]</sup> Also the cyclohexane ring protons yield three,  
47 rather than two, multiplets in <sup>1</sup>H NMR spectra. The same spectral features are observed in this  
48 work for NTBC-5-OH (see Experimental). It is interesting that in the spectra of the other  
49 NTBC metabolite only one proton pattern characteristic for six non-equivalent aliphatic  
50 protons and one set of six carbon lines for a cyclohexene ring are present (see Experimental).  
51 In view of slow exchange limit it proves that only one of two possible structures of this  
52 compound exists in the investigated solution. The quantum chemical calculations of the  
53 energy of the optimal molecular structures show that 2-acyl-3,6-dihydroxycyclohex-2-enone  
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(acyl = 4-CF<sub>3</sub>-2-NO<sub>2</sub>-C<sub>6</sub>H<sub>3</sub>CO-) is remarkably more stable than the appropriate 2-acyl-3,4-dihydroxycyclohex-2-enone (Fig. 3, Table 1). The same conclusion comes out from the analysis of the <sup>13</sup>C NMR chemical shifts (*vide infra*). It is the reason why we use the abbreviation NTBC-6-OH for this NTBC metabolite.

In the discussed system there is another tautomeric transformation in which the hydrogen atom that binds two oxygen atoms moves between two energy minima on the =O···H—O— ⇌ —O—H···O= path. This passage is accompanied by a due electron rearrangement in the chelate ring (Fig. 4). Such a transformation is believed to be extremely fast because of a low energy barrier.<sup>[21]</sup> As a result, averaged, population-weighted spectra are observed in NMR. This problem was widely discussed in the literature and also in several papers from our laboratory.<sup>[10,14,21-26]</sup> In ref. <sup>[14]</sup> it was shown (as opposite to ref. <sup>[10]</sup>) that in the case of NTBC the *endo*-enol structure prevails. The energy calculations (Table 1) and <sup>13</sup>C NMR chemical shift analysis performed in this work (*vide infra*) confirm this conclusion for NTBC and show that it is valid also for NTBC metabolites.

### Conformation of the system

The molecular structure optimizations have been performed for *endo*- and *exo*-enols of all three compounds investigated in this work using a DFT-based method. The energy minima have been found for conformations in which five carbons of the cyclohexene ring, C1, C2, C3, C4, C6 and all three carbon-oxygen bonds lie approximately on one plane, whereas C-5 deviates remarkably from it. Moreover, the aromatic ring assumes approximately perpendicular orientation to that plane (Fig. 5), presumably because of steric reasons. There are two such conformations, which will be further addressed as A and B type conformers, dependently on C-5 and -NO<sub>2</sub> being on the same or opposite sides of the main molecular plane. The calculated molecular energy differences (Table 1) between A and B conformers are very small and both of them are expected to be present in the investigated solutions in comparable amounts. Below we will see that the differences between the appropriate shielding constants are also small and so conformation populations cannot be reliably estimated on this basis.

The molecular structure described above actually denotes existence of four different conformers of the investigated compounds. In the case of NTBC they are pairwise enantiomeric: *R*-A, *S*-A, *R*-B and *S*-B). In the other two cases they are diastereomeric due to the axial or equatorial position of the -OH substituent (*ax*-A, *eq*-A, *ax*-B and *eq*-B). The

transitions between these conformers demand the aromatic ring rotation and/or cyclohexane ring inversion, both processes expected to be fast in NMR time scale at room temperature. Again, neither molecular energies nor  $^{13}\text{C}$  chemical shifts can be a basis for the evaluation of the populations of the axial and equatorial conformers. In this case, however, there is another source of reliable information at our disposal, namely, the vicinal spin-spin coupling constants between cyclohexene ring protons. Tables 2 and 3 contain experimental values of  $^1\text{H}$ - $^1\text{H}$  spin-spin coupling constants obtained from the analysis of spectral patterns occurring in aliphatic parts of  $^1\text{H}$  NMR spectra of both NTBC metabolites. These tables also contain theoretical coupling constants calculated for the selected conformers of the investigated compounds. In accord with the expectations, the vicinal coupling constants between aliphatic protons are strongly dependent on the appropriate dihedral angles.<sup>[27]</sup> On the other hand, the coupling constants are almost independent on  $\text{A} \rightleftharpoons \text{B}$  transition and only marginally dependent on  $\text{endo} \rightleftharpoons \text{exo}$  tautomerisation. Comparison of the experimental and theoretical data in Tables 2 and 3 shows that in the case of NTBC-6-OH the equatorial conformation highly predominates, whereas NTBC-5-OH is approximately equimolar mixture of *eq* and *ax* conformers. It is even possible to estimate conformer populations for NTBC-5-OH expressing measured coupling constants as population-weighted averages of values calculated for the appropriate *eq* and *ax* conformers (eq. 1) and assuming that the impact of small amounts of *exo*-enols present in the solution can be ignored.

$$J_{\text{exp}} = a(p_{\text{eq}}J_{\text{eq,calc}} + p_{\text{ax}}J_{\text{ax,calc}}) + b \quad (1)$$

In this equation *a* and *b* are adjustable parameters. The least-squares analysis yields:  $p_{\text{eq}} = 0.43 \pm 0.03$ ,  $p_{\text{ax}} = 0.57 \pm 0.03$ ,  $a = 0.988$ ,  $b = -0.3$  Hz. Thus, the result seems quite reasonable, as judged from *a* and *b* values and excellent reproduction of the experimental data (Fig. 6), though the error estimations are probably too optimistic. It is also noteworthy that the OH proton signal of NTBC-5-OH is a broadened singlet, whereas in the spectrum of NTBC-6-OH it is a doublet. Presumably, the intramolecular hydrogen bond in  $-\text{CHOH}-\text{C}(=\text{O})-$  fragment in the latter metabolite prevents the proton exchange. The small  $^3\text{J}(\text{HCOH})$  value agrees well with the calculated molecular structure in which the dihedral angle between the appropriate C-H and O-H vectors is close to  $90^\circ$ .

### Analysis of $^{13}\text{C}$ NMR chemical shifts

There is no need to substantiate that  $^{13}\text{C}$  NMR chemical shifts are an efficient source of valuable information on molecular structure. The usefulness of these relatively easily accessible and precise experimental data was enhanced in the last decade with the enhanced access to quantum chemical calculations of NMR parameters.<sup>[28-31]</sup> Numerous test calculations showed that the chemical shifts for the  $i$ -th carbon,  $\delta_i$ , in a compound existing in the investigated solution in several forms being in dynamic equilibrium (fast in NMR time scale), can be expressed in terms of the calculated shielding constants,  $\sigma_{i,j}$ , and species populations,  $p_j$ , by the equation:

$$\delta_i = a(p_1\sigma_{i,1} + p_2\sigma_{i,2} + \dots - b) \quad (2)$$

where  $a$  and  $b$  are compound-independent, adjustable parameters,  $a$  being a scaling factor, close to  $-1$ , and  $b$  - the shielding constant of the (virtual) chemical shift reference.<sup>[32-35]</sup> It was pointed out that in order to achieve a good experiment/theory correlation it is desired to use a sufficiently high and possibly the same level of theory in the molecular geometry optimization and NMR parameters calculation. The impact of the solvent should also be taken into account.<sup>[33,35]</sup> Obviously, all the abundant forms of the investigated compound present in the solution have to be considered. In the light of the above discussion, for NTBC there are four such species:  $A$  and  $B$  conformers of the *endo* and *exo* enols. In the case of NTBC-5-OH the number of species is doubled due to the distinction between axial and equatorial conformers. Finally, in the case of NTBC-6-OH metabolite, four species have to be considered, when assuming that only the equatorial conformers are present in the investigated solutions. Additionally, we performed calculations for four structures of the NTBC-4-OH topomers to check whether the chemical shifts could be used to distinguish between the NTBC-4-OH and NTBC-6-OH structures.

The assignment of the signals in the  $^{13}\text{C}$  NMR spectra for all three compounds has been a relatively easy task, when using 2D-HSQC and 2D-HMQC spectra, taking into account  $^{13}\text{C}$ - $^{19}\text{F}$  spin-spin couplings and characteristic broadening of C2' signal due to the scalar relaxation of the second kind. It is interesting that the assignments alone are insufficient to make the distinction between NTBC-4-OH and NTBC-6-OH structures.

Our experience has shown that applying the 6-311++G(2d,p) basis set in NMR parameter calculations usually yields good results. Unfortunately, however, application of the same basis in optimizations of all 20 structures considered would be computationally too demanding. Thus we have decided to use smaller basis in the first computation step. The

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3 results of magnetic shielding calculations are collected in Table 1S-4S (Supporting Materials).  
4 Examination of these data shows that  $A \rightleftharpoons B$  transformation affects the  $^{13}\text{C}$  shielding constants  
5 only marginally, which means that the determination of [A/B] molar ratio would be  
6 unreliable, if possible at all. In such a situation we decided to calculate the mean values of the  
7 appropriate shielding constants:  
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$$\sigma_{i,j} = 0.5(\sigma_{i,j(A)} + \sigma_{i,j(B)}) \quad (3)$$

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17 and to use them in correlations. In the case of NTBC-5-OH we are still left with four species:  
18 *ax/eq* conformers and *endo/exo* enols. The shielding constants for *ax* and *eq* conformers are  
19 again not very large and an attempt at determining the appropriate population parameters for  
20 the less populated *exo*-enol seems unrealistic. That is why we decided to average the  
21 appropriate shielding constants again. Finally, in the least-squares analysis of the chemical  
22 shift data we had 42 carbon chemical shifts and 9 unknowns ( $p_{endo}$  and  $p_{exo}$  for NTBC,  $p_{ax-endo}$ ,  
23  $p_{eq-endo}$  and  $p_{exo}$  for NTBC-5-OH,  $p_{eq-endo}$  and  $p_{eq-exo}$  for NTBC-6-OH, and a and b parameters.  
24 The results of this analysis are given in Table 4 and in Fig. 7.  
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32 The achieved overall fit between experiment and theory (RMSD = 1.87 ppm) is  
33 sufficiently good to interpret the determined numerical values of the population parameters. It  
34 may be noticed, however, that the fit in the aliphatic parts of the spectra (carbons 1 to 7) is  
35 excellent (RMSD = 0.75 ppm), whereas for the  $\text{CF}_3$  carbons and the aromatic ring carbons the  
36 deviations from the regression line are much larger and very similar for all three compounds  
37 (see the results for NTBC-6-OH in Fig. 8). The deviations for  $\text{CF}_3$  carbons could be a result of  
38 deficiencies of the theoretical method (an impact of electron correlations and ro-vibrational  
39 motions), while some test calculations (see Supplementary Materials) suggest that the  
40 deviations for the aromatic ring carbons are connected with large amplitude deformations of  
41 the equilibrium conformations, such as the rotation about C7–C1' bond, due to ground state  
42 internal motions and intermolecular collisions. It is noteworthy that the aromatic parts of the  
43 investigated molecules are identical and only marginally influenced by the substitution of the  
44 cyclohexane ring in NTBC-5-OH and NTBC-6-OH. This fact is manifested in very small  
45 chemical shift variations for aromatic carbons. Thus, almost all information on possible  
46 structural changes within the series should be cumulated in the data concerning the aliphatic  
47 parts of these compounds. Following this reasoning, we have performed the separate  
48 numerical analysis of the  $^{13}\text{C}$  NMR chemical shifts of C-1 to C-7 carbons and placed its  
49 results in Table 4. It is to be realised that error estimation in the problem in hand is very  
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3 difficult. The estimates given in Table 4 are purely 'statistical errors', having, unfortunately,  
4 an unclear meaning in the discussed case, where  $\sigma$  errors are hardly random.  
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7 The set of simplifying assumptions proposed above seems to be the most reasonable.  
8 Certainly, other approaches are possible and, actually, have been attempted. Nevertheless, our  
9 conclusion about small contributions (probably of the order of 0.1 or less) of *exo*-enols in  
10 tautomeric equilibriums for all three compounds seems to be unquestionable. In the case of  
11 NTBC-5-OH the axial and equatorial conformers seem to be present in comparable amounts,  
12 which is in agreement with the finding based on the values of  $^1\text{H}$ - $^1\text{H}$  coupling constants.  
13 Finally, the fit between experiment and theory for the correlation of the chemical shift data for  
14 the first NTBC metabolite, i.e. 6-hydroxy-2-[(2-nitro-4-trifluoromethyl)benzoyl]cyclohexane-  
15 1,3-dione has been examined, assuming for it the NTBC-4-OH or NTBC-6-OH structure. A  
16 better fit, especially in the aliphatic part of the spectrum, provides the additional argument  
17 towards the latter structure (Fig. 8).  
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## 28 Experimental

29 Preparation of organic extracts of urine samples was described previously.<sup>[18]</sup>  
30 Separation of NTBC metabolites from those extracts was performed using TLC glass plates  
31 20×20 cm with silica gel 60 F<sub>254</sub>, 0.25 mm and acetone-benzene mixture (1/1, v/v) as eluent.  
32 In these conditions  $R_f$  values for NTBC-5-OH and NTBC-6-OH were 0.15 and 0.25,  
33 respectively. All other compounds present in the urine sample were located at  $R_f$  larger than  
34 0.5.  
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42 NMR measurements were performed using 500 MHz (11.7 T) spectrometer equipped  
43 with a temperature controller.  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{19}\text{F}$  NMR spectra of the  $\text{CDCl}_3$  urine extracts or  
44 unprocessed urine samples after addition of *ca.* 5 %  $\text{D}_2\text{O}$  were recorded at 25 °C using 5 mm  
45 NMR tubes.  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts were referenced to the TMS scale using the residual  
46  $\text{CHCl}_3$  proton signal ( $\delta = 7.26$  ppm) or carbon  $\text{CDCl}_3$  signal ( $\delta = 77.0$  ppm). Fluorine spectra  
47 in  $\text{CDCl}_3$  were referenced to  $\text{CFCl}_3$  ( $\delta = 0.0$  ppm). 2-D  $^1\text{H}$ - $^{13}\text{C}$  correlations of the HSQC and  
48 HMQC type were recorded using the spectral widths  $\text{sw} = 6000$  Hz,  $\text{sw}_1 = 30000$  Hz, number  
49 of increments,  $\text{ni} = 512$ , number of scans,  $\text{ns} = 24$ , and the standard spectrometer software. 1-  
50 D NMR spectra were acquired using standard parameter sets adjusting the number of scans to  
51 achieve satisfying s/n for the signals of interest (typically  $\text{sw} = 10000$ , at = 5 s, digital  
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3 resolution < 0.1 Hz/point, ns = 64 for  $^1\text{H}$  NMR signals and sw = 36000, at = 5 s, digital  
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5 resolution ~ 0.15 Hz/point, ns = 1000 - 10000 for  $^{19}\text{F}$  NMR signals).

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7 **NTBC-5-OH:**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 16.31 (enol OH), 8.48 (H3'), 7.96 (H5'), 7.41 (H6'), 4.47  
8  
9 (H5), 3.49 (OH), 3.10 (H6), 2.93 (H6a), 2.66 (H4), 2.52 (H4a). For proton numbering and  
10  
11 coupling constants between aliphatic protons see Table 3.

12  
13  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ) 195.79 (C7), 192.5 (C1), 191.72 (C3), 145.60 (C2'), 139.35 (C1'), 132.33  
14  
15 (C4',  $J_{\text{C,F}} = 34.8$  Hz), 130.86 (C3',  $J_{\text{C,F}} = 3.5$  Hz), 127.89 (C6'), 122.58 (CF<sub>3</sub>,  $J_{\text{C,F}} = 275.4$   
16  
17 Hz), 121.20 (C5',  $J_{\text{C,F}} = 3.9$  Hz), 112.70 (C2), 63.51 (C5), 45.87 (C6), 39.86 (C4)

18  
19 HRMS:  $\text{C}_{14}\text{H}_{10}\text{F}_3\text{NO}_6$  requires  $[M-\text{H}]^\ominus$  344.0377, found 344.0391.

20  
21 **NTBC-6-OH:**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 16.19 (enol OH), 8.52 (H3'), 8.00 (H5'), 7.43 (H6'), 4.08  
22  
23 (H6), 3.49 (OH), 2.96 (H4), 2.91 (H4a), 2.43 (H5), 1.91 (H5a). For proton numbering and  
24  
25 coupling constants between aliphatic protons see Table 2.

26  
27  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ) 195.86 (C7), 195.05 (C1), 194.83 (C3), 145.68 (C2'), 138.71 (C1') 132.67  
28  
29 (C4'), 130.93 (C5',  $J_{\text{C,F}} = 3.3$  Hz), 128.07 (C6'), 121.36 (CF<sub>3</sub>,  $J_{\text{C,F}} = 273.0$  Hz), 121.20 (C3'),  
30  
31 110.20 (C2), 70.89 (C6), 29.88 (C4), 27.05 (C5).

32  
33 HRMS:  $\text{C}_{14}\text{H}_{10}\text{F}_3\text{NO}_6$  requires  $[M-\text{H}]^\ominus$  344.0377, found 344.0362.

34  
35 All the theoretical calculations of molecular structures and NMR parameters were performed  
36  
37 using Gaussian 03 program<sup>[36]</sup>. At both calculation stages the density functional theory (DFT)  
38  
39 with PBE1PBE functional<sup>[37]</sup> were used and the influence of the bulk solvent was taken into  
40  
41 account using the polarizable continuum model (PCM) of Tomasi et al.<sup>[38]</sup>, defining the cavity  
42  
43 according to the UFF scheme<sup>[39]</sup>. In the molecular geometry optimizations the 6-31+G\* basis  
44  
45 set was used, while for calculating NMR parameters the GIAO method and 6-311++G(2d,p)  
46  
47 basis set were exploited.

48  
49 The  $^1\text{H}$ - $^1\text{H}$  coupling constants between cyclohexene ring protons of NTBC-5-OH and NTBC-  
50  
51 6-OH were determined by the total lineshape analysis of the appropriate patterns in  $^1\text{H}$  NMR  
52  
53 spectra using a non-linear least-squares fitting procedure.

54  
55 The comparison of the experimental  $^1\text{H}$ - $^1\text{H}$  spin-spin coupling constants and  $^{13}\text{C}$  NMR  
56  
57 chemical shifts with the appropriate theoretical values was performed on the basis of eq. 1 and  
58  
59 eq. 2, respectively. The unknown parameters occurring in these equations (a, b,  $p_A$ ,  $p_B$ , ...) were  
60  
61 derived by a non-linear least-squares analysis of the experimental data set. The chemical  
62  
63 shift data for the whole series of investigated species were included into one data set. Such an  
64  
65 approach is advantageous as the parameters a and b are common for all three compounds, and  
66  
67 the total number of unknowns can be reduced.

**Acknowledgements**

This work was financially supported by Warsaw University of Technology.

**Supporting information**

Results of test calculation for 2-nitro-4-trifluoromethylbenzoyl chloride and carbon chemical shift values for NTBC, NTBC-5-OH and NTBC-6-OH and calculated shielding constants for particular conformers of these compounds.

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Table 1. The relative<sup>a</sup> total scf energies (in kJ/mol) after PCM corrections for various conformers of NTBC and its metabolites as calculated by DFT 6-311++G(2d,p)//PBE1PBE/6-31+G\* PCM(CHCl<sub>3</sub>) method.

Species, conformer	<i>endo</i> -A	<i>endo</i> -B	<i>exo</i> -A	<i>exo</i> -B
NTBC	2.6	0	12.2	11.4
NTBC-4-OH, <i>eq</i>	17.2	16.1	17.7	16.9
NTBC-5-OH, <i>ax</i>	10.7	6.5	16.6	14.3
NTBC-5-OH, <i>eq</i>	7.3	7.3	16.4	15.6
NTBC-6-OH, <i>eq</i>	0	0.8	9.6	10.7

<sup>a</sup> For NTBC and its metabolites the reference energies are -1268.8867 a.u. and -1344.0593 a.u., respectively.

Table 2. The experimental values of spin-spin coupling constants [Hz] for NTBC-6-OH and the values calculated for its selected conformers.

Coupling constant	Experimental	<i>eq-endo-A</i>	<i>eq-endo-B</i>	<i>eq-exo-A</i>
$^2J_{H4,H4a}$	-19.4	-19.7	-19.8	-19.2
$^2J_{H5,H5a}$	-12.9	-13.2	-13.1	-13.2
$^3J_{H4a,H5}$	2.3	1.9	2.0	2.1
$^3J_{H4,H5}$	5.4	5.4	5.3	5.4
$^3J_{H4a,H5a}$	4.9	4.5	4.4	4.2
$^3J_{H4,H5a}$	13.0	11.4	11.4	12.1
$^3J_{H5,H6}$	5.5	5.5	5.5	5.6
$^3J_{H5a,H6}$	13.1	11.3	11.3	11.2
$^3J_{H6,OH}$	1.7	0.8	0.2	1.1
$^4J_{H4,H6}$	-	0.3	0.2	0.0
$^4J_{H4a,H6}$	-	-1.0	-0.5	-0.6

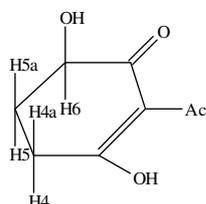


Table 3. The experimental values of spin-spin coupling constants [Hz] for NTBC-5-OH and the values calculated for its selected conformers.

Coupling constant	<i>ax</i> -B	Experimental	<i>eq</i> -B
$^2J_{H4,H4a}$	-19.1	-18.3	-18.2
$^2J_{H6,H6a}$	-17.8	-16.7	-16.7
$^3J_{H4,H5}$	2.6	5.7	9.5
$^3J_{H4a,H5}$	3.0	4.2	4.4
$^3J_{H5,H6a}$	3.4	3.5	4.2
$^3J_{H5,H6}$	2.9	6.7	10.8
$^4J_{H4,H6}$	2.0	1.5	-0.1
$^4J_{H4a,H6a}$	-0.1	0.7	1.7
$^4J_{H4,H6a}$	-0.3	0	~-0.6
$^4J_{H4a,H6}$	-0.3	~0	-0.6

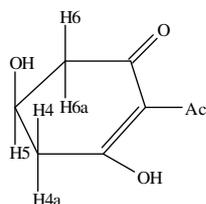


Table 4. Results of the analysis of  $^{13}\text{C}$  NMR chemical shift data on the basis of eq. 2.

Compound	Parameter	All data points	Aliphatic carbon atoms
NTBC	<i>p</i> <sub>endo</sub>	0.84±0.12	0.93±0.05
	<i>p</i> <sub>exo</sub>	0.16±0.12	0.07 ± 0.05
NTBC-5-OH	<i>p</i> <sub>ax-endo</sub>	0.51±0.71	0.36±0.36
	<i>p</i> <sub>eq-endo</sub>	0.38±0.76	0.64 ± 0.39
	<i>p</i> <sub>exo</sub>	0.11±0.12	0.01 ± 0.06
NTBC-6-OH	<i>p</i> <sub>endo</sub>	0.88±0.13	0.97±0.06
	<i>p</i> <sub>exo</sub>	0.12±0.13	0.03 ± 0.06
Total data set	a	-0.966± 0.006	-0.966±0.003
	b [ppm]	186.0±0.8	186.6±0.4
	RMSD [ppm]	1.87	0.75

## Figure captions

Fig.1. Chemical formulas of NTBC and its metabolites.

Fig. 2. Fragment of  $^{19}\text{F}$  NMR spectrum of urine sample (5% of  $\text{D}_2\text{O}$  was added for lock purpose): a. original, b. after NTBC addition, c. after NTBC and NTBC-6-OH addition.

Fig. 3. Topomeric structures resulting from rotation about C2-C7 bond.

Fig. 4. *Endo-exo* tautomerisation.

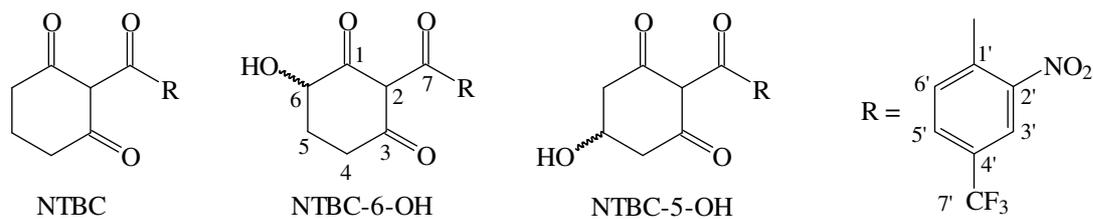
Fig. 5. Definition of conformer types A and B.

Figure 6. Reproduction of the experimental  $^1\text{H}$ - $^1\text{H}$  spin-spin coupling constants for NTBC-5-OH, using Equation 1 and theoretical values of the appropriate coupling constants calculated for axial and equatorial conformers of the *endo*-enol of this metabolite ( $p_{eq} = 0.43 \pm 0.03$ ,  $p_{ax} = 0.57 \pm 0.03$ ,  $a = 0.988$ ,  $b = -0.3$  Hz, RMSD = 0.23 Hz).

Figure 7. Reproduction of the experimental  $^{13}\text{C}$  NMR chemical shifts for NTBC, NTBC-5-OH and NTBC-6-OH using Equation 2 and theoretical values of the appropriate magnetic shielding constants calculated for various conformers of the *endo*- and *exo*-enols of these compounds ( $a = -0.966$ ,  $b = 186.0$  ppm, RMSD = 1.87 ppm, population parameters given in Table 4).

Figure 8. Comparison of  $\delta_{\text{exp}}/\sigma_{\text{calc}}$  correlations for NTBC-6-OH assuming 2-acyl-3,4-dihydroxycyclohex-2-enone and 2-acyl-3,6-dihydroxycyclohex-2-enone structures (the deviations [ppm] from the regression lines are given).

Fig. 1



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Fig. 2

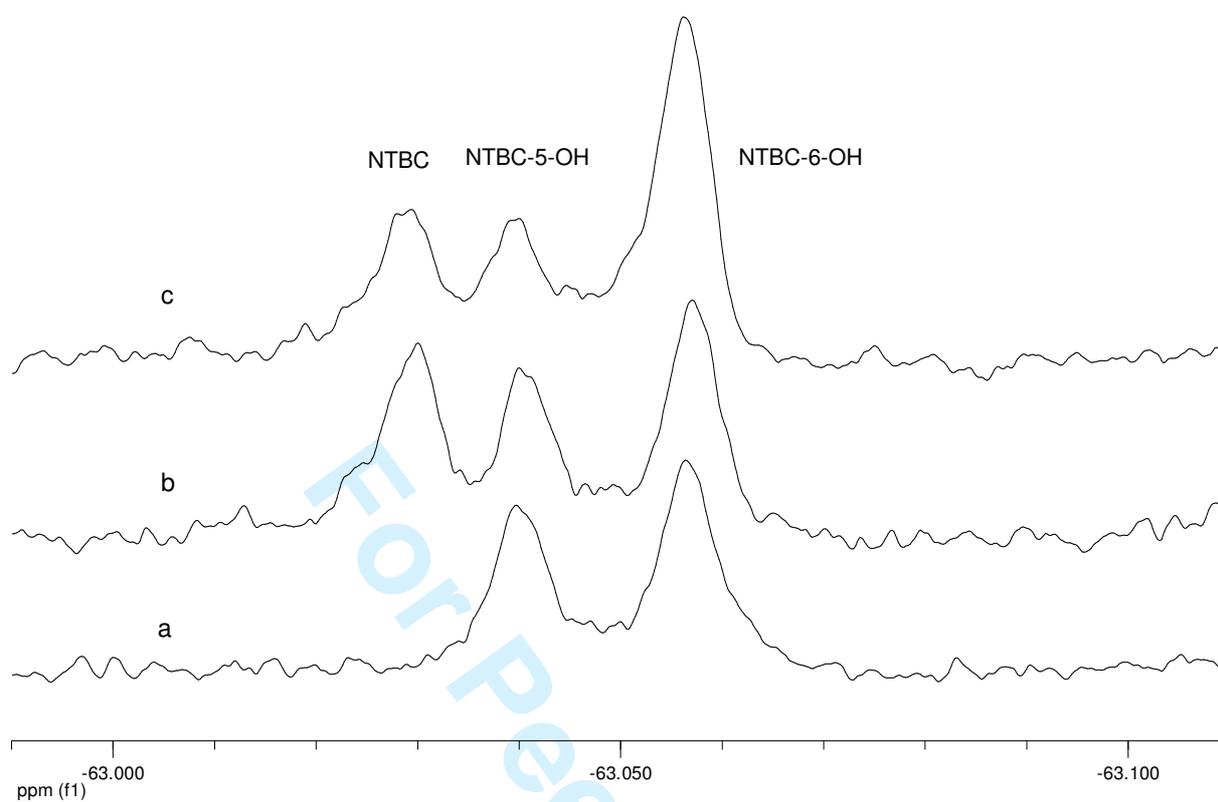


Fig. 3

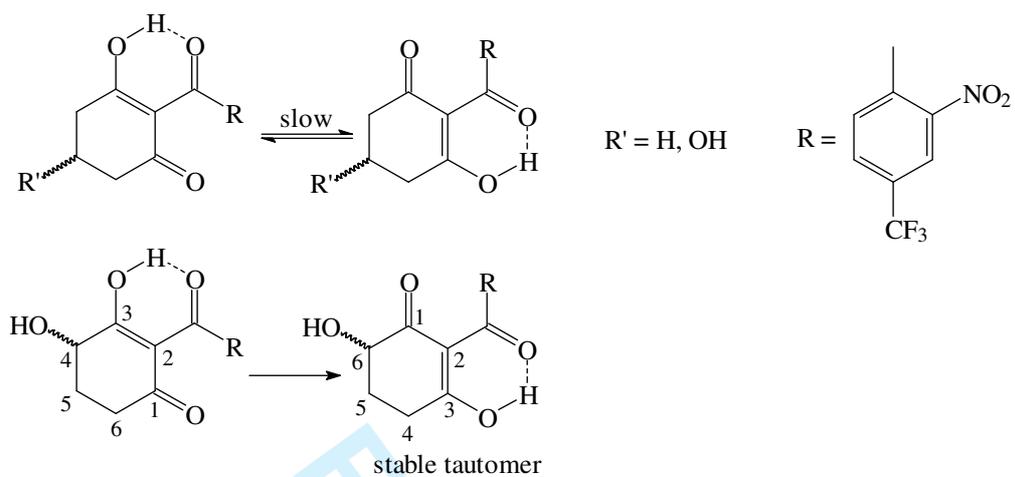


Fig. 4

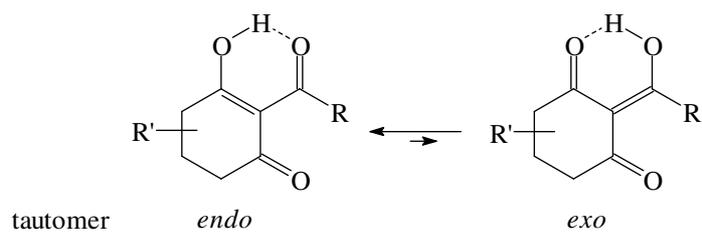


Fig. 5

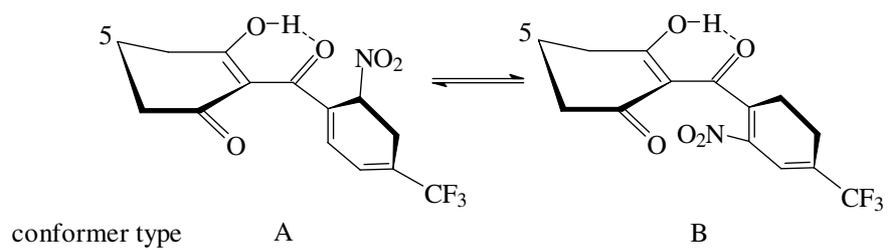


Fig. 6

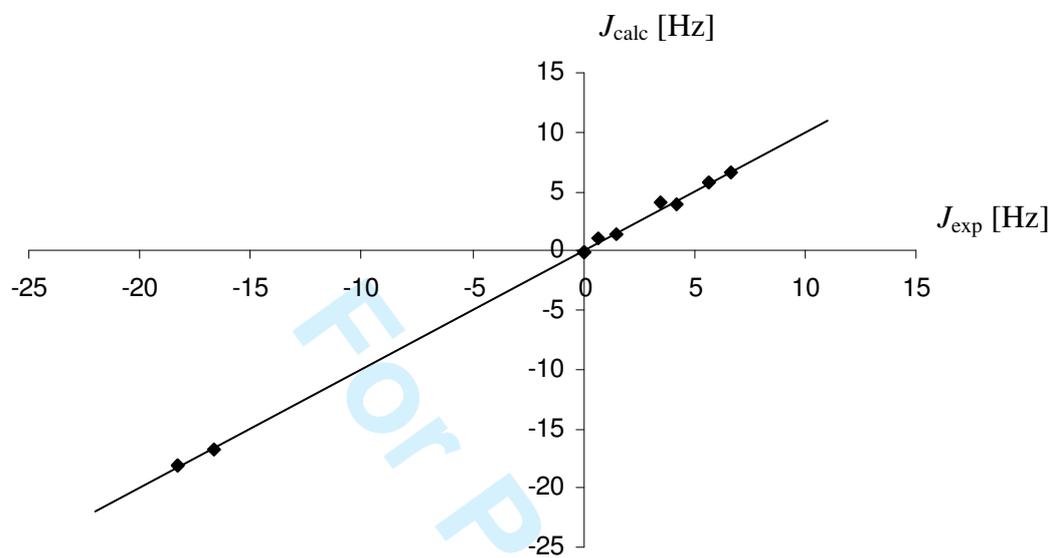


Fig. 7

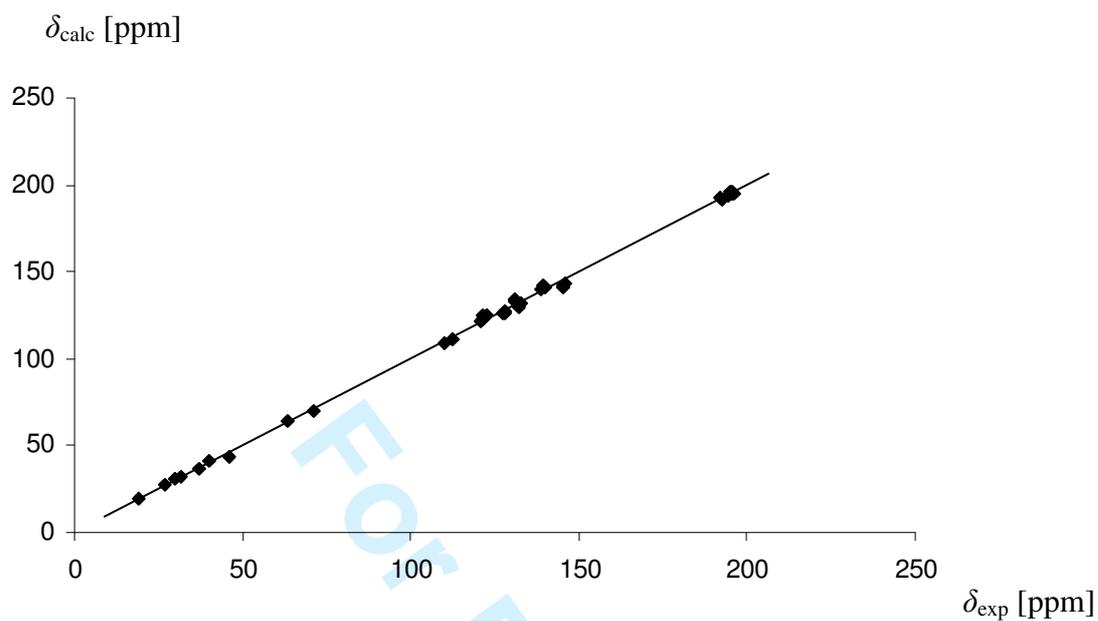
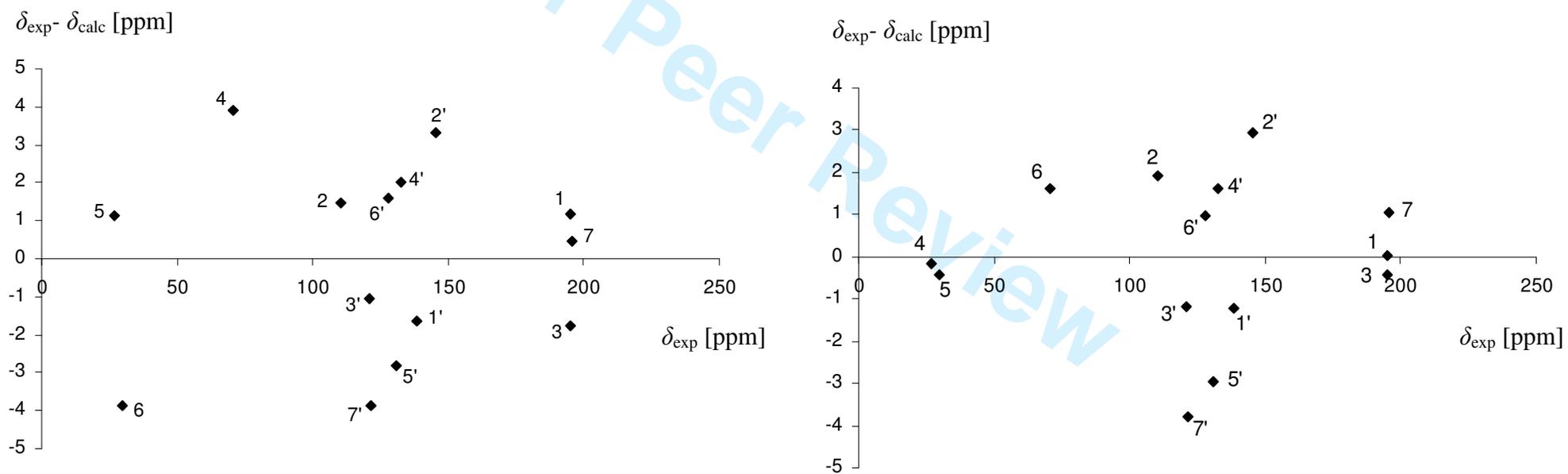


Fig. 8



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## Supplementary material

**NMR and DFT calculation Study on Structures of 2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC) and Its Two Metabolites Isolated from Urine of Patients Suffering from Tyrosinemia Type I.**

**Adam Gryff-Keller\*<sup>[a]</sup>, Przemysław Szczeciński<sup>[a]</sup> and Anna Kraska-Dziadecka<sup>[a]</sup>**

**Results of test calculations for 2-nitro-4-trifluoromethylbenzoyl chloride.**

It was noticed that in the final chemical shifts/shielding constants correlation the fit for aromatic ring carbons (C1'÷C7') was much poorer than that for C1÷C7 and that the deviations from the regression line were very similar for all three investigated compounds. This latter fact was not surprising as the aromatic parts of the investigated molecules were identical. The larger deviations observed for the aromatic ring carbons could be caused by large amplitude deformations of the equilibrium structures, due to the librations about C7–C1' bond. Such movements could be a result of ground state vibrations and intermolecular collisions. We performed test calculations for a model compound, 4-trifluoromethyl-2-nitrobenzoyl chloride, starting from its equilibrium geometry, in which NO<sub>2</sub> group was coplanar with and COCl group perpendicular to the aromatic ring. For this geometry the deviations from the  $\delta/\sigma$  regression line for aromatic carbons were almost identical to those for NTBC derivatives. On the other hand, the deviations could be almost completely compensated when it was assumed that in reality we deal with 85 % of conformer with NO<sub>2</sub> group forming a 30 deg angle with the aromatic ring plane and 15 % of conformer with perpendicular NO<sub>2</sub> group (Fig S1). The assumed (during molecular geometry optimization) twist of the NO<sub>2</sub> group obviously resulted in a due twist of the COCl group.

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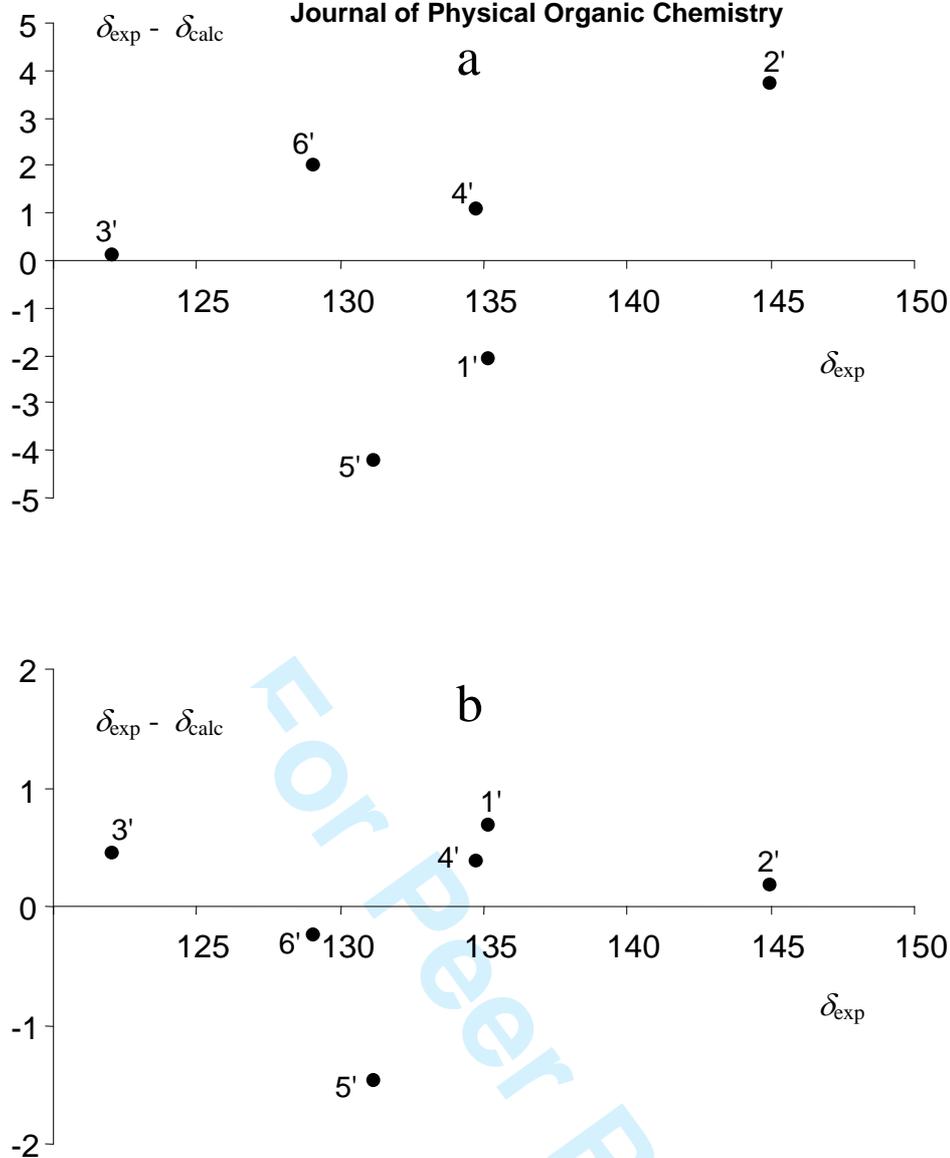


Figure S1. Comparison of  $\delta_{\text{exp}}$  vs.  $\sigma_{\text{calc}}$  correlations for the aromatic ring carbons of 4-trifluoromethyl-2-nitrobenzoyl chloride assuming equilibrium (a, RMSD = 2.62 ppm) and twisted (b, RMSD = 0.72 ppm) molecular geometries (the deviations [ppm] from the regression lines are given).

Table S1. The experimental  $^{13}\text{C}$  NMR chemical shifts and magnetic shielding constants calculated for A and B conformers of *endo*- and *exo*-enols of NTBC.

Carbon	$\delta_{\text{exp}}$	$\sigma_{\text{endo,A}}$	$\sigma_{\text{endo,B}}$	$\sigma_{\text{exo,A}}$	$\sigma_{\text{exo,B}}$
7	196.27	-17.66	-16.30	-7.62	-8.33
3	195.76	-15.56	-15.10	-27.14	-27.13
1	194.12	-15.05	-12.85	-16.02	-15.95
2'	145.49	38.96	40.83	37.80	38.68
1'	139.66	38.05	39.75	44.11	43.83
4'	132.02	51.64	52.56	50.09	50.31
5'	130.78	47.67	48.55	49.11	47.37
6'	127.66	55.37	56.62	55.16	54.05
7'	122.57	55.99	57.80	56.17	56.18
3'	121.11	59.86	60.52	57.08	58.66

2	112.65	70.08	71.72	70.94	71.01
6	37.26	147.86	148.96	146.72	147.35
4	31.62	154.04	154.61	148.04	147.62
5	19.05	165.25	166.51	165.64	165.67

Table S2. The experimental  $^{13}\text{C}$  NMR chemical shifts and magnetic shielding constants calculated for equatorial and axial A and B conformers of *endo*- and *exo*-enols of NTBC-5-OH.

Carbon	$\delta_{exp}$	<i>Endo</i> -tautomers				<i>Exo</i> -tautomers			
		<i>S,ax,A</i>	<i>S,ax,B</i>	<i>S,eq,A</i>	<i>S,eq,B</i>	<i>S,ax,A</i>	<i>S,ax,B</i>	<i>S,eq,A</i>	<i>S,eq,B</i>
7	195.80	-17.32	-17.68	-17.37	-17.38	-6.55	-6.22	-8.09	-8.13
1	192.50	-12.59	-12.45	-12.54	-12.76	-13.67	-14.01	-13.37	-13.45
3	191.72	-11.72	-11.74	-12.62	-12.47	-24.88	-25.03	-23.72	-23.69
2'	145.60	39.43	38.81	39.97	38.45	39.57	37.02	39.46	38.29
1'	139.35	38.23	38.78	38.19	38.54	43.78	44.22	44.27	44.14
4'	132.33	51.63	50.84	51.26	51.44	50.45	50.43	49.83	50.27
5'	130.86	47.29	47.12	47.33	48.99	47.49	49.62	47.05	49.27
6'	127.89	55.18	54.97	55.83	56.03	53.15	55.42	53.15	55.14
7'	122.58	55.98	56.12	56.10	56.09	56.13	56.23	56.25	56.24
3'	121.20	59.21	59.38	59.08	58.45	58.70	57.59	58.35	56.93
2	112.70	69.48	70.64	71.19	70.41	70.01	70.90	73.05	72.37
5	63.51	119.83	119.36	120.20	120.81	119.60	119.81	120.87	120.97
6	45.87	142.51	140.26	139.54	139.86	141.65	141.00	139.47	139.08
4	39.86	145.06	145.29	143.52	143.47	139.33	139.82	137.56	137.36

Table S3. The experimental  $^{13}\text{C}$  NMR chemical shifts and magnetic shielding constants calculated for equatorial A and B conformers of *endo*- and *exo*-enols of NTBC-6-OH.

Carbon	$\delta_{exp}$	$\sigma_{endo,A}$	$\sigma_{endo,B}$	$\sigma_{exo,A}$	$\sigma_{exo,B}$
7	195.86	-16.63	-17.58	-8.10	-9.04
1	194.83	-15.79	-16.09	-16.38	-16.73
3	195.05	-15.57	-15.18	-25.91	-25.88
2'	145.68	36.40	39.66	36.45	38.15
1'	138.71	40.69	39.55	46.27	45.75
4'	132.67	49.77	50.63	49.04	49.13
5'	130.93	47.63	46.36	48.33	47.85
6'	128.07	54.51	54.02	54.35	53.00
7'	121.36	56.16	56.13	56.38	56.33
3'	121.20	59.30	59.22	57.77	57.42
2	110.20	74.11	73.17	73.74	74.26
6	70.89	114.25	114.35	112.58	113.13
4	29.88	155.05	155.23	150.02	149.77
5	27.05	157.41	157.88	157.84	158.37

Table S4. The magnetic shielding constants of carbon nuclei calculated for equatorial A and B conformers of *endo*- and *exo*-enols of NTBC-4-OH.

Carbon	$\sigma_{endo,A}$	$\sigma_{endo,B}$	$\sigma_{exo,A}$	$\sigma_{exo,B}$
7	-18.53	-18.61	-7.88	-7.70
1	-13.71	-13.92	-14.99	-14.85
3	-13.98	-13.80	-25.09	-25.24
2'	39.08	38.52	38.22	38.07
1'	39.16	39.10	44.84	44.84
4'	50.95	51.00	49.45	49.65
5'	47.29	47.22	48.84	46.98
6'	55.25	54.96	54.36	54.01
7'	56.15	56.04	56.18	56.24
3'	59.69	59.65	57.00	58.74
2	72.22	73.57	73.64	73.32
4	116.80	116.52	113.21	113.20
6	150.44	149.73	148.40	149.37
5	157.75	157.61	158.22	157.93