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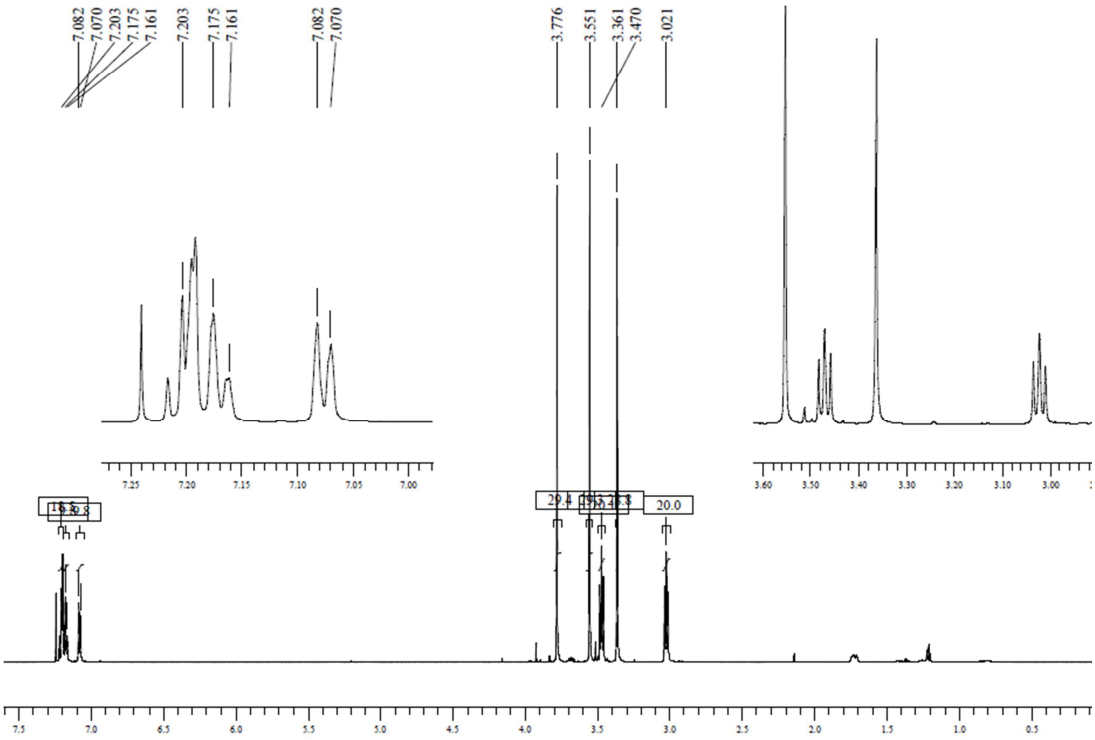
Addendum

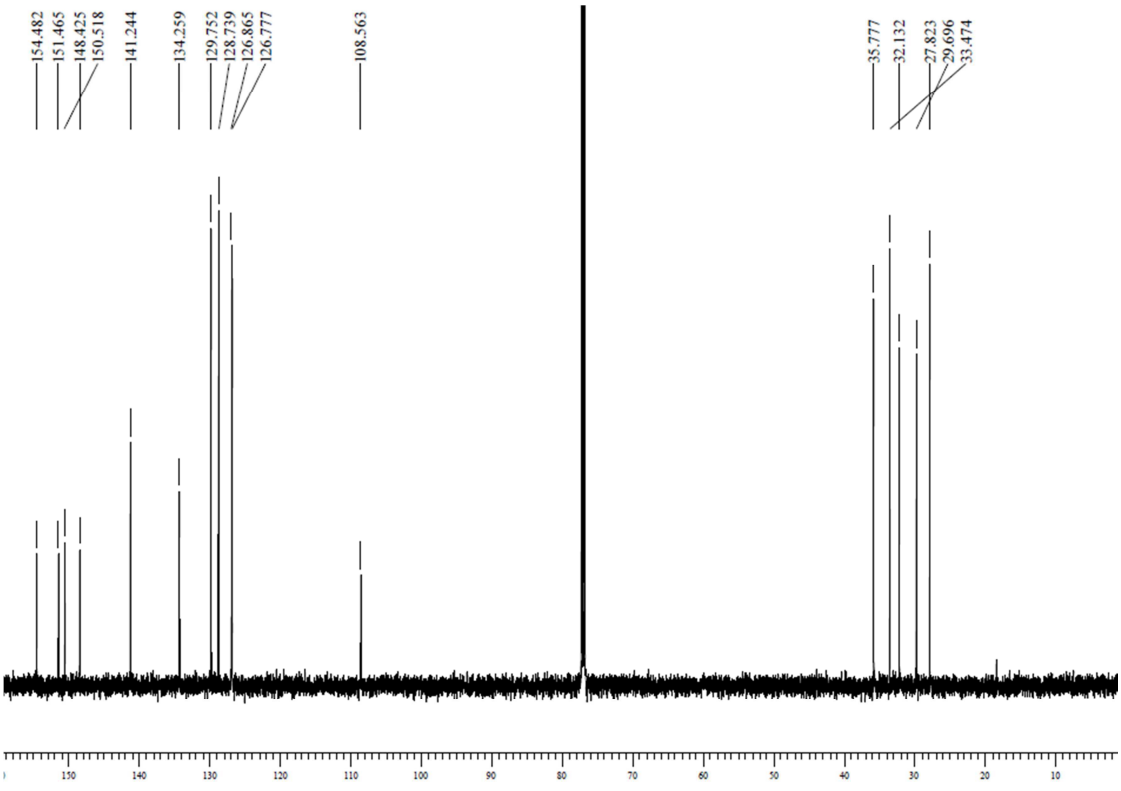
List of ^{1}H and ^{13}C NMR spectra

List of HPLC chromatograms

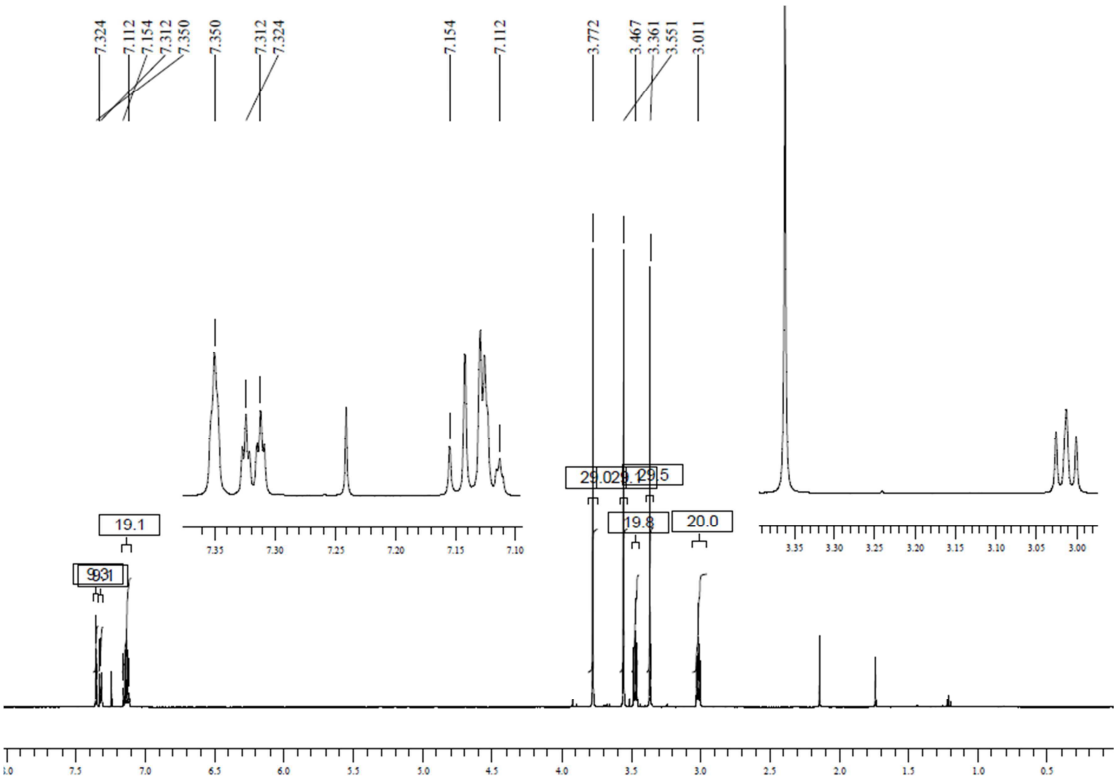
List of mass spectra

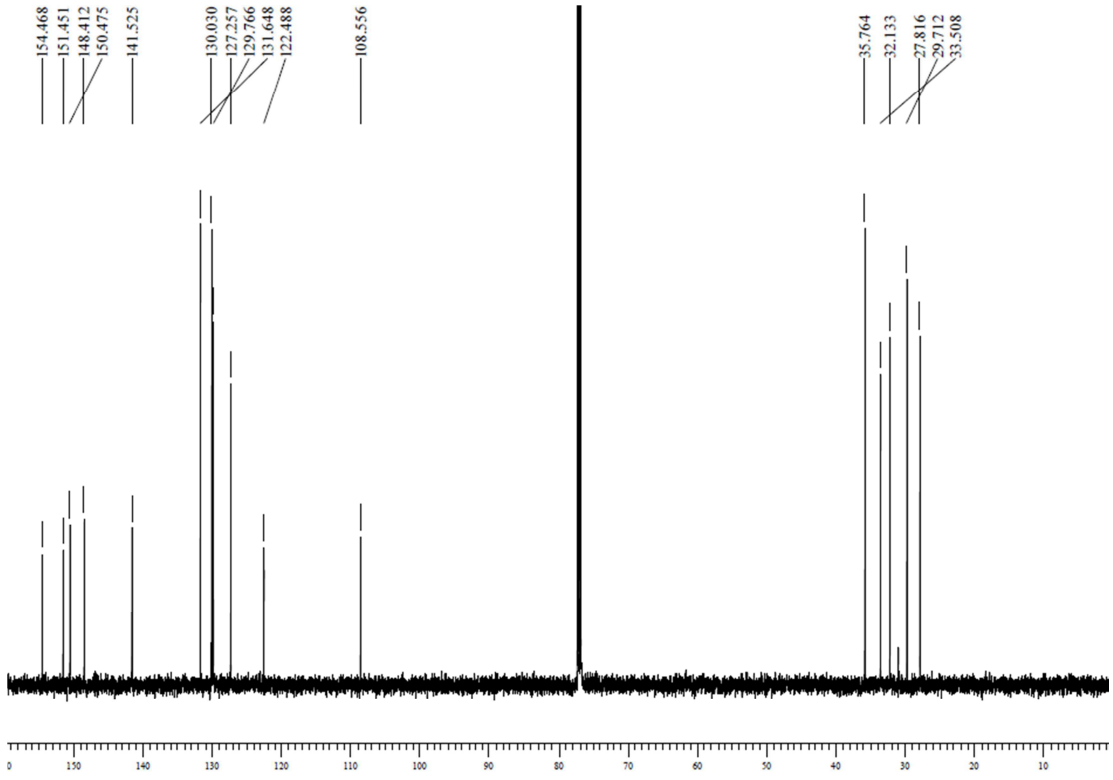
8-{[2-(3-Chlorophenyl)ethyl]sulfanyl}caffeine (3a)



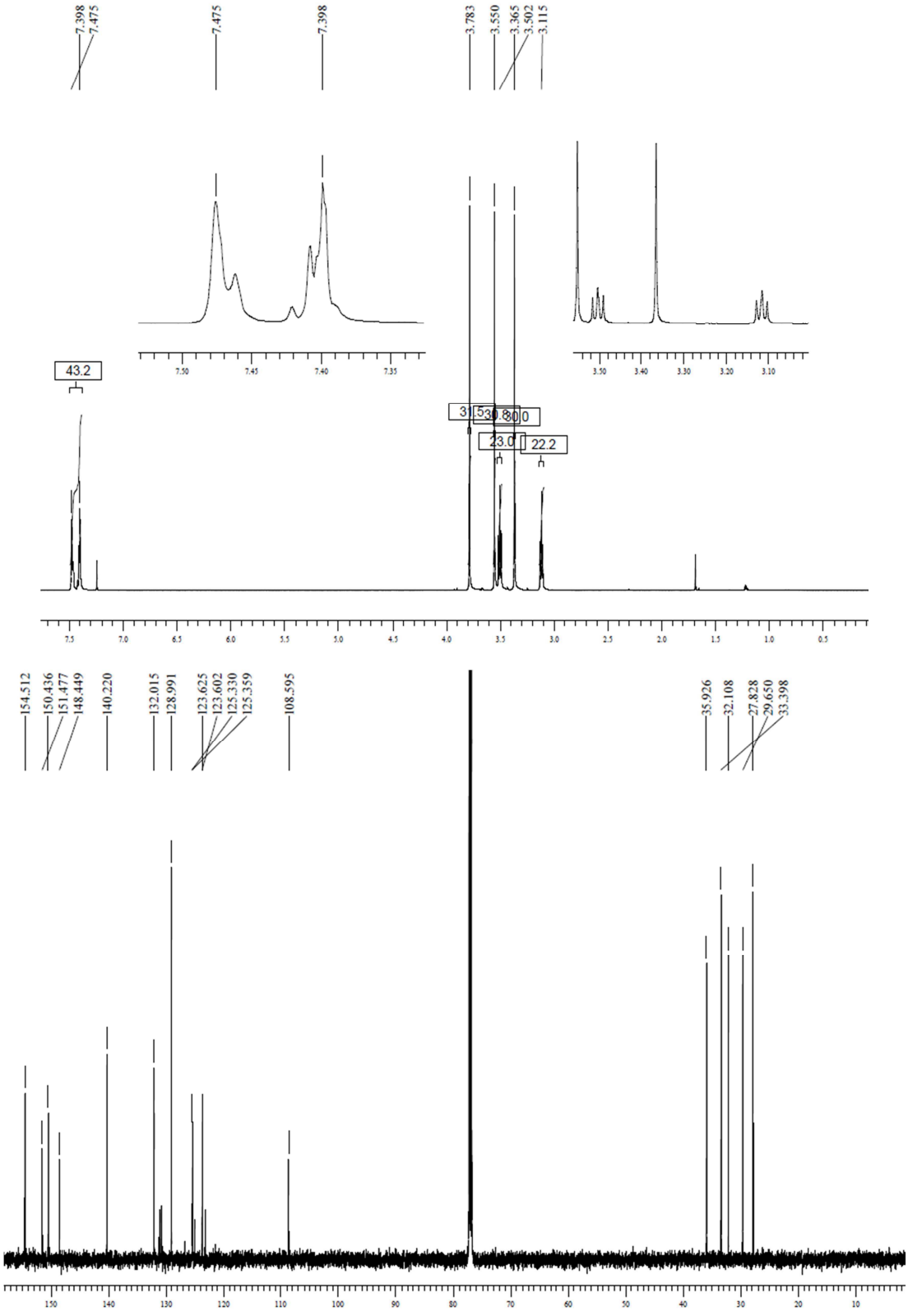


8-{[2-(3-Bromophenyl)ethyl]sulfanyl}caffeine (3b)

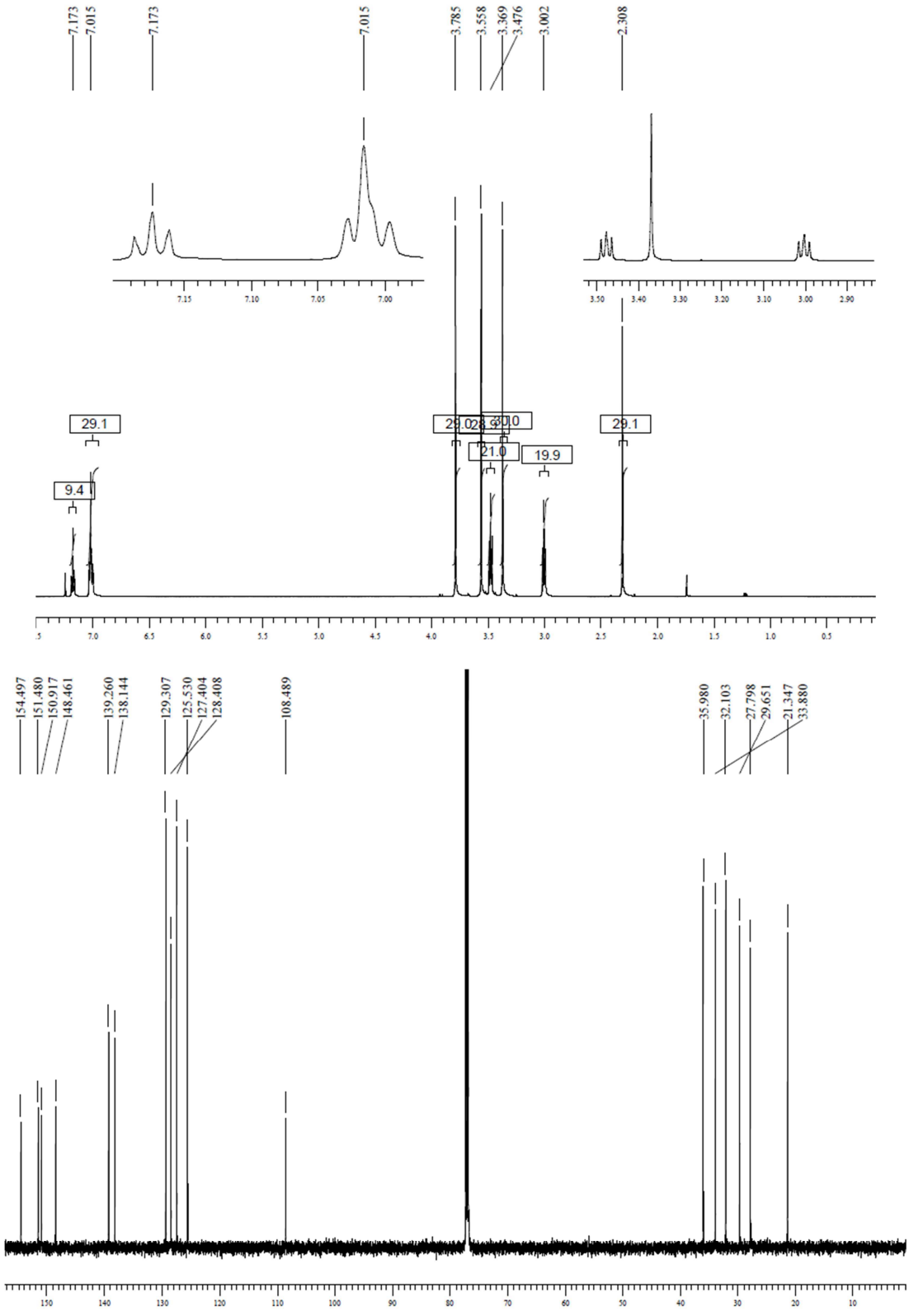




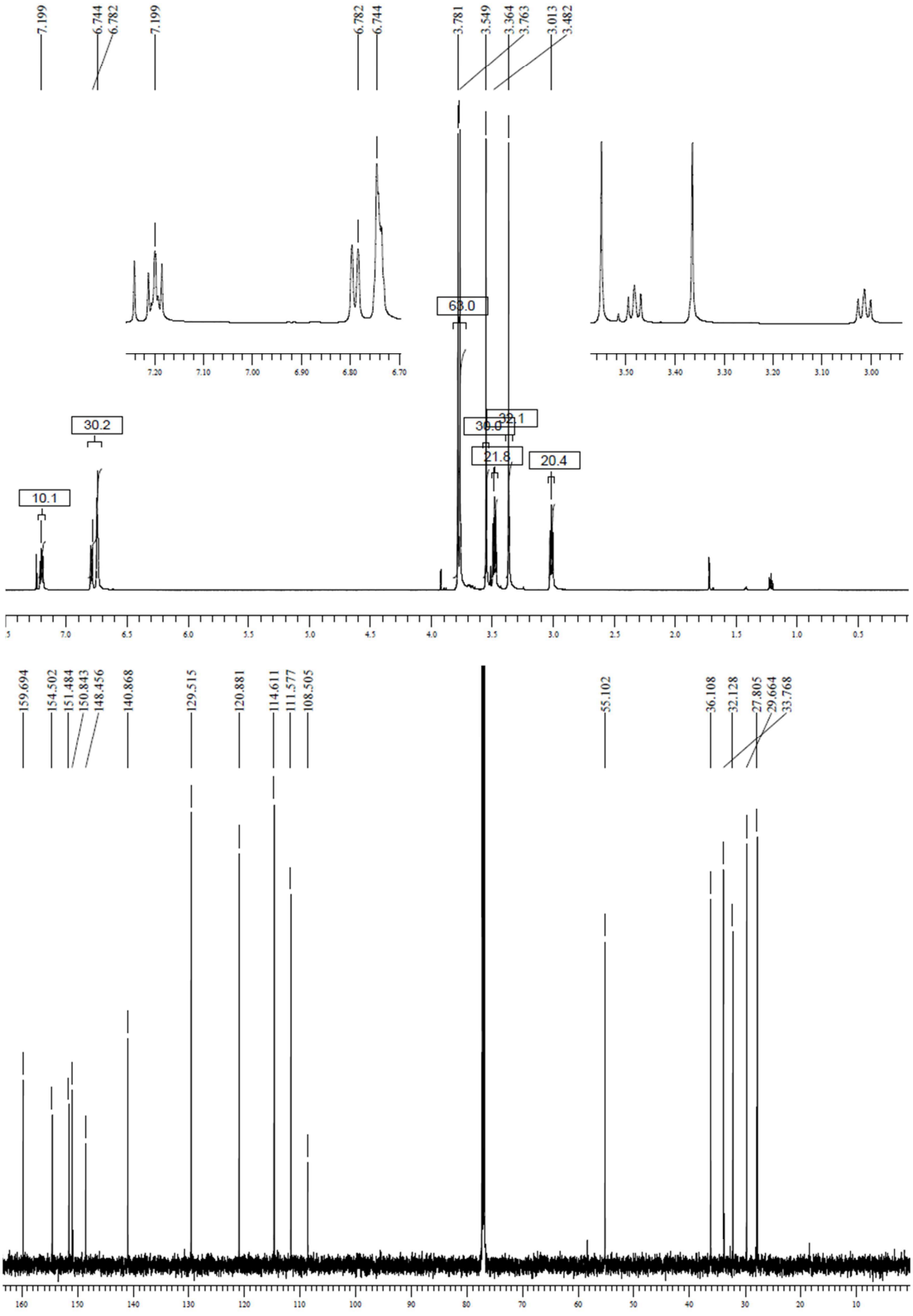
8-{[2-(3-(Trifluoromethyl)phenyl)ethyl]sulfanyl}caffeine (3c)



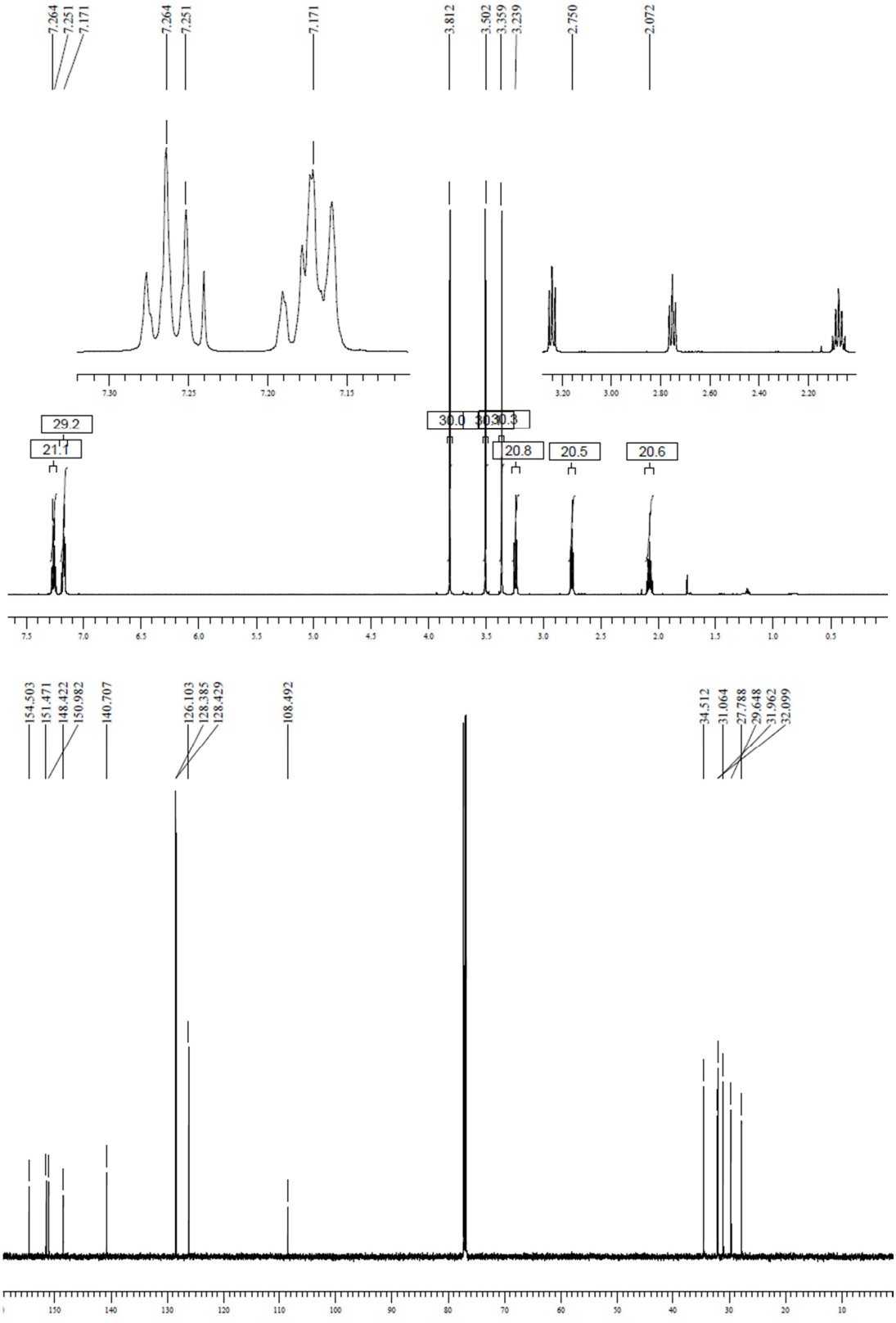
8-{[2-(3-Methylphenyl)ethyl]sulfanyl}caffeine (3d)



8-{[2-(3-Methoxyphenyl)ethyl]sulfanyl}caffeine (3e)



8-[(3-Phenylpropyl)sulfanyl]caffeine (4a)



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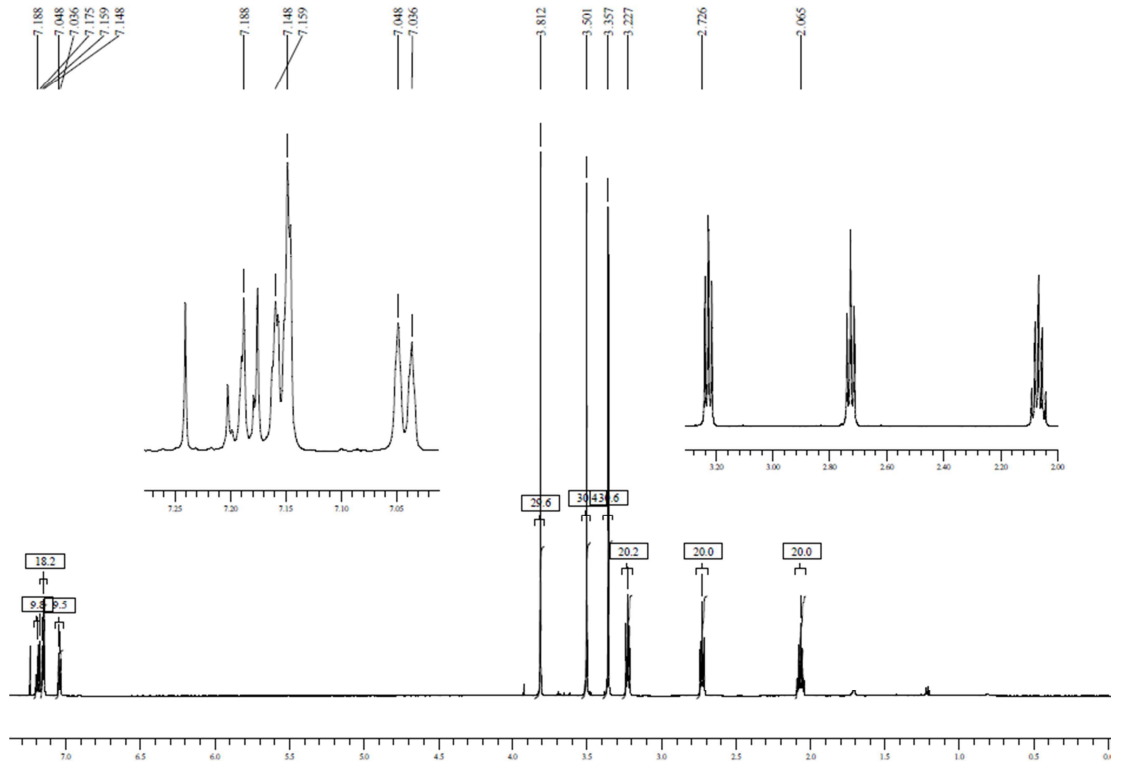

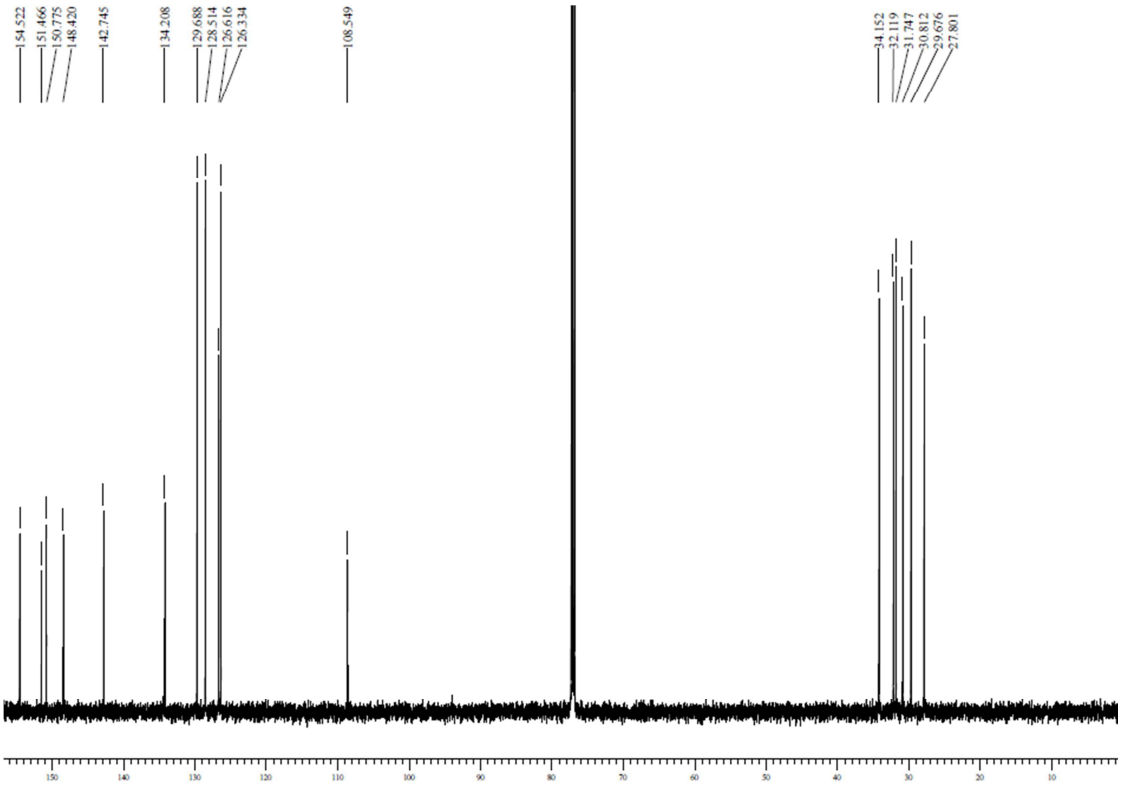

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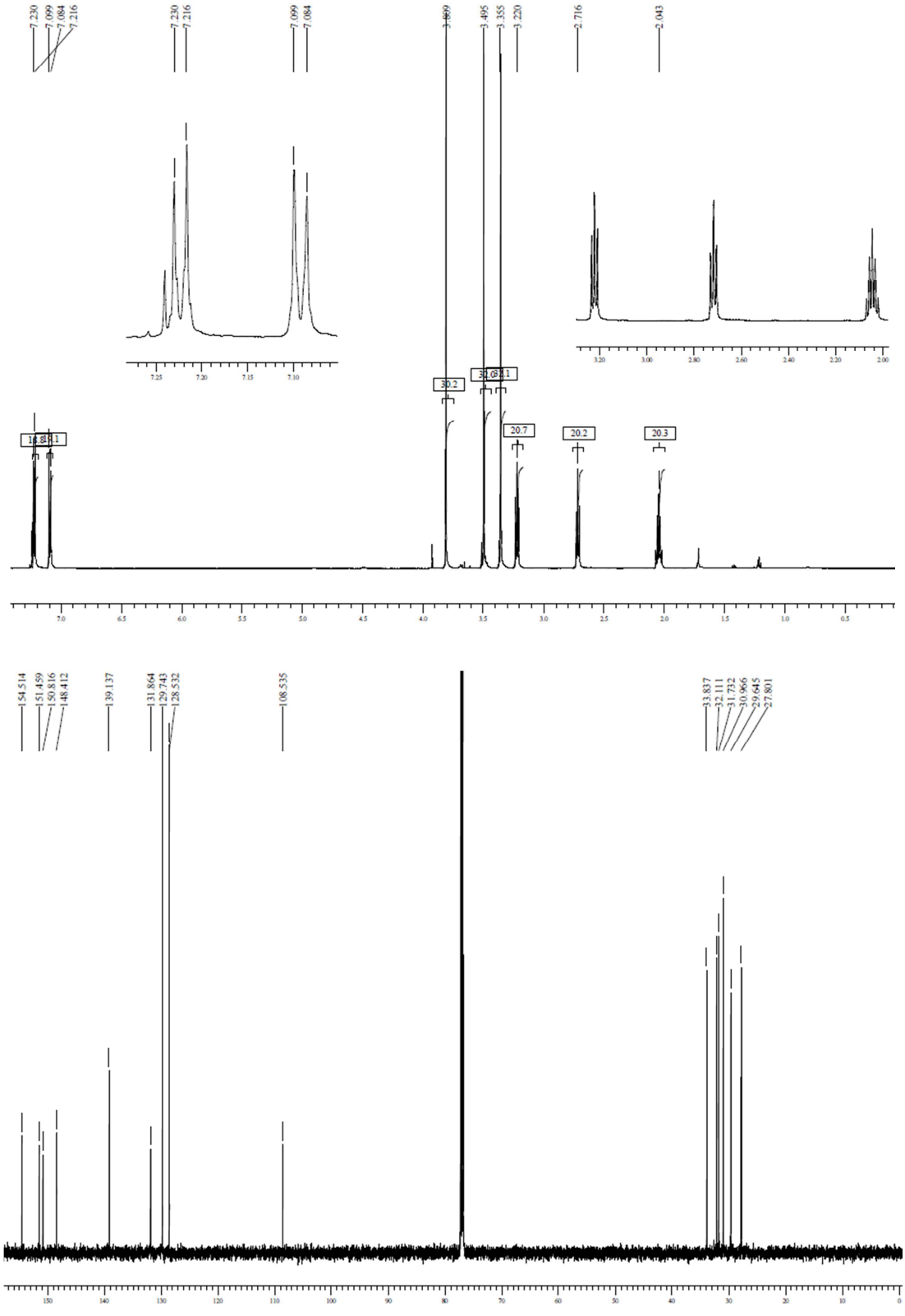

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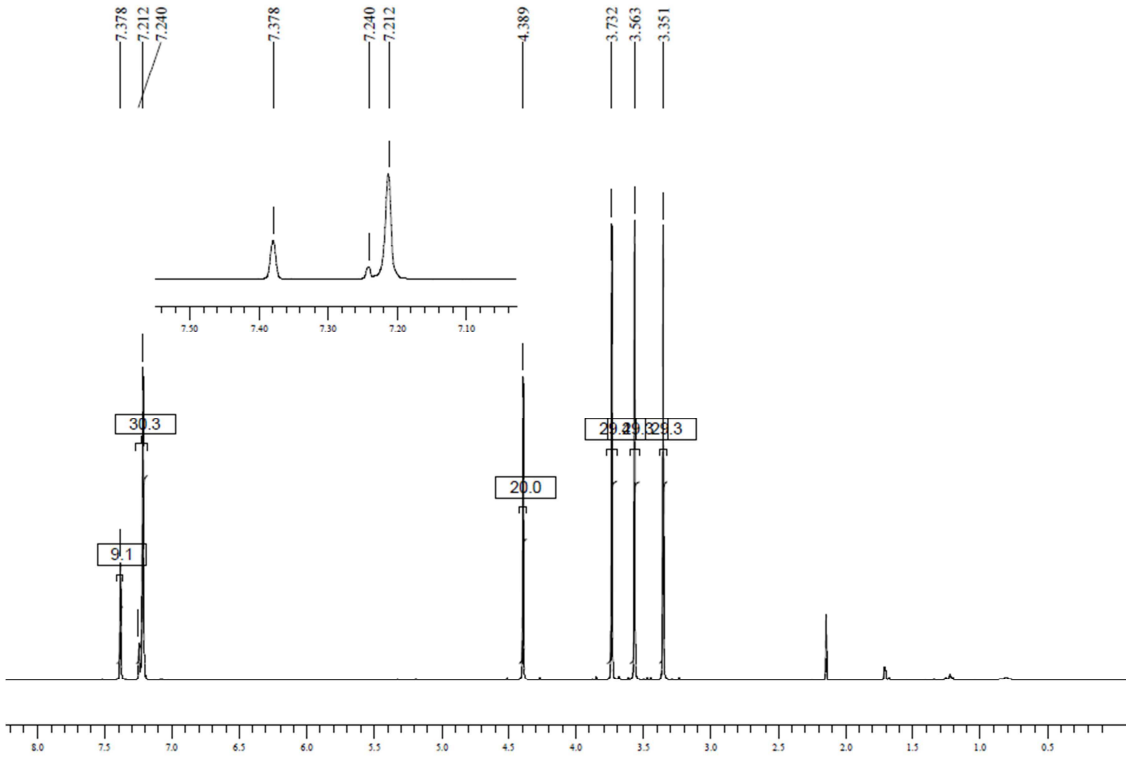

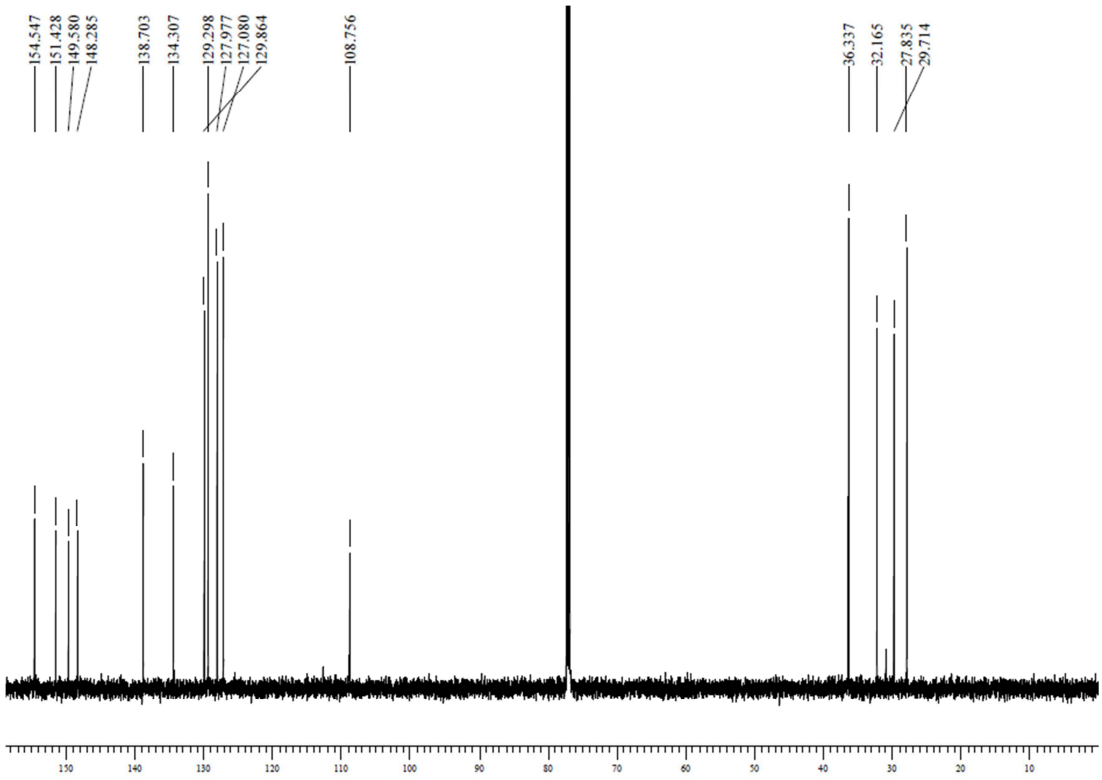

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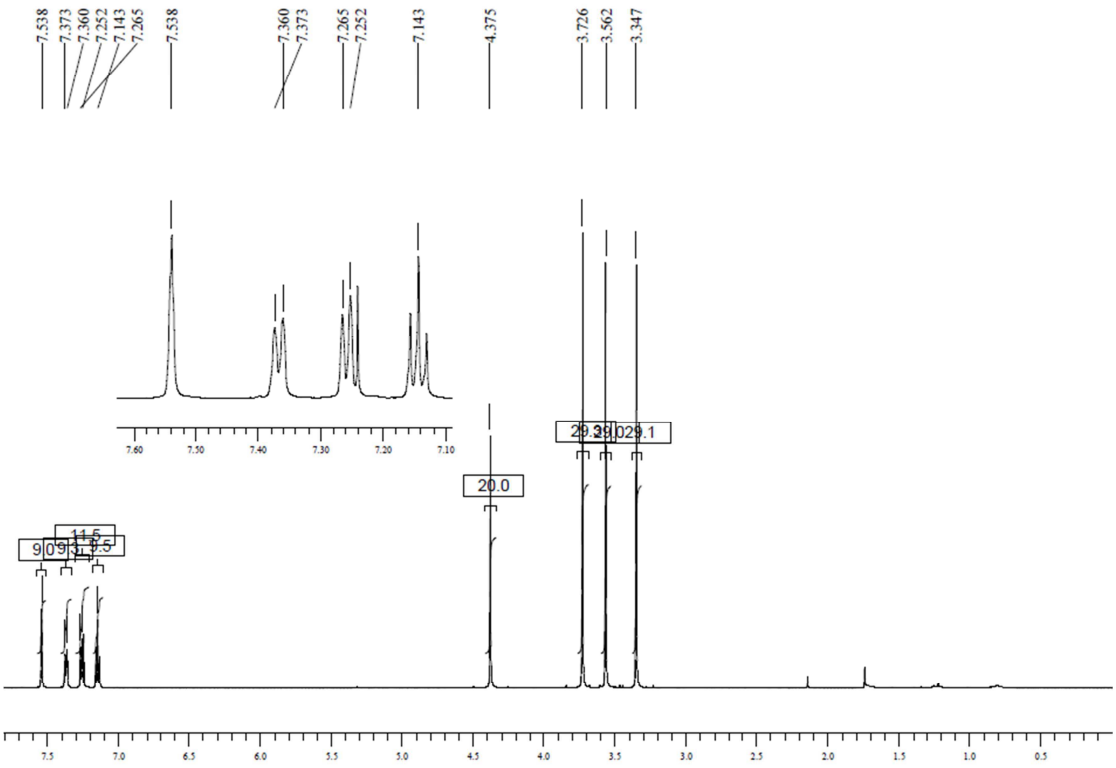

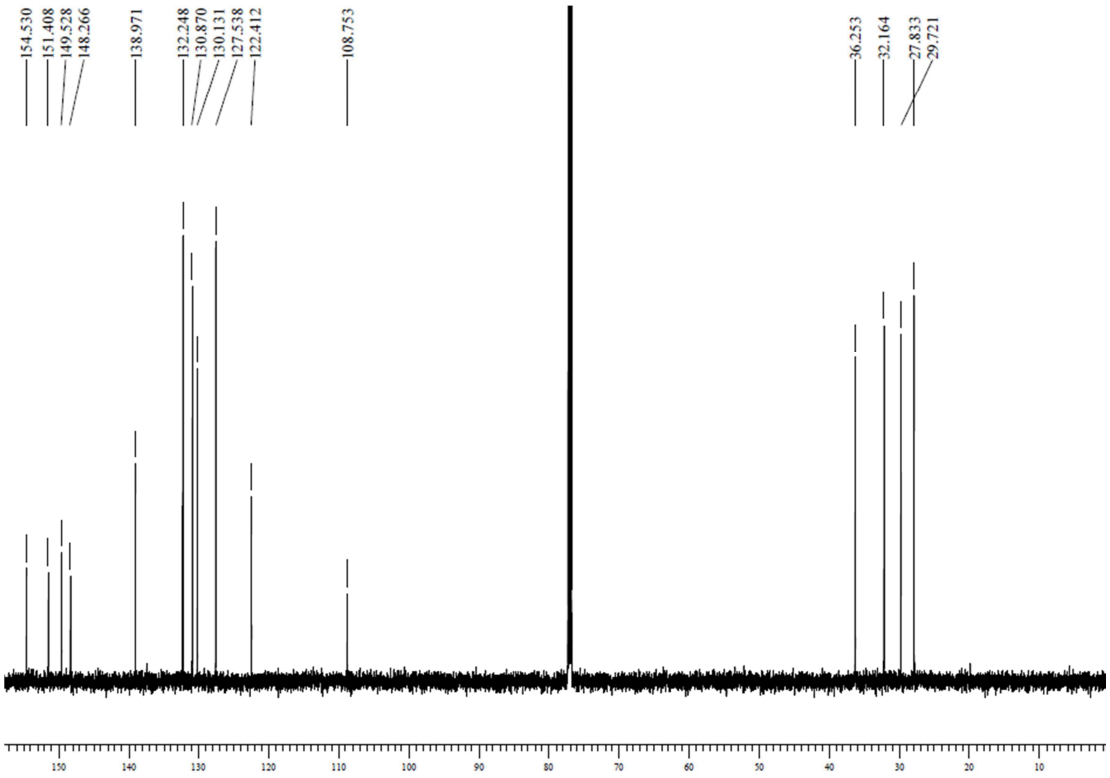

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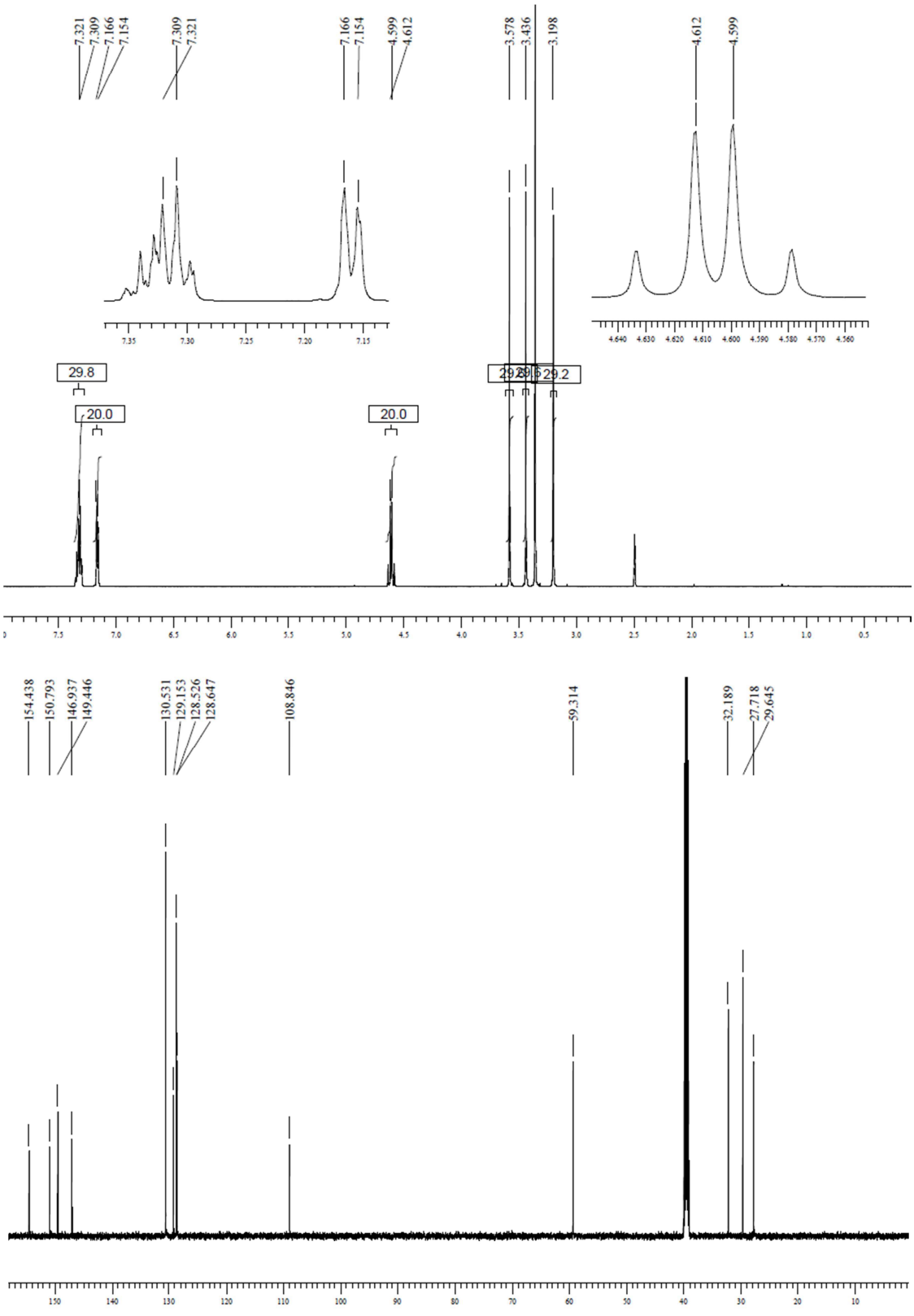

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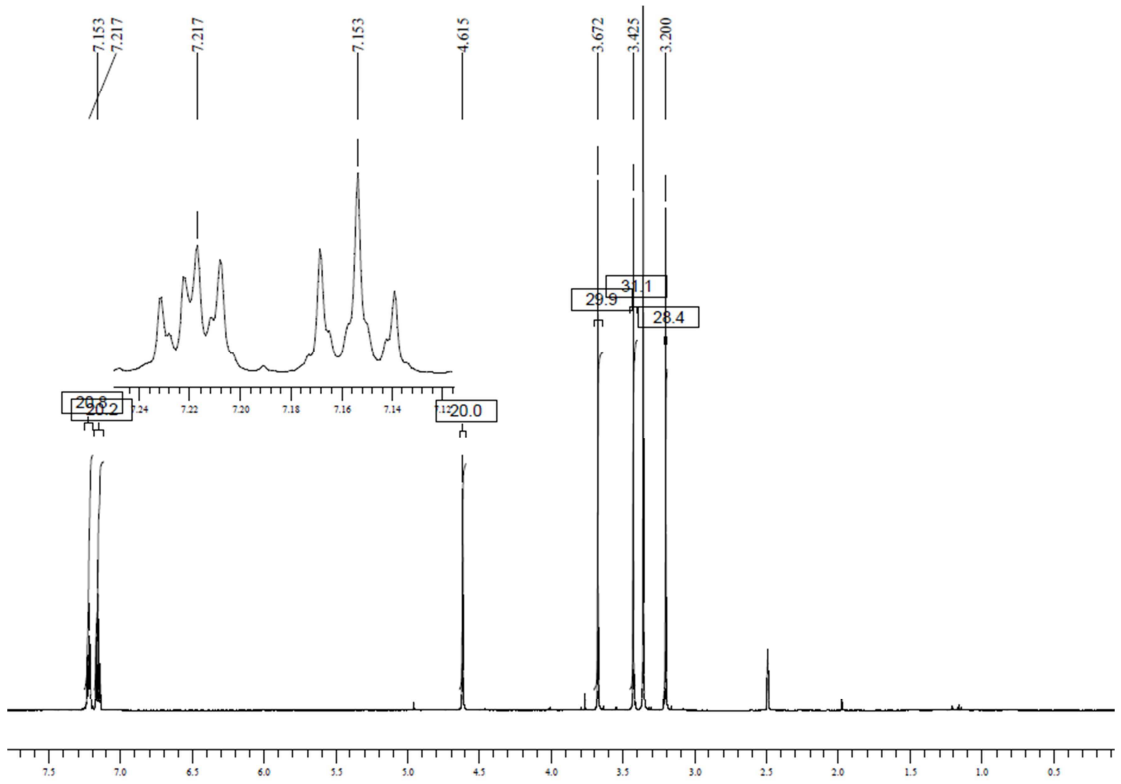

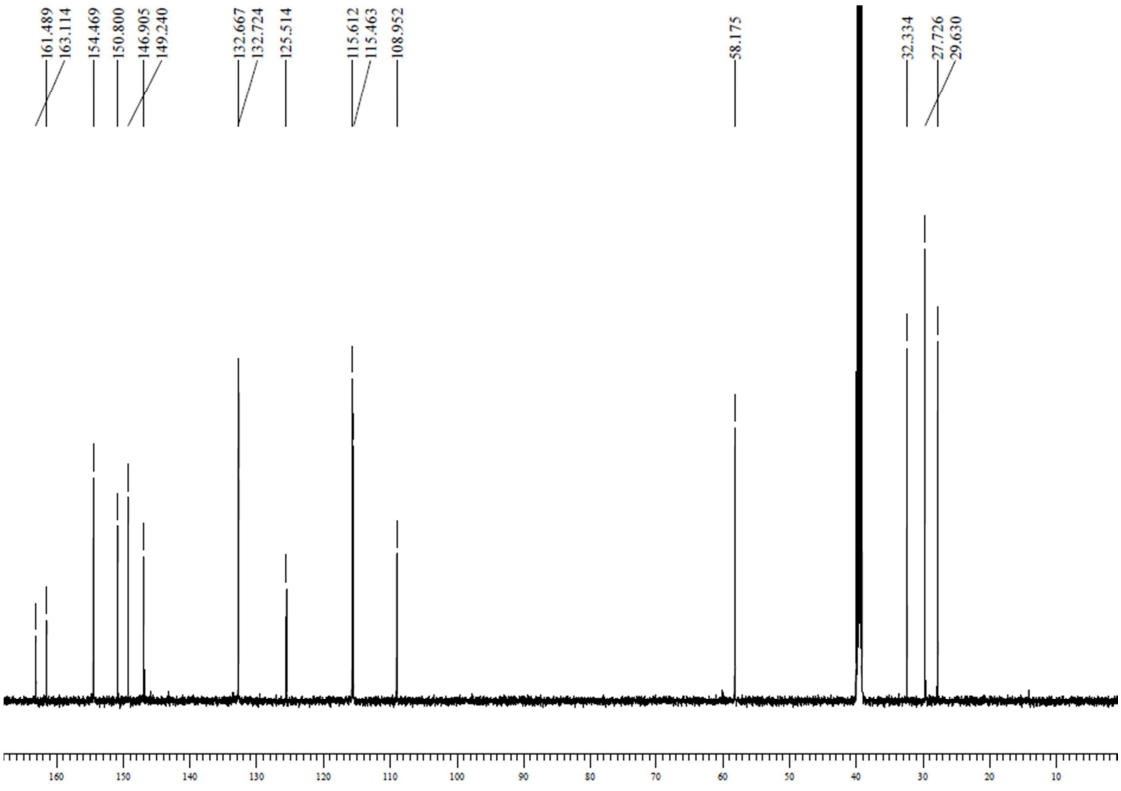

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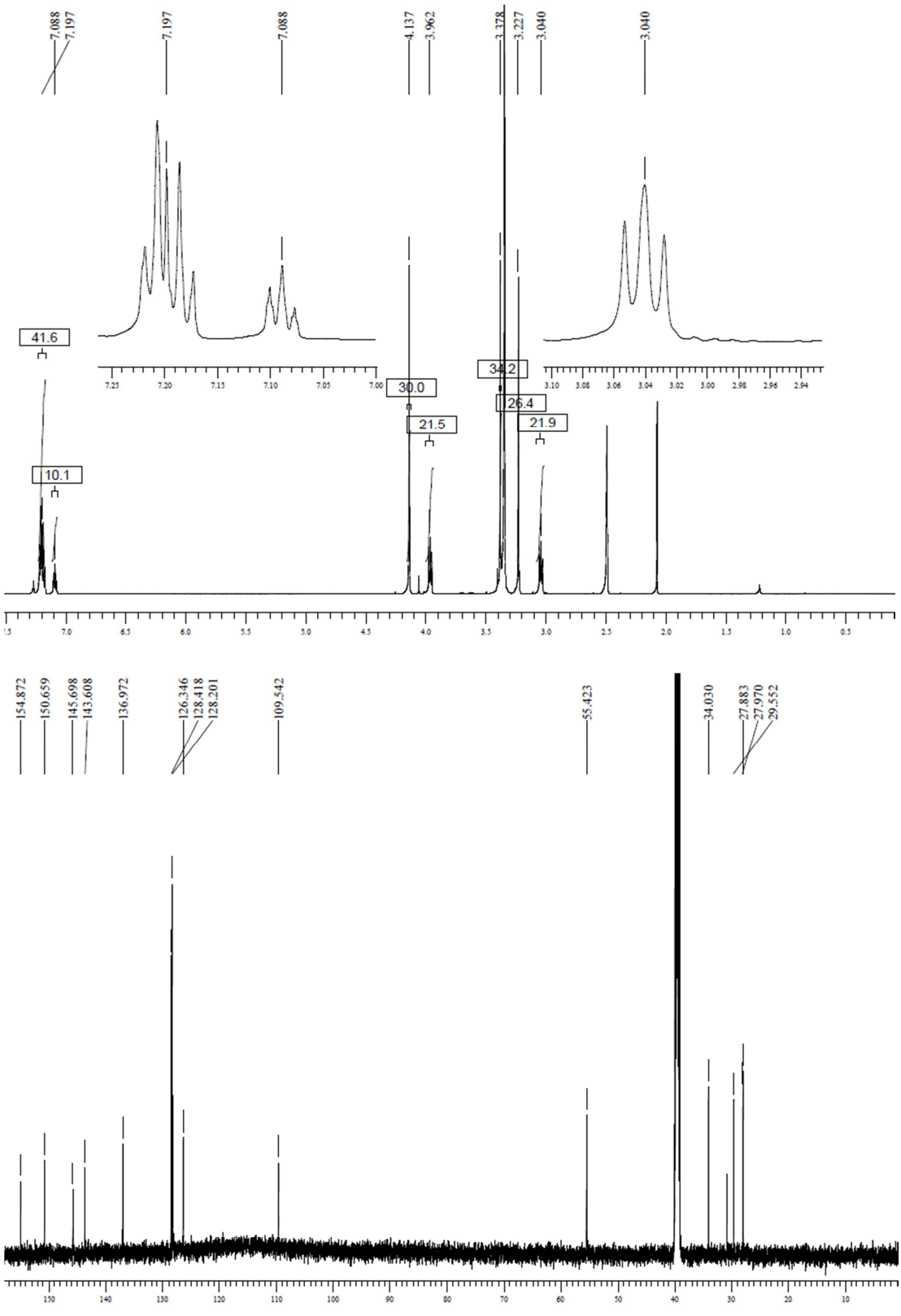

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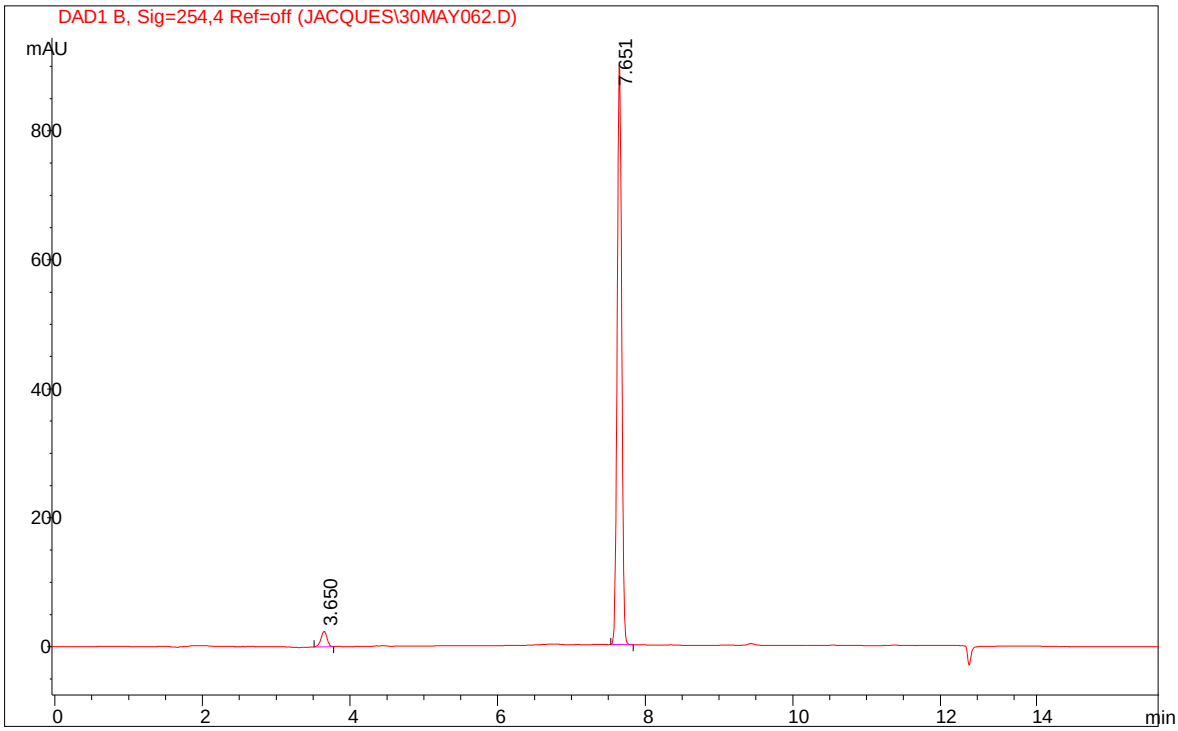

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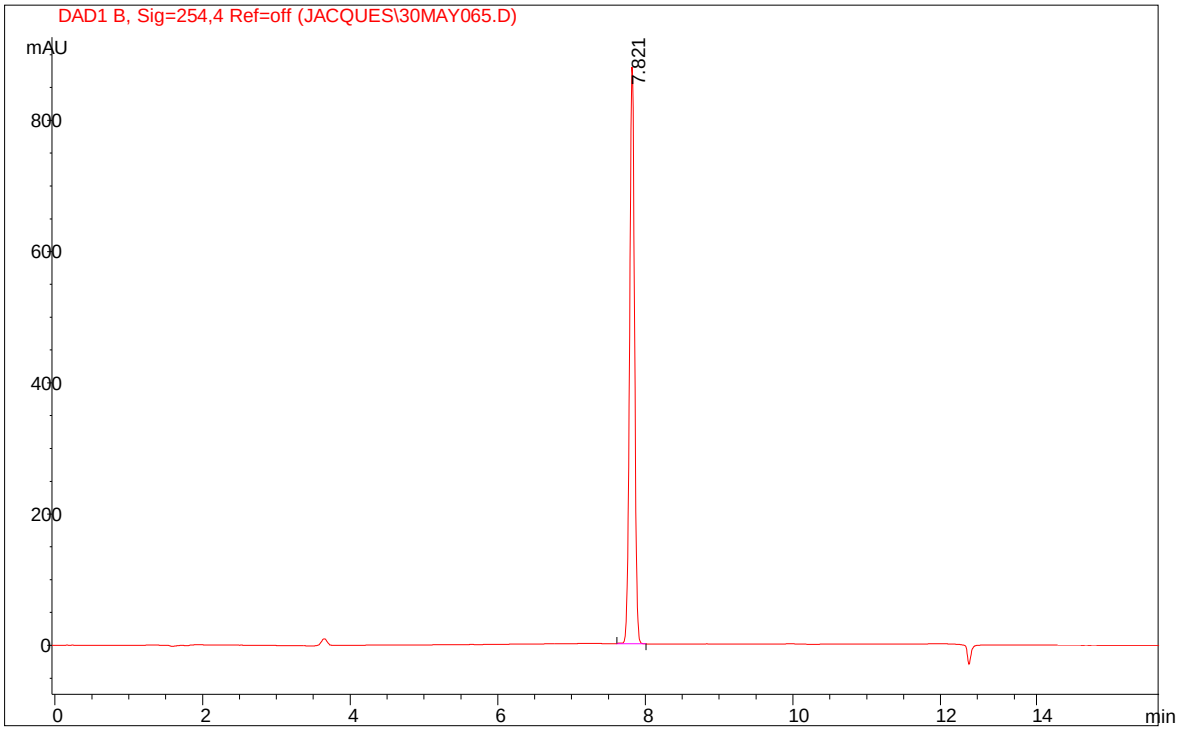

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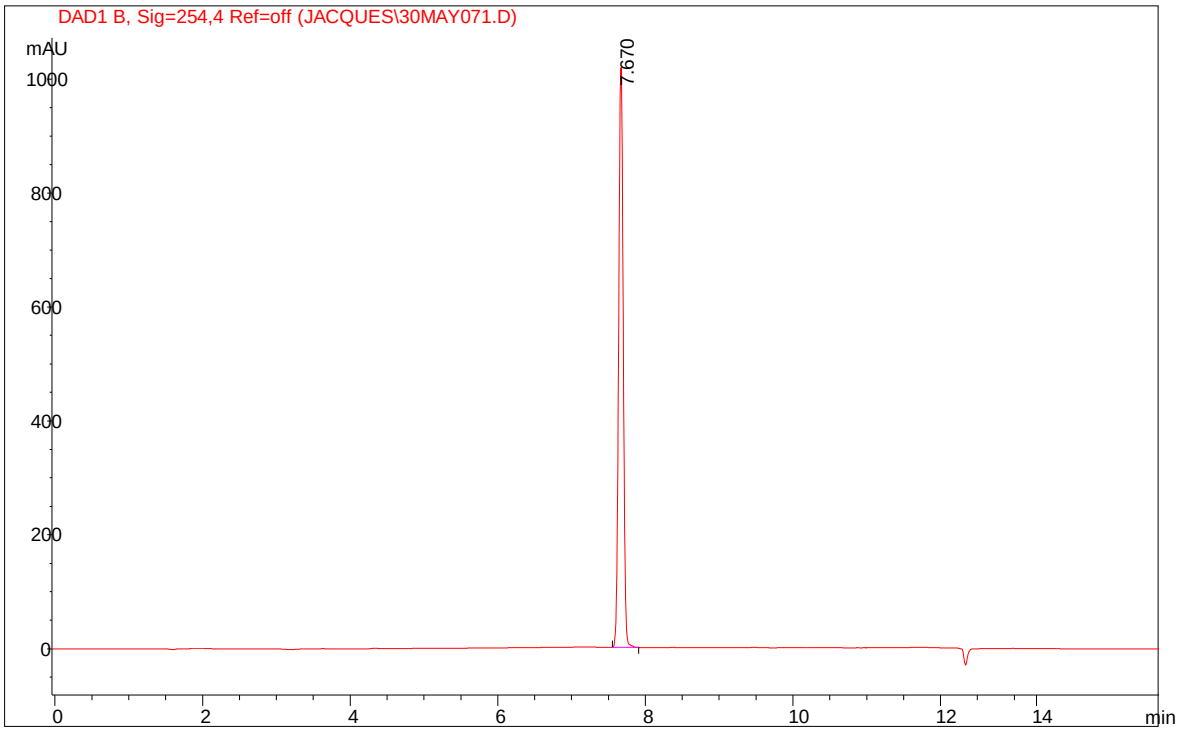

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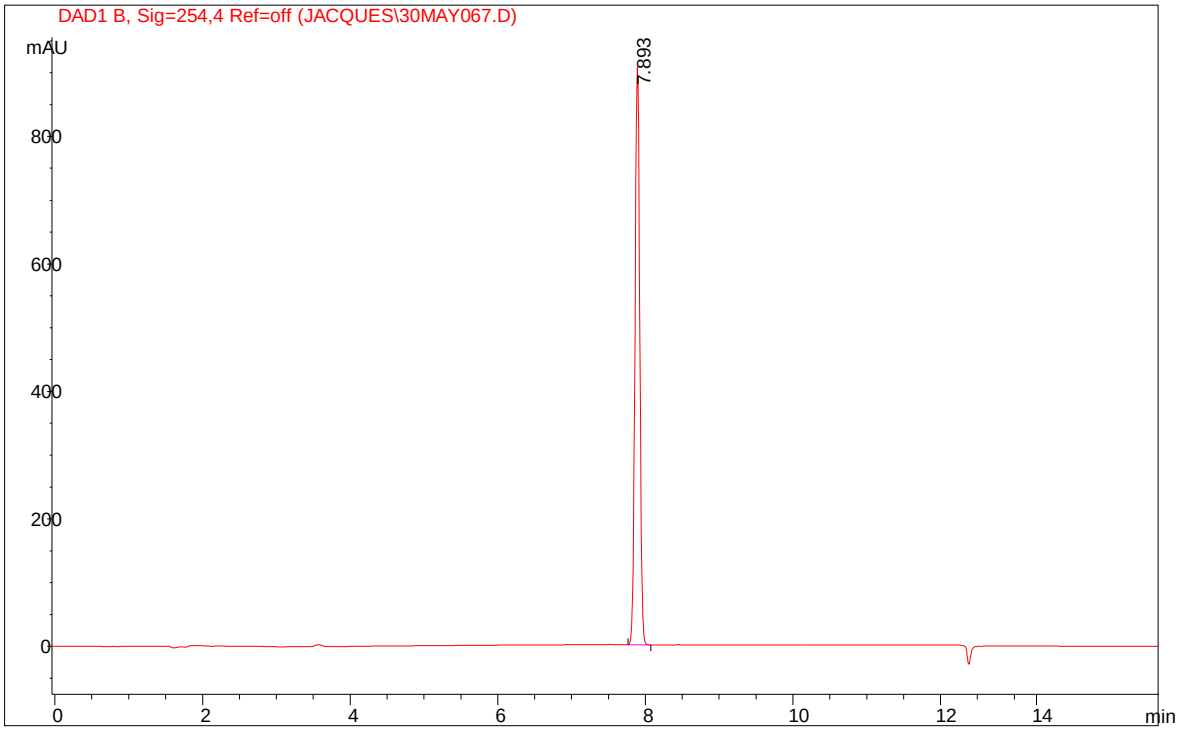

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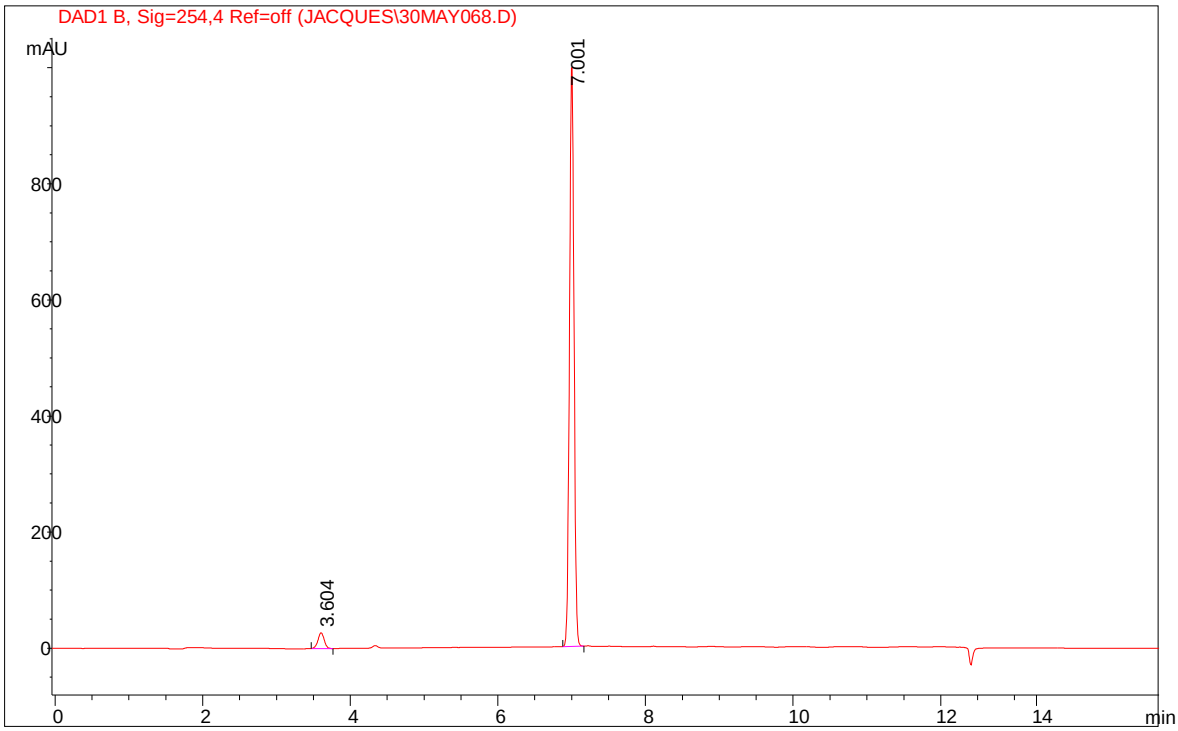

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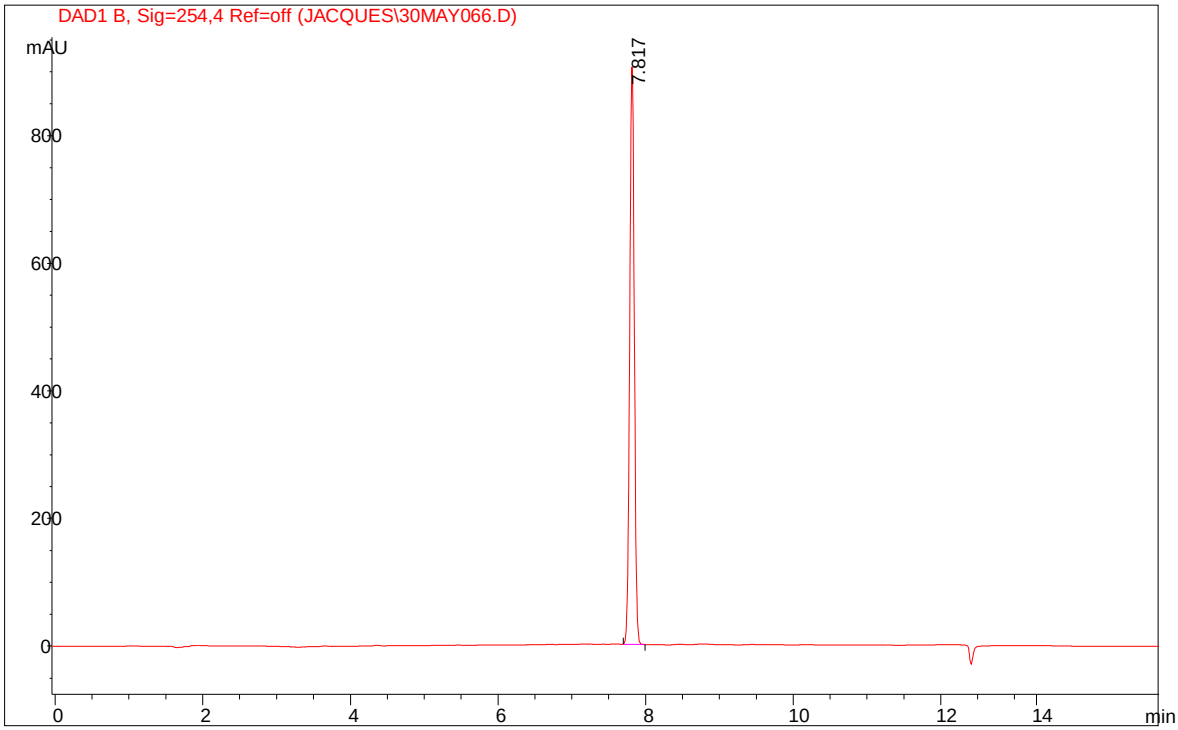

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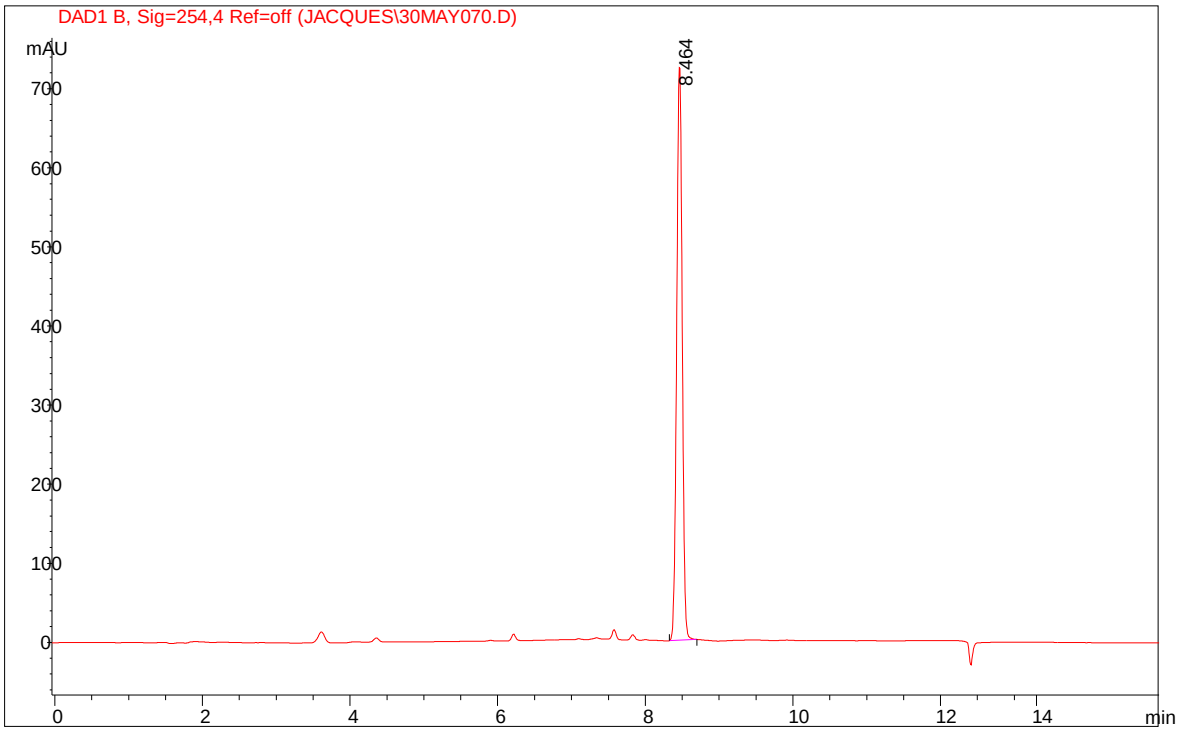

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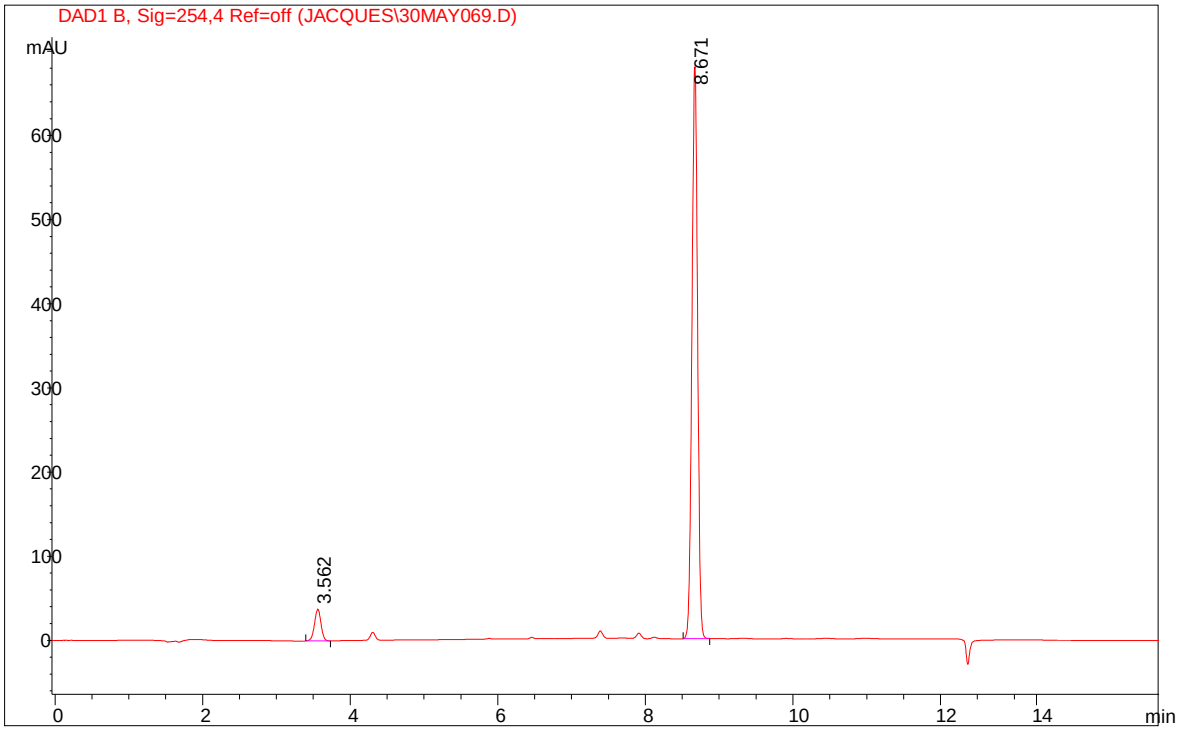

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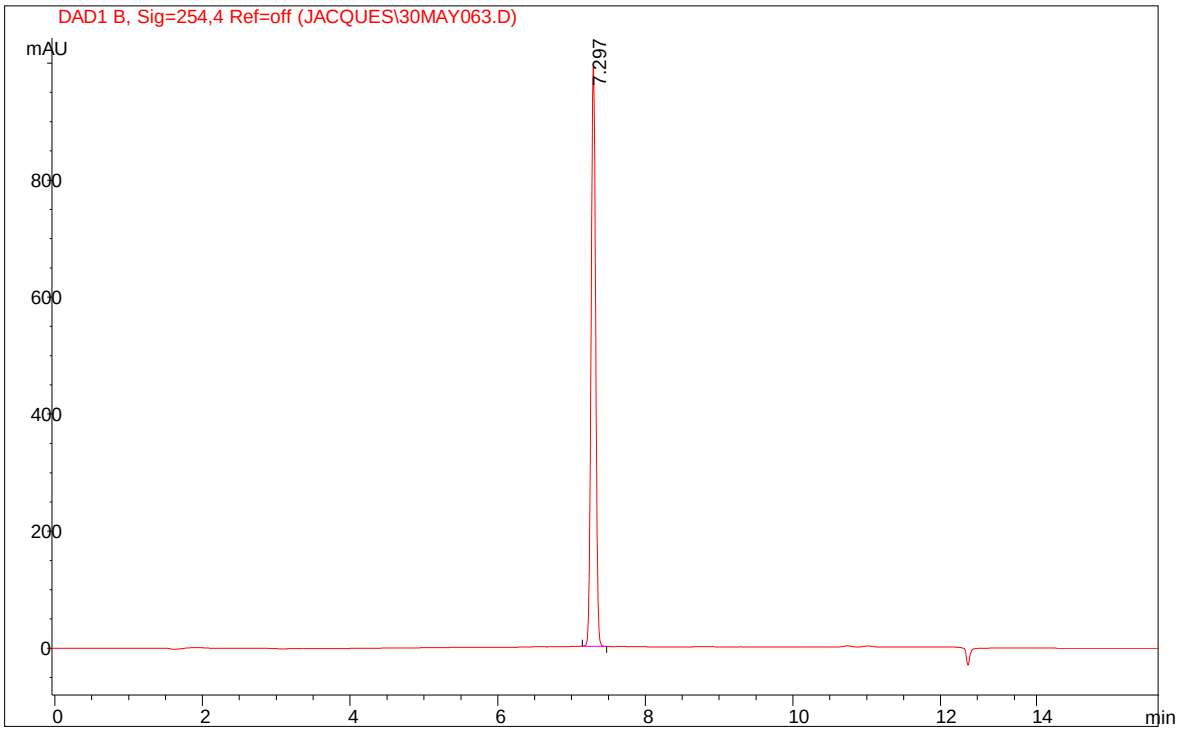

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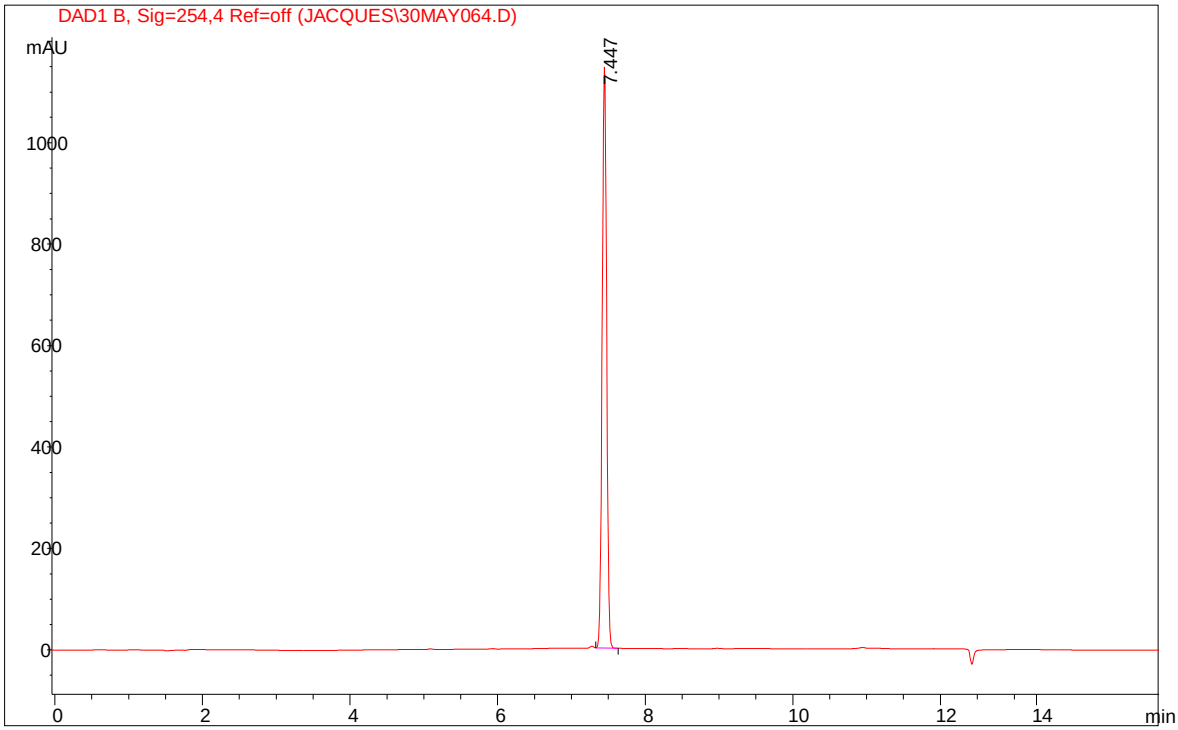

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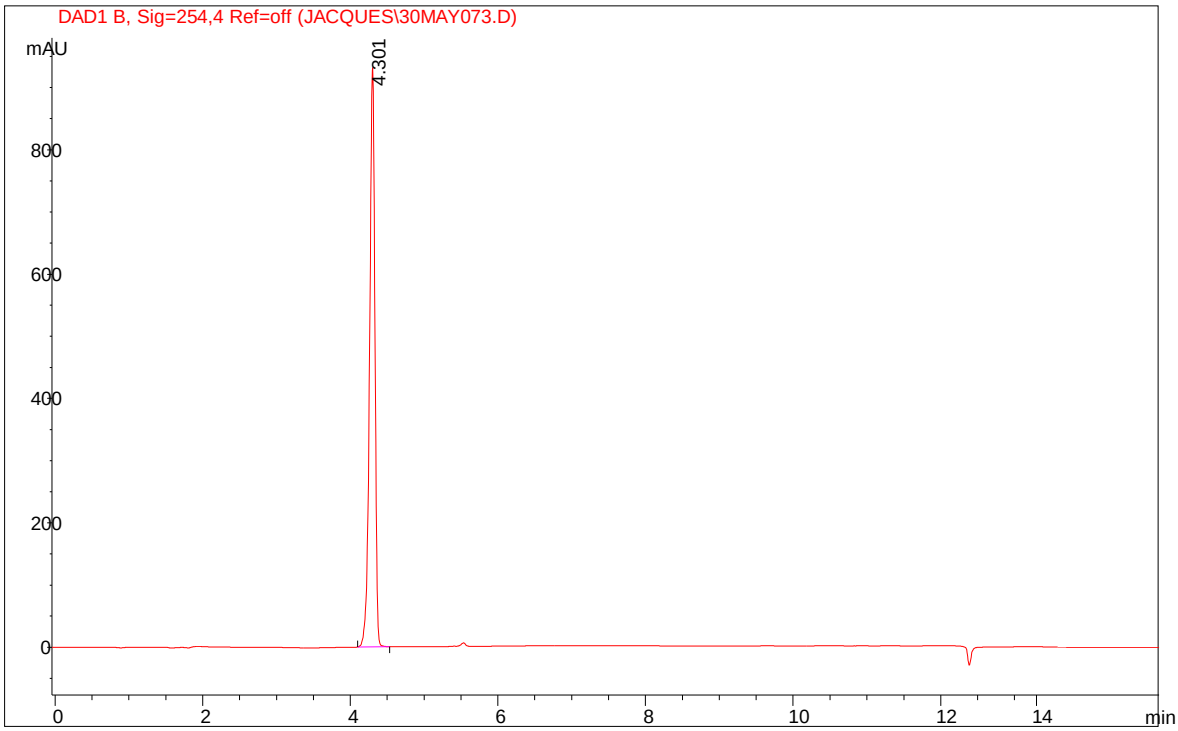

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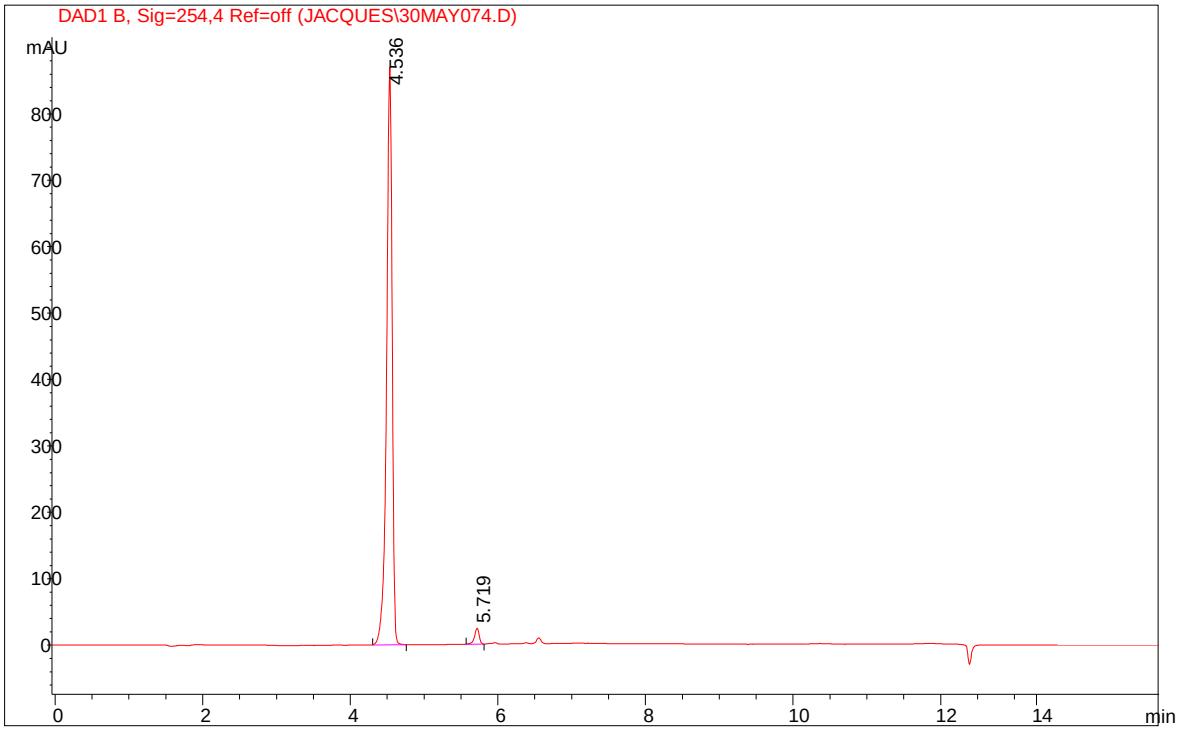

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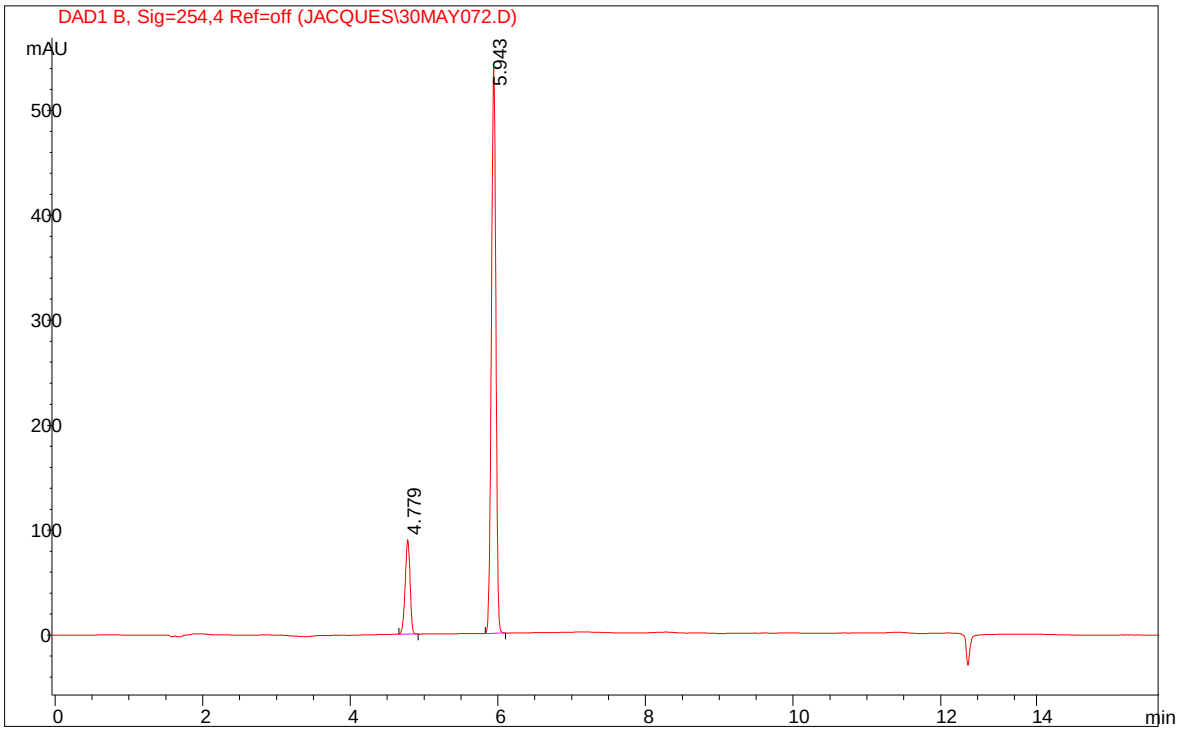

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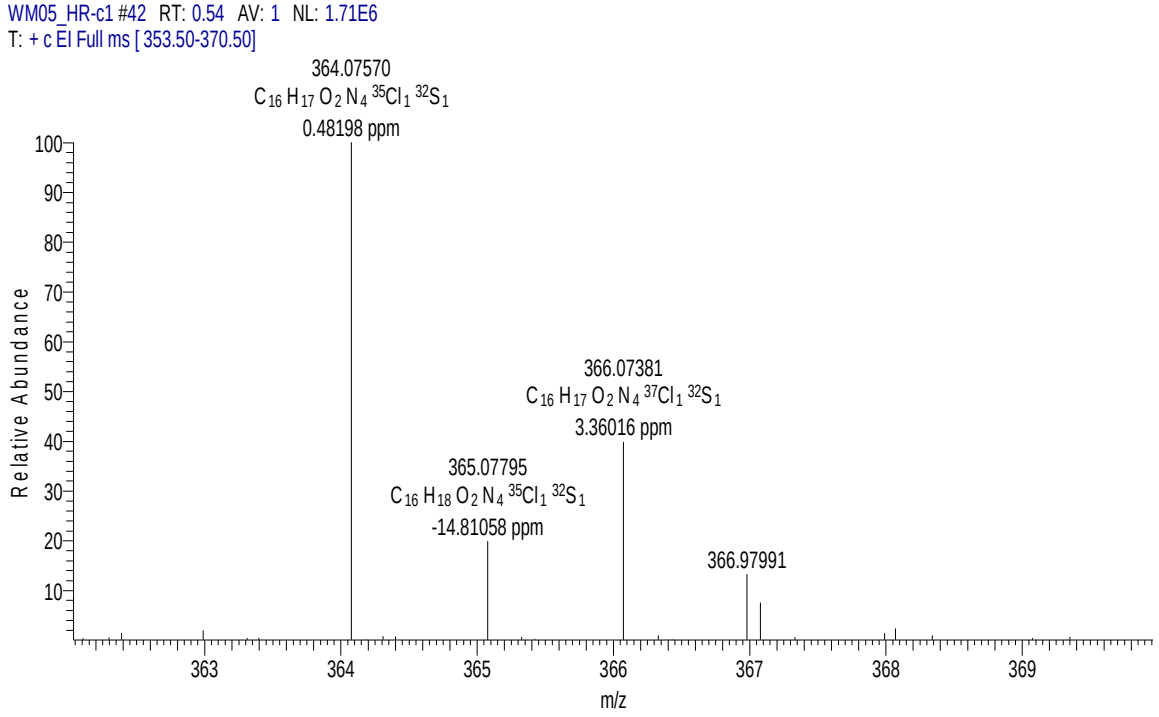

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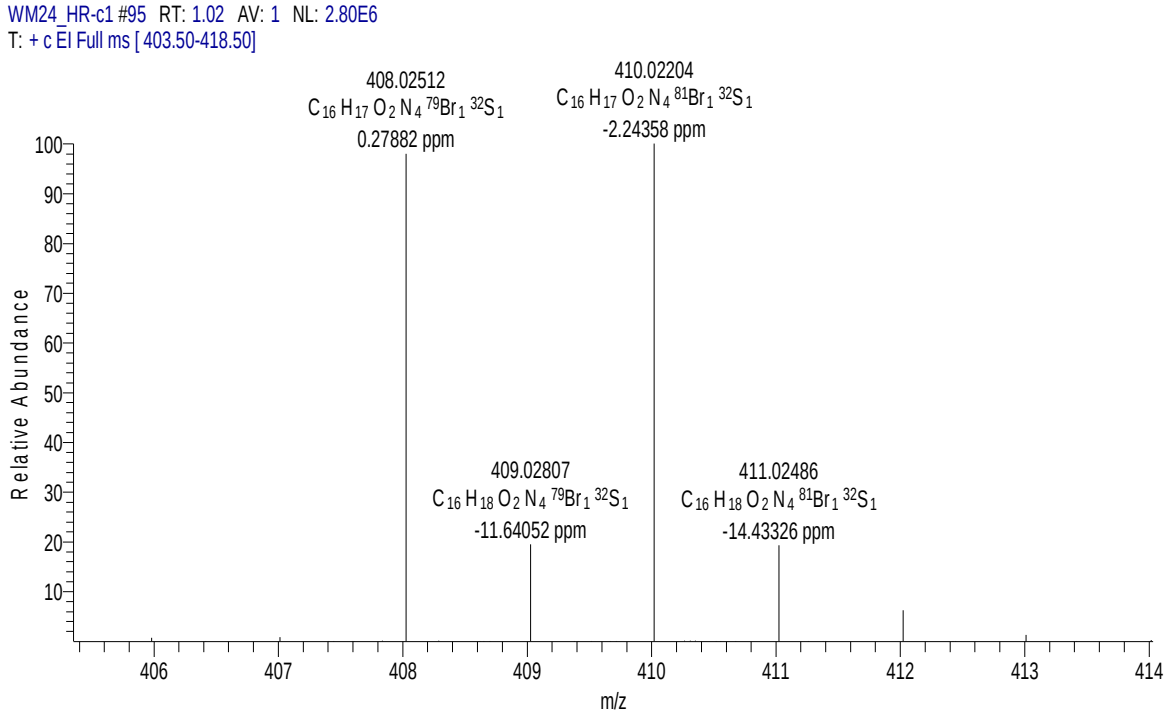

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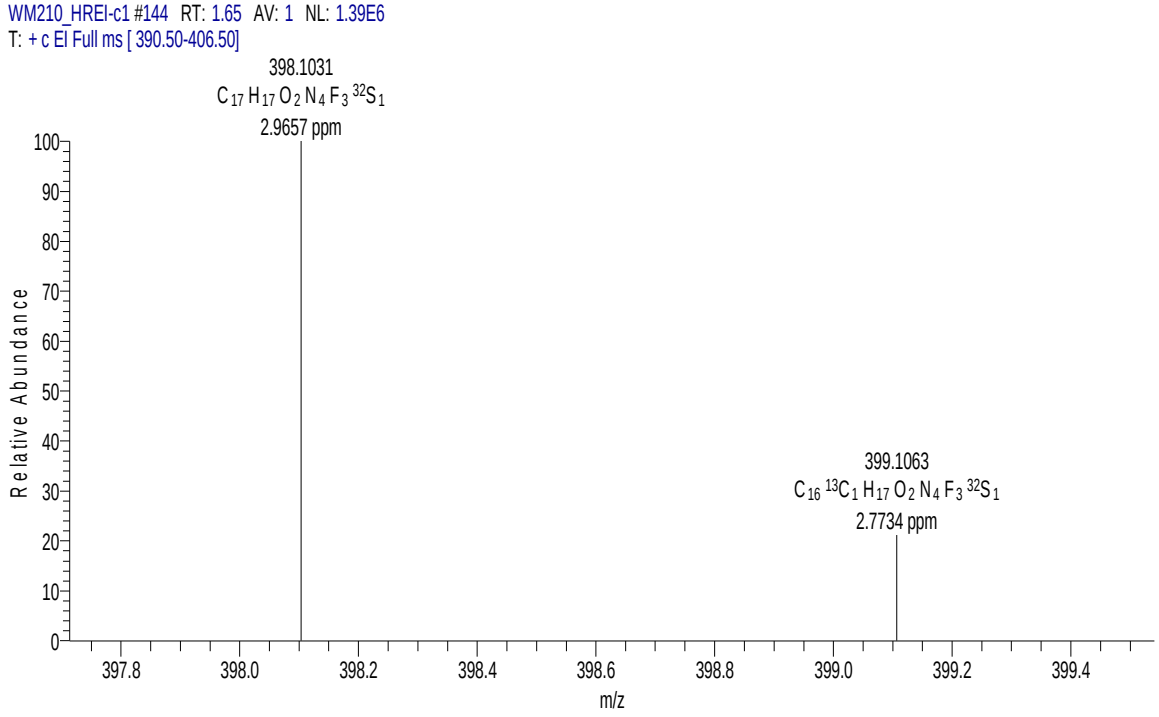

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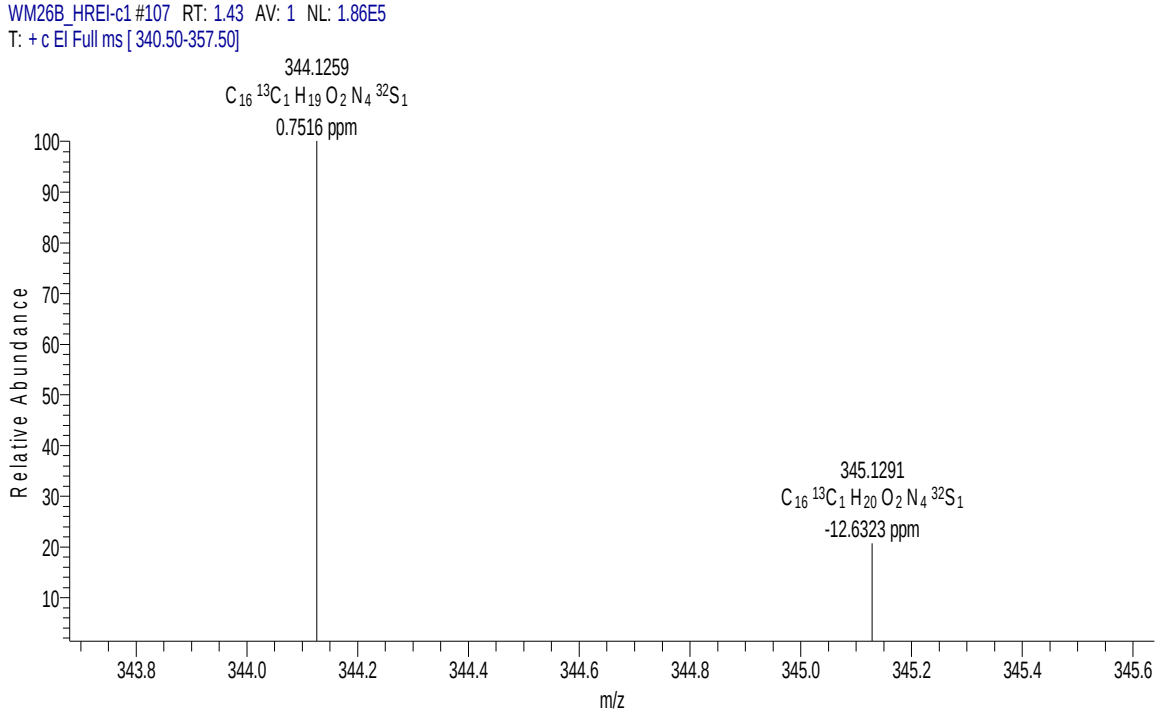

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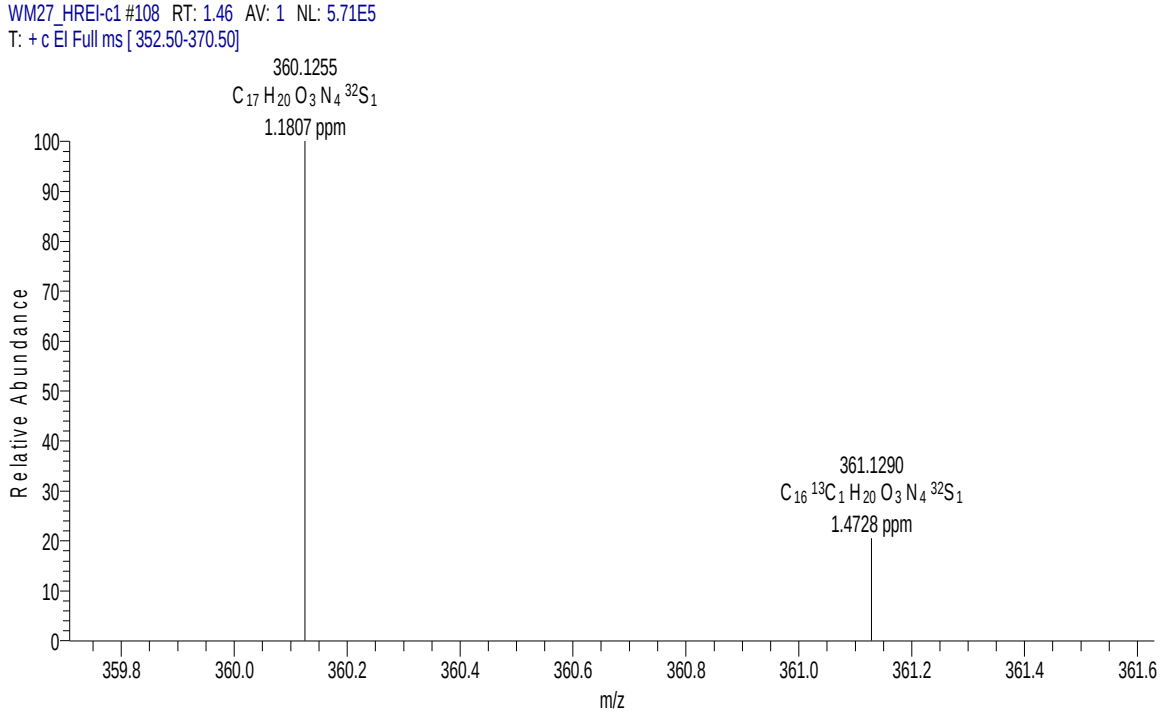

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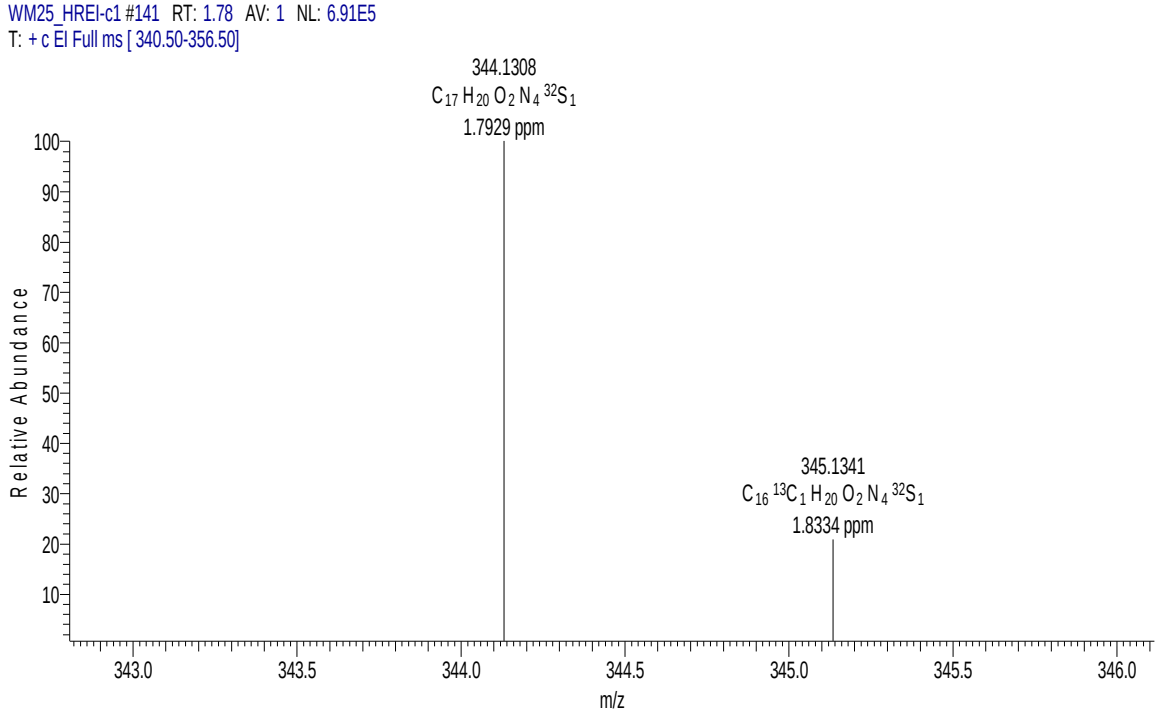

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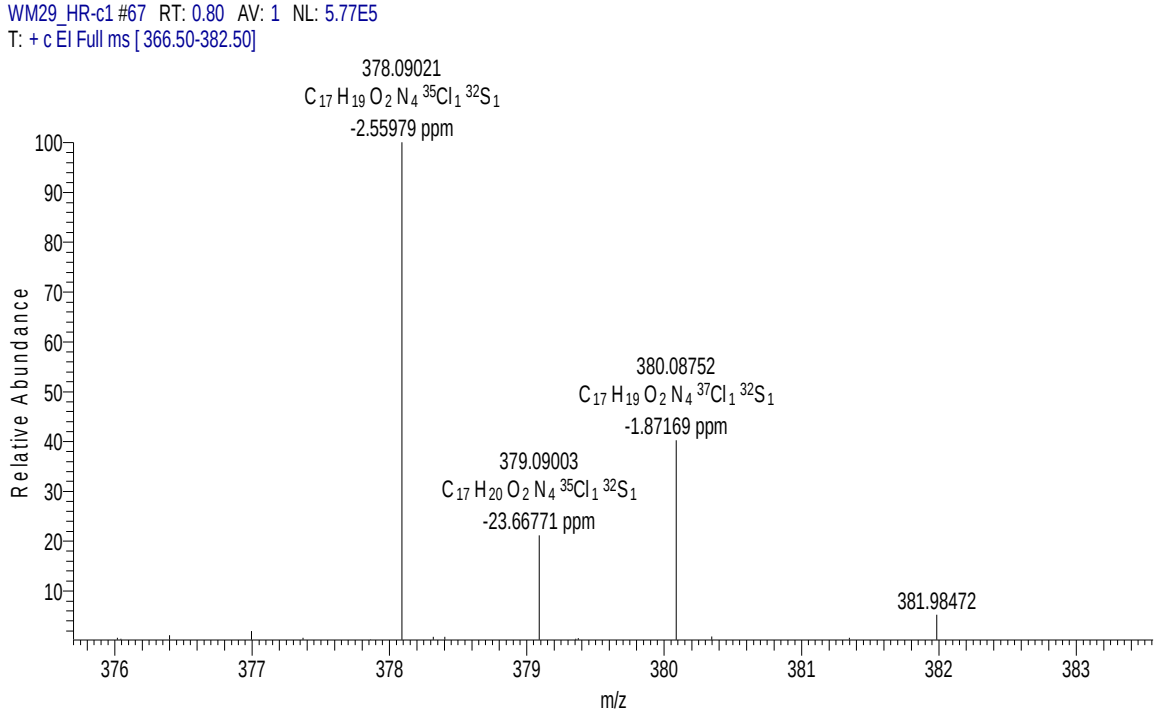

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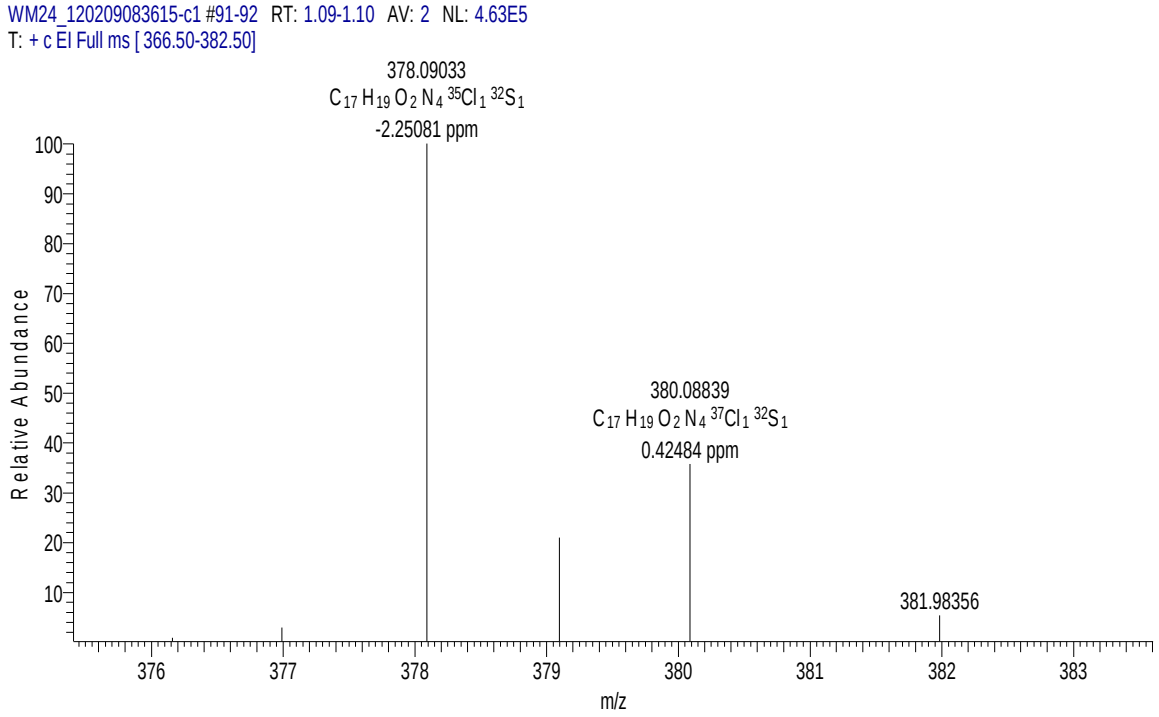

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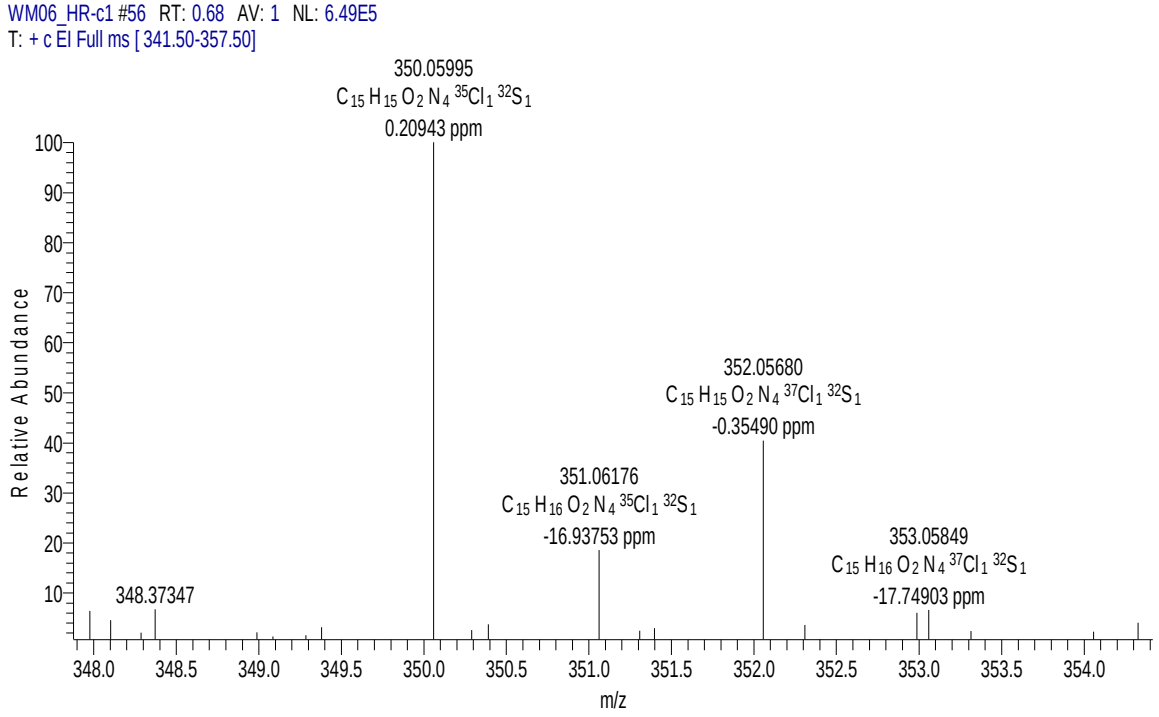

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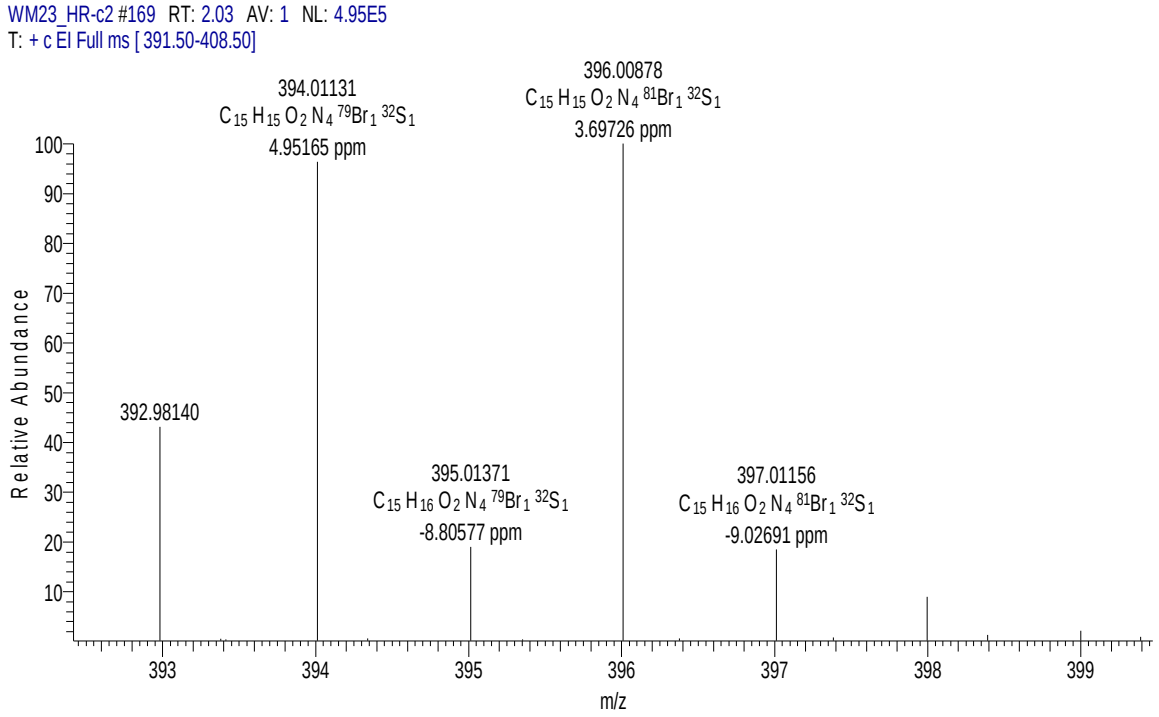

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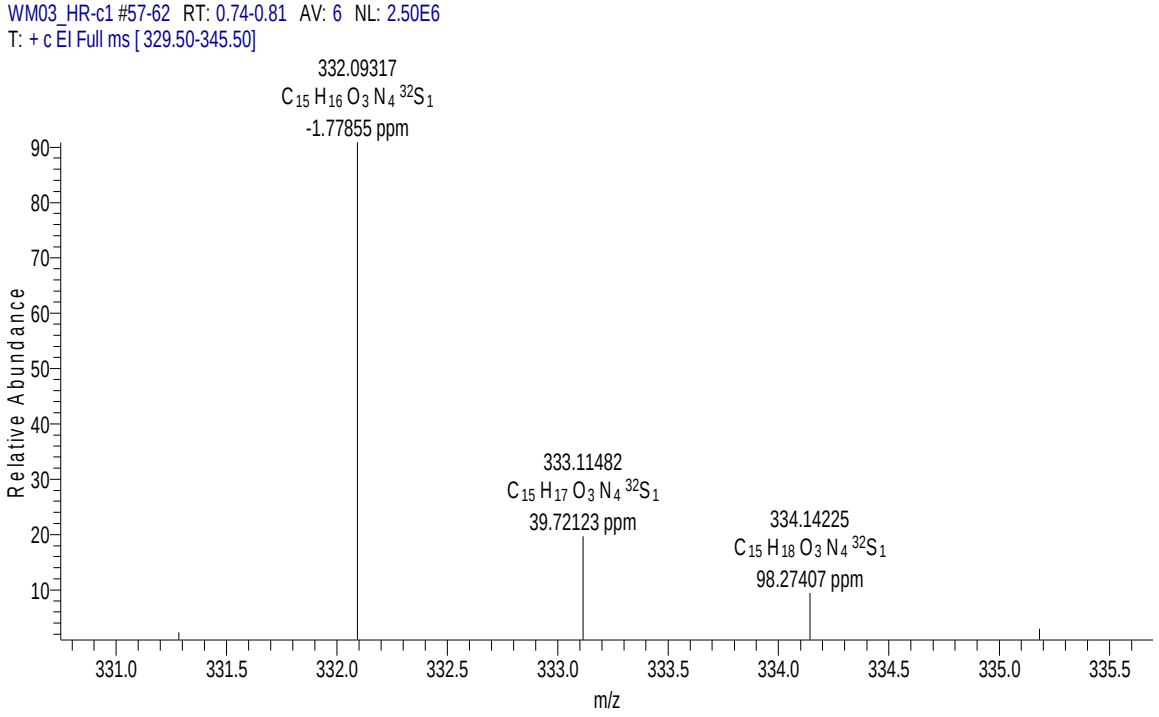

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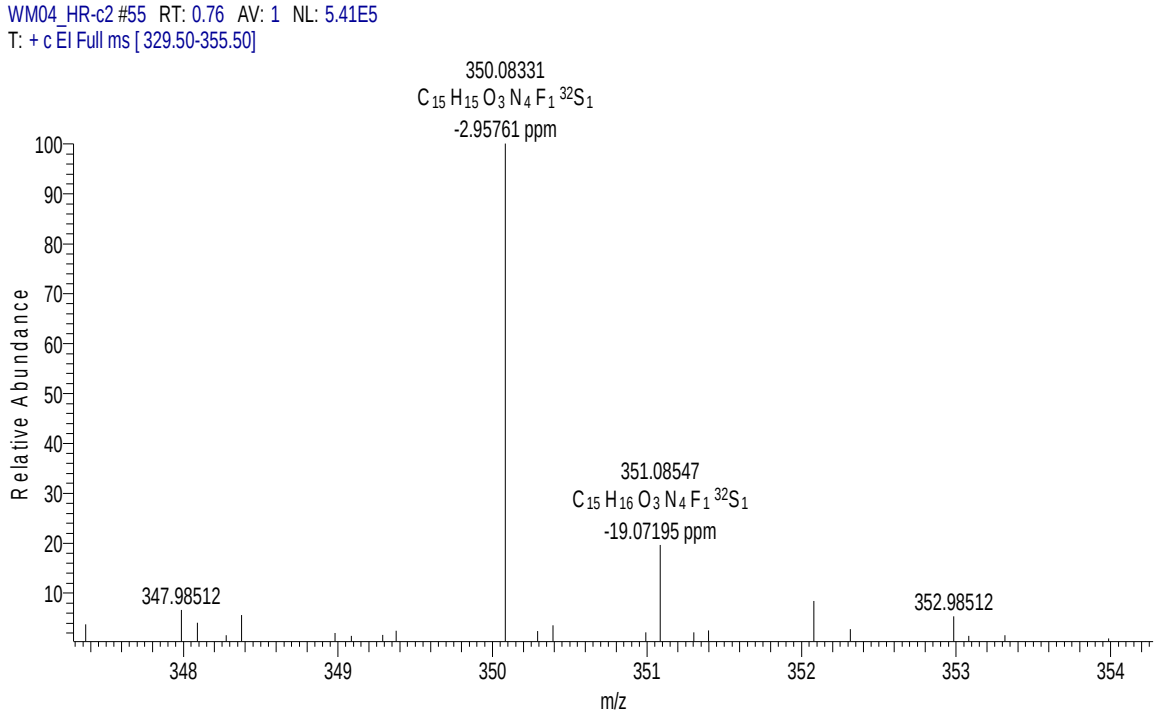

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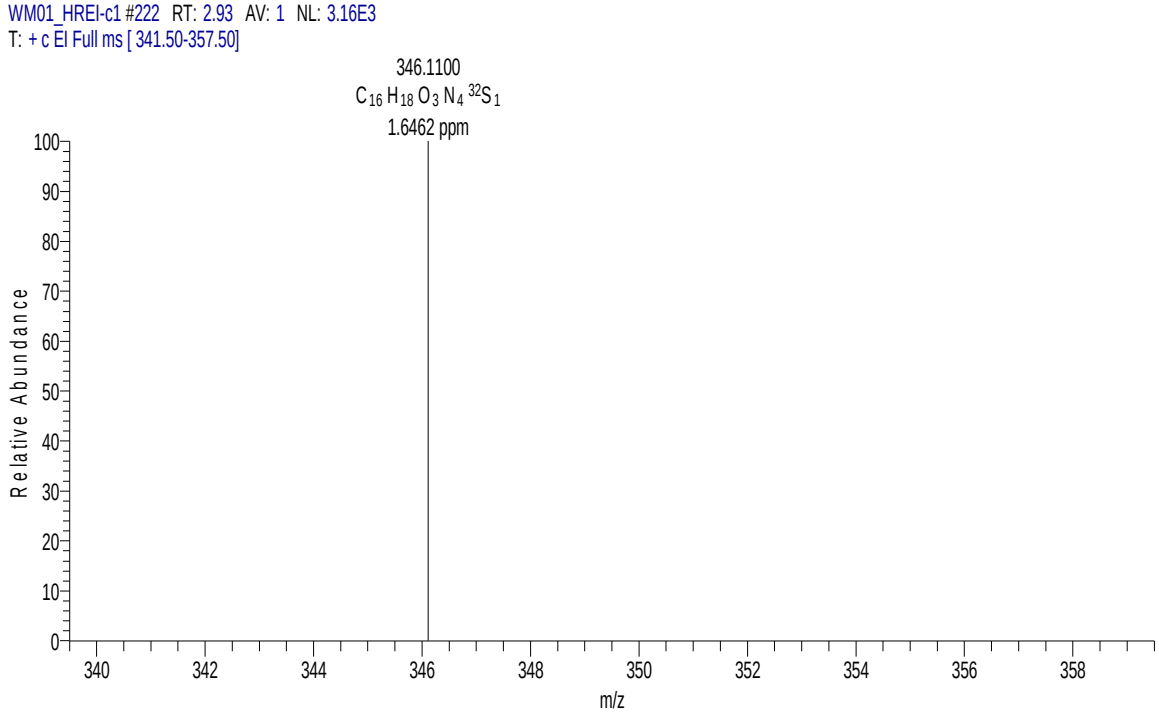

Inhibition of monoamine oxidase by 8-[(phenylethyl)sulfanyl]caffeine analogues

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Abstract—In a previous study we have investigated the monoamine oxidase (MAO) inhibitory properties of a series of 8-sulfanylcaffeine analogues. Among the compounds studied, 8-[(phenylethyl)sulfanyl]caffeine (IC_{50} = 0.223 µM) was found to be a particularly potent inhibitor of the type B MAO isoform. In an attempt to discover potent MAO inhibitors and to further examine the structure-activity relationships (SAR) of MAO inhibition by 8-sulfanylcaffeine analogues, in the present study a series of 8-[(phenylethyl)sulfanyl]caffeine analogues were synthesized and evaluated as inhibitors of human MAO-A and –B. The results document that substitution on C3 and C4 of the phenyl ring with alkyl groups and halogens yields 8-[(phenylethyl)sulfanyl]caffeine analogues which are potent and selective MAO-B inhibitors with IC_{50} values ranging from 0.017–0.125 µM. The MAO inhibitory properties of a series of 8-sulfinylcaffeine analogues were also examined. The results show that compared to the corresponding 8-sulfanylcaffeine analogues, the 8-sulfinylcaffeins are weaker MAO-B inhibitors. Both the 8-sulfanylcaffeine and 8-sulfinylcaffeine analogues were found to be weak MAO-A inhibitors. This study also reports the MAO inhibition properties of selected 8-[(phenylpropyl)sulfanyl]caffeine analogues.

Keywords: Monoamine oxidase; Sulfanylcaffeine; Sulfinylcaffeine; Reversible inhibition; Selective inhibition; Caffeine; Structure-activity relationship.

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1. Introduction

The monoamine oxidases (MAO) A and B are flavin adenine dinucleotide (FAD) containing enzymes which are bound to the outer membranes of mitochondria.[1] Although the two MAO isoforms are distinctive enzymes and products of separate genes,[2] they exhibit several similarities. MAO-A and –B have approximately 70% identity at the amino acid level and their crystallographic structures show that the amino acid residues comprising their active sites and their relative geometries are highly similar.[2] Only 6 of the 16 active site amino acids differ between the two enzymes.[3–5] In spite of these similarities, MAO-A and –B exhibit different substrate and inhibitor specificities. For example, serotonin acts as a MAO-A selective substrate while the arylalkylamines, benzylamine and β-phenethylamine, are MAO-B selective substrates. Dopamine, epinephrine and norepinephrine are employed by both MAO isoforms as substrates.[6]

Based on their roles in the degradation of neurotransmitter amines, MAO-A and –B have attracted attention as pharmacological targets for the treatment of neurological and neuropsychiatric disorders.[6] Inhibitors of MAO-A are used in the management of anxiety disorder and depression while MAO-B inhibitors have been employed as antiparkinsonian drugs. Examples of MAO inhibitors that are currently in clinical use are the reversible MAO-A inhibitor, moclobemide, and the irreversible MAO-B inhibitors, (*R*)-deprenyl and rasagiline.[7,8] This study is particularly interested in the discovery of new MAO-B inhibitors. In Parkinson's disease therapy, MAO-B inhibitors are frequently combined with the dopamine precursor, levodopa.[8] Inhibition of the MAO-catalyzed catabolism of dopamine is thought to lead to a dopamine sparing effect in the brain and subsequently a symptomatic benefit for Parkinson's disease patients.[9] Furthermore, inhibitors of MAO-B may enhance an elevation of the concentration of dopamine derived from levodopa and thus allow for a reduction of the dosage of levodopa that is necessary for a therapeutic response.[10] A reduction of the dosage of levodopa is also expected to diminish levodopa associated side effects.[11] By inhibiting the degradation of β-phenethylamine, MAO-B inhibitors may also indirectly induce an increase of extracellular dopamine concentrations.[12] This effect may possibly be attributed to the release of dopamine and inhibition of active dopamine uptake by β-phenethylamine.[13] Although MAO-A may also metabolize dopamine in the primate and possibly human brain,[10] in the aged brain MAO-B is thought to be the major dopamine metabolizing enzyme.[14,15] One reason for this is that the activity and density of MAO-B increase in most brain regions with age while MAO-A activity remains unchanged.[16,17] In the aged parkinsonian brain, MAO-B is therefore considered

to be the principal drug target for reducing the oxidative metabolism of dopamine. Furthermore, the clinical use of MAO-A inhibitors have declined in recent years because of concerns of severe side effects that may arise from the indirectly-acting sympathomimetic amine, tyramine. Intestinal MAO-A metabolizes tyramine, which is present in certain foods, and thus reduces the amount of tyramine that enters the systemic circulation. MAO-A inhibitors may enhance tyramine blood levels and lead to a tyramine-induced release of norepinephrine from peripheral neurons.[18] It should, however, be noted that, in contrast to irreversible inhibitors, reversible MAO-A inhibitors do not, in general, elevate tyramine levels to such an extent as to result in sympathomimetic side effects.[19]

MAO-B inhibitors may also have significance in the treatment of neurodegenerative disorders due to a putative neuroprotective effect. In the central nervous system, MAO-B inhibitors are thought to reduce the formation of aldehydes and hydrogen peroxide, which are produced by the MAO-B-catalyzed oxidation of amines.[20–23] These harmful metabolic by-products may lead to neurotoxicity and accelerate the neurodegenerative processes associated with Parkinson's disease. The aldehydic product derived from the MAO-catalyzed oxidation of dopamine has been implicated in the aggregation of α-synuclein, a process which is associated with the pathogenesis of Parkinson's disease.[24] Hydrogen peroxide, in turn, causes oxidative damage and promotes apoptotic signaling events.[25] Considering that MAO-B activity increases in the brain with age,[16,17] the generation of these metabolic by-products by the MAO-B isoform may be especially relevant in Parkinson's disease. Although irreversible MAO-B inhibitors have been used in the therapy of Parkinson's disease, these compounds may have certain disadvantages. Among these are slow and variable rates of enzyme recovery following withdrawal of the irreversible inhibitor.[26] For example, the turnover rate for the biosynthesis of MAO-B in the human brain may be as much as 40 days.[27] In contrast, following withdrawal of a reversible inhibitor, enzyme activity is recovered when the inhibitor is eliminated from the tissues. For these reasons the discovery of new reversible inhibitors may be of value.

As mentioned above, the present study aims to discover new reversible inhibitors of the MAO enzymes, particularly the MAO-B isoform. This study is a continuation of an investigation of the MAO inhibitory properties of caffeine derived compounds.[28,29] We have recently reported that a series of 8-sulfanylcaffeine analogues acts as selective inhibitors of human MAO-B.[30] Among the compounds examined, 8-[(phenylethyl)sulfanyl]caffeine (**1a**) was found to be a particularly potent MAO-B inhibitor with an IC_{50} value of 0.223 μM (Fig. 1). In an attempt to further enhance the MAO-B inhibition potency of **1a**, and possibly to discover highly potent MAO-B inhibitors, a

series of 8-[(phenylethyl)sulfanyl]caffeine analogues (**1a**–**l**) was synthesized and evaluated as inhibitors of human MAO-A and –B. For the purpose of this study 8-[(phenylethyl)sulfanyl]caffeine homologues containing C3 and C4 alkyl (CF_3, CH_3, OCH_3) and halogen (Cl, Br, F) substituents on the phenyl ring were considered. Similar substitution of a series of 8-benzyloxycaffeine analogues has previously been shown to be beneficial for MAO-B inhibition.[28,29] For the purpose of this study, substitution on C3 and C4 of the phenyl ring was considered since literature reports that the MAO inhibitory activities of 8-benzyloxycaffeine and 8-sulfanylcaffeine analogues may be significantly attenuated with substituents at these positions.[28–30] Furthermore, a series of 8-sulfinylcaffeine analogues (**2a**–**d**) was synthesized and their MAO inhibitory potencies were measured (Fig. 2). The purpose with these compounds was to compare the MAO inhibitory properties of the 8-sulfinylcaffeine analogues (**2**) with those of the 8-sulfanylcaffeine analogues (**1**). This study also reports the MAO inhibition properties of selected 8-[(phenylpropyl)sulfanyl]caffeine (**3a**–**c**) and 8-(benzylsulfanyl)caffeine analogues (**4a**–**b**).

2. Results

2.1. Chemistry

The target 8-sulfanylcaffeine analogues, compounds **1a**–**l**, **3a**–**c** and **4a**–**b** were synthesized according to the literature procedure as shown in Scheme 1.[31] 8-Chlorocaffeine (**5**) was reacted with an appropriate mercaptan (**6**) in the presence of NaOH, with 50% aqueous ethanol serving as solvent. This gave the target 8-sulfanylcaffeine analogues in yields of 6.4–83%. 8-Chlorocaffeine, in turn, was conveniently synthesized in high yield by reacting chlorine with caffeine in chloroform.[32] In certain instances, the mercaptan starting materials were not commercially available and were thus synthesized according the literature procedure.[33] For this purpose an appropriate alkylbromide was reacted with thiourea (**7**) in ethanol (Scheme 2). The resulting thiouronium salt (**8**) was hydrolyzed in the presence of NaOH to yield the target mercaptan (**6**). The 8-sulfinylcaffeine analogues, **2a**–**d**, were synthesized by reacting the 8-sulfanylcaffeines with H_2O_2 in the presence of glacial acetic acid and acetic anhydride (Scheme 3).[31] Both MS and NMR indicated that the structures of **2a**–**d** were those of the 8-sulfinylcaffeines and not the corresponding 8-sulfonylcaffeines. This was apparent from the ^{1}H NMR spectra which yielded two distinctive signals, multiplets integrating for 1 proton each, for the protons of the –S-CH_2– moiety of **2a**–**c**. For the 8-sulfonylcaffeines, these protons are expected to be equivalent and would lead to a single ^{1}H NMR signal, a triplet integrating for 2

protons. The ^{1}H NMR signal of the benzylic CH_2 of **2d** is a multiplet, indicating nonequivalence of these protons. With the 8-sulfonylcaffeine homologue, the benzylic protons are expected to be equivalent and would lead to a singlet. The purities of the target compounds were estimated via HPLC analysis.

2.2. MAO inhibition studies

The MAO inhibitory properties of the test compounds were examined using the recombinant human enzymes. The mixed MAO-A/B substrate, kynuramine, was employed as substrate for both enzymes. Kynuramine exhibits similar K_m values for the two isozymes of 16.1 µM and 22.7 µM, respectively.[28] Kynuramine is oxidized by the MAOs to yield 4-hydroxyquinoline, a fluorescent compound, which is readily measurable in the presence of the non-fluorescent kynuramine and the test inhibitors investigated in the current study.

2.2.1. Inhibition of MAO-B

The IC_{50} values for the inhibition of human MAO by the 8-sulfanylcaffeines **1a**–**l** are given in Table 1. As shown, the lead compound for this study, compound **1a**, inhibits MAO-B with an IC_{50} value of 0.271 µM. This value is similar to that previously reported by us (IC_{50} = 0.223 µM).[30] The results further show that substitution on the phenyl ring of **1a** leads to a considerable enhancement of its MAO-B inhibition potency, with all of the substituted homologues exhibiting more potent MAO-B inhibition than **1a**. The IC_{50} values recorded for these homologues (**1b**–**l**) ranged from 0.017–0.125 µM, making them twofold to 16-fold more potent MAO-B inhibitors than the lead compound. For comparison, the reversible MAO-B selective inhibitor, lazabemide, exhibits an IC_{50} value of 0.091 µM under the same conditions (unpublished data from our laboratory). Interestingly, both alkyl (CF_3, CH_3, OCH_3) and halogen (Cl, Br, F) substitution lead to highly potent MAO-B inhibition. It may therefore be concluded that substitution on C3 and C4 is a general strategy to enhance the MAO-B inhibition potency of 8-[(phenylethyl)sulfanyl]caffeine (**1a**). This result is in agreement with a previous observation that the human MAO-B inhibition potency of 8-(benzylsulfanyl)caffeine (**4c**; IC_{50} = 1.86 µM) may be improved with halogen (Cl, Br and F) substitution on the *para* position of the benzyl ring, yielding compounds with IC_{50} values ranging from 0.167–0.348 µM.[30] The present study also shows that *meta* substitution with chlorine (**4a**; IC_{50} = 0.227 µM) and bromine (**4b**; IC_{50} = 0.199 µM) enhances the MAO-B inhibition potency of 8-(benzylsulfanyl)caffeine (**4c**). The 8-[(phenylethyl)sulfanyl]caffeine analogues are, however, significantly more potent MAO-B inhibitors than the corresponding 8-(benzylsulfanyl)caffeines. For example, the 8-

[(phenylethyl)sulfanyl]caffeine analogues substituted with chlorine on the *meta* (**1h**; IC_{50} = 0.043 µM) and *para* (**1b**; IC_{50} = 0.020 µM) positions of the phenyl ring are fivefold and ninefold, respectively, more potent than the corresponding *meta* (**4a**; IC_{50} = 0.227 µM) and *para* (IC_{50} = 0.192 µM)[30] chlorine substituted 8-(benzylsulfanyl)caffeines. Similarly, the 8-[(phenylethyl)sulfanyl]caffeines containing bromine on the *meta* (**1i**; IC_{50} = 0.040 µM) and *para* (**1c**; IC_{50} = 0.019 µM) positions of the phenyl ring are fivefold and eightfold, respectively, more potent than the corresponding *meta* (**4b**; IC_{50} = 0.199 µM) and *para* (IC_{50} = 0.167 µM)[30] bromine substituted 8-(benzylsulfanyl)caffeines. Based on these analyses, it may be concluded that 8-[(phenylethyl)sulfanyl]caffeines with substituents on the phenyl ring are exceptionally potent MAO-B inhibitors and suitable lead compounds for the design of novel inhibitors of this enzyme.

The IC_{50} values for the inhibition of human MAO by the 8-sulfinylcaffeines **2a**–**d** are given in Table 2. The results document that the 8-sulfinylcaffeines also are inhibitors of MAO-B with IC_{50} values of 0.471–131 µM. Compared to the 8-sulfanylcaffeines, these homologues are, however, weaker inhibitors. For example, the most potent 8-sulfinylcaffeine inhibitor, compound **2b** (IC_{50} = 0.471 µM), is approximately 25-fold weaker as a MAO-B inhibitor than its corresponding 8-sulfanylcaffeine homologue, compound **1c** (IC_{50} = 0.019 µM). Similarly, the 8-sulfinylcaffeine **2a** (IC_{50} = 0.781 µM) is 39-fold weaker as a MAO-B inhibitor than its corresponding 8-sulfanylcaffeine homologue, compound **1b** (IC_{50} = 0.02 µM). It may, therefore, be concluded that 8-sulfinylcaffeines are comparatively weak MAO-B inhibitors and less suited for the design of high potency MAO-B inhibitors.

This study also examined the MAO inhibitory properties of a limited series of 8-[(phenylpropyl)sulfanyl]caffeine analogues (**3a**–**c**). The results are given in Table 3, and shows that these compounds are also inhibitors of MAO-B with IC_{50} values of 0.061–0.500 µM. Those homologues substituted with chlorine on the *para* and *meta* positions of the phenyl ring, compounds **3b**–**c**, were found to be exceptionally potent inhibitors with IC_{50} values of 0.061 µM and 0.062 µM, respectively. These compounds are slightly less active than the corresponding 8-[(phenylethyl)sulfanyl]caffeine homologues, compounds **1h** (IC_{50} = 0.043 µM) and **1b** (IC_{50} = 0.020 µM). It may therefore be concluded that, although less active than the 8-[(phenylethyl)sulfanyl]caffeines, 8-[(phenylpropyl)sulfanyl]caffeine substituted on the phenyl ring may represent suitable lead compounds for the design of MAO-B inhibitors.

2.2.2. Inhibition of MAO-A

The results of the human MAO inhibition studies show that the 8-sulfanylcaffeines **1a**–**l** are relatively weak MAO-A inhibitors with IC_{50} values of 5.66–168 µM (Table 1). Compound **1e** did not exhibit inhibition towards MAO-A. Interestingly, those compounds substituted on the *meta* position (**1h**–**1l**) of the phenyl ring were more potent MAO-A inhibitors than the corresponding homologues substituted on the *para* (**1b**–**1g**) position. For example, the *meta* substituted chlorine and bromine homologues, compounds **1h** (IC_{50} = 5.66 µM) and **1i** (IC_{50} = 5.70 µM), were 1.5-fold and 19-fold, respectively, more potent than the corresponding *para* substituted homologues, compounds **1b** (IC_{50} = 8.46 µM) and **1c** (IC_{50} = 108 µM). As evident from the selectivity indices (SI values), compounds **1a**–**l** were all selective inhibitors of the MAO-B isoform. Five compounds (**1c**, **1e**, **1f**, **1g**, and **1j**) exhibited SI values in excess of 1000. Since these compounds are also highly potent MAO B inhibitors, they represent suitable leads for the design of potent and selective MAO-B inhibitors.

The results document that the 8-sulfinylcaffeines **2a**–**d** are weak MAO-A inhibitors with IC_{50} values of 57.3–250 µM (Table 2). The SI values demonstrate that these compounds are MAO-B selective inhibitors, although to a lesser degree than the 8-sulfanylcaffeines **1a**–**l**. Table 3 shows that 8-[(phenylpropyl)sulfanyl]caffeines **3a**–**c** are also MAO-A inhibitors with IC_{50} values of 0.708–6.48 µM. It is noteworthy that **3b**–**c** are the most potent MAO-A inhibitors among the compounds evaluated in this study. In fact, **3b** (IC_{50} = 0.708 µM) is the only compound with an IC_{50} value for the inhibition of MAO-A in the submicromolar range. Although more potent as MAO-A inhibitors, compounds **3b**–**c** are still MAO-B selective inhibitors with SI values of 12 and 57, respectively. The 8-[(phenylpropyl)sulfanyl]caffeines **3a**–**c** display lower degrees of selectivity for MAO-B than the corresponding 8-[(phenylethyl)sulfanyl]caffeines. For example, compounds **3a**–**c** exhibit SI values of 12–57, while the equivalently substituted 8-[(phenylethyl)sulfanyl]caffeine homologues, compounds **1a**, **1b** and **1h**, display SI values of 69–423.

2.3. Reversibility of inhibition

8-(Benzylsulfanyl)caffeine analogues have previously been shown to interact reversibly with human MAO-A and –B.[30] This is advantageous since the activity of the MAO enzymes are recovered when treatment is terminated and the inhibitor is cleared from the tissues. For irreversible inhibitors, de novo synthesis is required for enzyme activity to recover. Although it is likely that 8-[(phenylethyl)sulfanyl]caffeines also interact reversibly with MAO-A and –B, this

study verified this expectation with a representative inhibitor, compound **1b**. In order to evaluate reversibility, the time-dependence of MAO inhibition was examined. Compound **1b**, at concentrations approximately twofold its measured IC_{50} values, was preincubated with human MAO-A or –B for 0, 15, 30 and 60 min. Following these preincubations the reactions were diluted twofold, to yield concentrations of the inhibitor that are equal to its IC_{50} values for the inhibition of the respective enzymes. The residual MAO catalytic activities were subsequently measured. The results of these reversibility studies are shown in Figure 3. As shown, when **1b** is preincubated with MAO-A no time-dependent reductions of the catalytic activities are observed, even following a period of 60 min preincubation with the enzymes. After preincubation with MAO-B, only a slight time-dependent reduction of catalysis is observed. These data are consistent with a reversible interaction of **1b** with both MAO enzymes.

The reversible interaction of **1b** with MAO-B was further investigated by evaluating the recovery of the enzymatic activity after dilution of the enzyme-inhibitor complex. For this purpose, MAO-B was preincubated with compound **1b** at concentrations of 10 × IC_{50} and 100 × IC_{50} for 30 min. The reactions were subsequently diluted 100-fold to 0.1 × IC_{50} and 1 × IC_{50}, respectively. The results are given in Figure 4 and show that, after dilution to 0.1 × IC_{50} and 1 × IC_{50}, the MAO-B catalytic activities are recovered to 58% and 26%, respectively, of the control value. For reversible enzyme inhibition, the enzyme activities are expected to recover to levels of approximately 90% and 50%, respectively, after 100-fold dilution of the preincubations containing inhibitor concentrations of 10 × IC_{50} and 100 × IC_{50}. After preincubation of MAO-B with the irreversible inhibitor (R)-deprenyl (at 10 × IC_{50}), and dilution of the resulting complex to 0.1 × IC_{50}, MAO-B activity is not recovered (3.0% of control). These data indicate that **1b** interacts reversibly with MAO-B. Interestingly, after dilution, the enzyme activities are not recovered to 90% and 50%, respectively, as expected. This result suggests that the binding of **1b** may possess a quasi-reversible or tight-binding component. This behavior was also observed with other 8-[(phenylethyl)sulfanyl]caffeines. For example, after similar preincubation of compound **1j** with MAO-B and dilution of the resulting enzyme-inhibitor complexes, the MAO-B catalytic activities are recovered to only 35% and 22%, respectively, of the control value (Fig. 4). This also supports a tight-binding mechanism, and further investigation is necessary to clarify this point. The interaction of inhibitors via a potential tight-binding mechanism has been reported previously. For example, chromone carboxamides display a quasi-reversible binding to human MAO-B,[34,35] while oxadiazolones are reported to bind reversibly and time-dependently to

rat brain MAO-B.[36] Mazouz and colleagues has also described a series of reversible and extremely tight-binding MAO-B specific inhibitors.[37]

The reversibility of MAO-B inhibition by compound **1b** was further demonstrated by constructing a set of Lineweaver–Burk plots for the inhibition of MAO-B. For this purpose, the initial catalytic rates were measured in the presence of three different concentrations of **1b**, and in the absence of inhibitor. These measurements were carried out using four different concentrations of the substrate, kynuramine (15–90 µM). Figure 5 illustrates the set of Lineweaver–Burk plots that were obtained from these studies. The results show that the Lineweaver–Burk plots are linear and intersect at y = 0. This indicates that the inhibition of MAO-B by **1b** is competitive, and is further evidence that **1b** is a reversible inhibitor of MAO-B. From a replot of the slopes of the Lineweaver-Burk plots versus inhibitor concentration, a K_i value of 0.013 µM was obtained for the inhibition of MAO-B by **1b**. Since this inhibitor may exhibit tight-binding to MAO-B, the inhibition data were analyzed according to the equation by Morrison.[38] This analysis gave a K_i value of 0.0098 ± 0.002 nM, a value that is similar to that obtained from the Lineweaver-Burk plots.

2.4. Lipophilicity

To further examine the potential of 8-[(phenylethyl)sulfanyl]caffeines to serve as lead compounds for the treatment of central nervous system disorders, the octanol/water partition coefficients (P) of selected homologues were measured. The partition coefficient is a measure of the lipophilicity of a compound, a property which has a major effect on the permeability of a compound through biological membranes. Lipophilicity estimates may thus be used to predict the likelihood of a compound to penetrate the blood-brain barrier. As shown in Table 4, all of the evaluated compounds may be viewed as lipophilic since they display LogP values in excess of 2.55. As a general guide, LogP values ranging from 0–3 are optimal for passive diffusion permeability and such compounds are expected to display good oral bioavailability and blood-brain barrier permeation.[39] While the measurement of additional properties such as solubility, permeability, metabolism and protein binding will provide a clearer picture, these preliminary results suggest that particularly compounds **1c** and **1g** may possess suitable properties to gain access in the central nervous system. These compounds are of further interest since they also display potent MAO-B inhibition.

3. Discussion and conclusion

The present study examines the possibility of improving the MAO inhibitory properties of **1a**, a compound previously shown to be a potent and selective MAO-B inhibitor.[30] The results show that substitution on the phenyl ring of **1a** leads to an enhancement of MAO-B inhibition potency. The most potent inhibitor of this study, the 3-CF_3 substituted homologue (**1j**), is approximately 16-fold more potent as a MAO-B inhibitor than **1a**, and exhibits a higher degree of selectivity for MAO-B. A variety of substituents, however, yields homologues with potent MAO-B inhibition activities. In this respect *para* substitution generally leads to slightly more potent MAO-B inhibitors than *meta* substitution. Although the MAO-B inhibition potencies of the 8-[(phenylethyl)sulfanyl]caffeine analogues are very similar, the following additional structure-activity relationships (SARs) are apparent: (1) substitution on both C3 and C4 of the phenyl ring yields improved inhibitory potency; (2) methoxy substitution on C3 yields the weakest MAO-B inhibitor of the series, compound **1l** with an IC_{50} value of 0.125 µM; (3) in general, both halogen and alkyl substitution yields highly potent MAO-B inhibitors. For the inhibition of MAO-A, no clear SAR trends is apparent. It is however noteworthy that the most potent MAO-A inhibitors among the 8-[(phenylethyl)sulfanyl]caffeine analogues are the C3 chlorine and bromine substituted homologues. The relatively large degree of tolerance for different substituents and substitution patterns makes 8-[(phenylethyl)sulfanyl]caffeine analogues good candidates for the design of MAO-B inhibitors since structural modifications that may lead to better drug properties are less likely to be associated with a loss of activity. This study also shows that 8-[(phenylethyl)sulfanyl]caffeine analogues are significantly more potent as MAO-B inhibitors than the corresponding 8-(benzylsulfanyl)caffeines investigated previously.[30]

Interestingly 8-[(phenylpropyl)sulfanyl]caffeine analogues (**3a**–**c**) are also highly potent MAO-B inhibitors with comparable potencies to those of the 8-[(phenylethyl)sulfanyl]caffeines. Compounds **3a**–**c** are, however, less selective for MAO-B. In fact, **3b** is the most potent MAO-A inhibitor among the investigated compounds, and the only inhibitor with an IC_{50} value in the submicromolar range. Although dopamine is metabolized by both MAO-A and –B in the human brain, the inhibition of MAO-A is associated with potentially dangerous side effects, and highly selective MAO-B inhibitors may therefore be more desirable for the treatment of Parkinson's disease. Furthermore, the combination of MAO-A inhibitors and levodopa in PD therapy should be avoided since this may lead to a hypertensive response.[40] Based on this analysis, 8-[(phenylethyl)sulfanyl]caffeines may be better suited as antiparkinsonian drugs than 8-[(phenylpropyl)sulfanyl]caffeines, since several 8-[(phenylethyl)sulfanyl]caffeines are highly

potent (IC_{50} < 0.05 μM) MAO-B inhibitors with SI values in excess of 1000. The 8-sulfinylcaffeine analogues on the other hand exhibited comparatively weak MAO-B inhibition and are thus not suited for the design of MAO inhibitors.

4. Experimental section

4.1. Chemicals and instrumentation

Unless otherwise noted, all starting materials and reagents were obtained from Sigma-Aldrich and were used without further purification. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a Bruker Avance III 600 spectrometer at frequencies of 600 MHz and 150 MHz, respectively, with $CDCl_3$ serving as NMR solvent. All chemical shifts are reported in parts per million (δ) downfield from the signal of $Si(CH_3)_4$ added to the $CDCl_3$. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), qn (quintet) or m (multiplet). High resolution mass spectra (HRMS) were recorded on a DFS high resolution magnetic sector mass spectrometer (Thermo Electron Corporation) in electron ionization (EI) mode or on a Bruker micrOTOF-Q II mass spectrometer in atmospheric-pressure chemical ionization (APCI) mode. Melting points (mp) were determined with a Buchi M-545 melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was carried out using silica gel 60 (Merck) with UV254 fluorescent indicator. The purities of the synthesized compounds were estimated via HPLC analyses. For this purpose an Agilent 1100 HPLC system equipped with a quaternary pump and an Agilent 1100 series diode array detector were employed (see Supplementary Material). HPLC grade acetonitrile (Merck) and Milli-Q water (Millipore) were used for the chromatography. The mercaptan starting materials (**6**), which were not commercially available, were synthesized according to the literature procedure.[33] Fluorescence spectrophotometry was carried out with a Varian Cary Eclipse fluorescence spectrophotometer while UV-VIS spectrophotometry was carried out with a Shimadzu MultiSpec-1501 UV-VIS photodiode array spectrophotometer. Microsomes from insect cells containing recombinant human MAO-A and –B (5 mg/mL), and kynuramine.2HBr were obtained from Sigma-Aldrich.

4.2. Synthesis of 8-sulfanylcaffeine analogues (1, 3 and 4)

To a solution of NaOH (4 mmol) in 3.5 mL water was added a volume of 3.5 mL ethanol. The mixture was cooled in an ice bath and an appropriate thiol (4 mmol) was added. While the reaction mixture was stirred, 8-chlorocaffeine (4 mmol) was added in a single portion to yield a suspension. The reaction was heated under reflux for 60 min and then cooled to room

temperature. The white precipitate present in the reaction was collected by filtration and washed with 30 mL ethanol. The product was recrystallized from 30 mL ethanol at room temperature and the crystals were washed with 30 mL ethanol.[31] TLC was conducted using silica gel sheets (Merck) and ethylacetate/n-hexane (60:30) as mobile phase. (*Rf* ~ 0.45).

4.2.1. 8-[(Phenylethyl)sulfanyl]caffeine (1a)

The title compound was prepared from 2-phenylethane-1-thiol in a yield of 83%: mp 95 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 3.04 (t, 2H, J = 7.5 Hz), 3.36 (s, 3H), 3.48 (t, 2H, J = 7.5 Hz); 3.55 (s, 3H), 3.78 (s, 3H), 7.21 (m, 3H), 7.28 (m, 2H); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.8, 29.6, 32.1, 33.8, 36.0, 108.5, 126.7, 128.5, 139.3, 148.4, 150.8, 151.4, 154.5; EI-HRMS m/z: calcd for $C_{16}H_{18}N_4O_2S$, 330.1151, found 330.1147; Purity (HPLC): 98%.

4.2.2. 8-{[2-(4-Chlorophenyl)ethyl]sulfanyl}caffeine (1b)

The title compound was prepared from 2-(4-chlorophenyl)ethane-1-thiol in a yield of 69%: mp 164 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) *δ* 3.01 (t, 2H, J = 7.5 Hz), 3.35 (s, 3H), 3.45 (t, 2H, J = 7.5 Hz); 3.54 (s, 3H), 3.78 (s, 3H), 7.12 (d, 2H, J = 8.3 Hz), 7.23 (d, 2H, J = 8.3 Hz) ; ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) *δ* 27.8, 29.6, 32.1, 33.6, 35.4, 108.5, 128.6, 129.9, 132.5, 137.7, 148.4, 150.5, 151.4, 154.5; EI-HRMS *m*/*z*: calcd for $C_{16}H_{17}ClN_4O_2S$, 364.0761, found 364.0756; Purity (HPLC): 99%.

4.2.3. 8-{[2-(4-Bromophenyl)ethyl]sulfanyl}caffeine (1c)

The title compound was prepared from 2-(4-bromophenyl)ethane-1-thiol in a yield of 38%: mp 165 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) *δ* 3.01 (t, 2H, J = 7.5 Hz), 3.37 (s, 3H), 3.45 (t, 2H, J = 7.5 Hz); 3.54 (s, 3H), 3.79 (s, 3H), 7.07 (d, 2H, J = 8.7 Hz), 7.39 (d, 2H, J = 8.3 Hz); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) *δ* 27.8, 29.7, 32.1, 33.6, 35.5, 108.6, 120.6, 130.3, 131.6, 138.2, 148.4, 150.5, 151.5, 154.5; EI-HRMS *m*/*z*: calcd for $C_{16}H_{17}BrN_4O_2S$, 408.0256, found 408.0258; Purity (HPLC): 97%.

4.2.4. 8-{[2-(4-Fluorophenyl)ethyl]sulfanyl}caffeine (1d)

The title compound was prepared from 2-(4-fluorophenyl)ethane-1-thiol in a yield of 53%: mp 145 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 3.01 (t, 2H, J = 7.5 Hz), 3.36 (s, 3H), 3.45 (t, 2H, J = 7.5 Hz); 3.55 (s, 3H), 3.78 (s, 3H), 6.97 (t, 2H, J = 8.7 Hz), 7.16 (m, 2H); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.8, 29.7, 32.1, 33.8, 35.2, 108.6, 115.4, 130.0, 135.0, 135.1, 148.4, 150.7, 151.4, 154.5, 160.9, 162.5; EI-HRMS *m*/*z*: calcd for $C_{16}H_{17}FN_4O_2S$, 348.1056, found 348.1045; Purity (HPLC): 99%.

4.2.5. 8-{[2-(4-(Trifluoromethyl)phenyl)ethyl]sulfanyl}caffeine (1e)

The title compound was prepared from 2-[4-(trifluoromethyl)phenyl]ethane-1-thiol in a yield of 37%: mp 178 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 3.12 (t, 2H, J = 7.5 Hz), 3.36 (s, 3H), 3.49 (t, 2H, J = 7.5 Hz); 3.54 (s, 3H), 3.79 (s, 3H), 7.33 (d, 2H, J = 7.9 Hz), 7.54 (d, 2H, J = 8.2 Hz); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.8, 29.6, 32.1, 33.3, 35.8, 108.6, 121.4, 123.2, 125.0, 125.5 (q), 126.8, 128.7, 128.9, 129.2, 129.4, 143.3, 148.4, 150.4, 151.4, 154.5; EI-HRMS *m*/*z*: calcd for $C_{17}H_{17}F_3N_4O_2S$, 398.1024, found 398.1007; Purity (HPLC): 99%.

4.2.6. 8-{[2-(4-Methylphenyl)ethyl]sulfanyl}caffeine (1f)

The title compound was prepared from 2-(4-methylphenyl)ethane-1-thiol in a yield of 36%: mp 122 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 2.29 (s, 3H), 2.99 (t, 2H, J = 7.5 Hz), 3.36 (s, 3H), 3.46 (t, 2H, J = 7.5 Hz), 3.55 (s, 3H), 3.78 (s, 3H), 7.09 (m, 4H); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 21.0, 27.8, 29.6, 32.1, 34.0, 35.6, 108.4, 128.4, 129.2, 136.3, 148.4, 150.9, 151.5, 154.5; EI-HRMS *m*/*z*: calcd for $C_{17}H_{20}N_4O_2S$, 344.1307, found 344.1307; Purity (HPLC): 97%.

4.2.7. 8-{[2-(4-Methoxyphenyl)ethyl]sulfanyl}caffeine (1g)

The title compound was prepared from 2-(4-methoxyphenyl)ethane-1-thiol in a yield of 45%: mp 125 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 2.98 (t, 2H, J = 7.5 Hz), 3.36 (s, 3H), 3.44 (t, 2H, J = 7.5 Hz); 3.55 (s, 3H), 3.76 (s, 3H), 3.79(s, 3H), 6.82 (d, 2H, J = 8.7 Hz), 7.11 (d, 2H, J = 8.7 Hz); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.8, 29.7, 32.1, 34.1, 35.2, 55.2, 108.5, 113.9, 129.5, 131.4, 148.5, 151.0, 151.5, 154.5, 158.4; EI-HRMS *m*/*z*: calcd for $C_{17}H_{20}N_4O_3S$, 360.1256, found 360.1248; Purity (HPLC): 99%.

4.2.8. 8-{[2-(3-Chlorophenyl)ethyl]sulfanyl}caffeine (1h)

The title compound was prepared from 2-(3-chlorophenyl)ethane-1-thiol in a yield of 6.4%: mp 125.5–126.1 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 3.02 (t, 2H, J = 7.2 Hz), 3.36 (s, 3H), 3.47 (t, 2H, J = 7.2 Hz); 3.55 (s, 3H), 3.78 (s, 3H), 7.08 (d, 1H, J = 7.5 Hz), 7.16–7.22 (m, 3H); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.8, 29.7, 32.1, 33.5, 35.8, 108.6, 126.8, 126.9, 128.7, 129.7, 134.3, 141.2, 148.4, 150.5, 151.5, 154.5; EI-HRMS m/z: calcd for $C_{16}H_{17}ClN_4O_2S$, 364.0761, found 364.0757; Purity (HPLC): 97%.

4.2.9. 8-{[2-(3-Bromophenyl)ethyl]sulfanyl}caffeine (1i)

The title compound was prepared from 2-(3-bromophenyl)ethane-1-thiol in a yield of 32%: mp 122.6–124.3 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 3.01 (t, 2H, J = 7.2 Hz), 3.36 (s, 3H), 3.47 (t, 2H, J = 7.2 Hz); 3.55 (s, 3H), 3.77 (s, 3H), 7.11–7.15 (m, 2H), 7.31–7.32 (m, 1H), 7.35 (m, 1H); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.8, 29.7, 32.1, 33.5, 35.8, 108.6, 122.5, 127.3, 129.8, 130.0, 131.7, 141.5, 148.4, 150.5, 151.5, 154.5; EI-HRMS m/z: calcd for $C_{16}H_{17}BrN_4O_2S$, 408.0256, found 408.0251; Purity (HPLC): 99%.

4.2.10. 8-{[2-(3-(Trifluoromethyl)phenyl)ethyl]sulfanyl}caffeine (1j)

The title compound was prepared from 2-[3-(trifluoromethyl)phenyl]ethane-1-thiol in a yield of 18%: mp 130.4–132.7 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 3.12 (t, 2H, J = 7.5 Hz), 3.37 (s, 3H), 3.50 (t, 2H, J = 7.5 Hz), 3.55 (s, 3H), 3.78 (s, 3H), 3.40–3.42 (m, 2H), 3.46–3.48 (m, 2H; ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ27.8, 29.7, 32.12, 33.4, 35.9, 108.6, 123.1 (d), 125.3 (d), 129.0, 130.9 (q), 132.0, 140.2, 148.4, 150.4, 151.5, 154.5; EI-HRMS m/z: calcd for $C_{17}H_{17}F_3N_4O_2S$, 398.1024, found 398.1031; Purity (HPLC): 99%.

4.2.11. 8-{[2-(3-Methylphenyl)ethyl]sulfanyl}caffeine (1k)

The title compound was prepared from 2-(3-methylphenyl)ethane-1-thiol in a yield of 28%: mp 110.5–112.0 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 2.31 (s, 3H), 3.00 (t, 2H, J = 7.5 Hz), 3.37 (s, 3H), 3.48 (t, 2H, J = 7.5 Hz), 3.56 (s, 3H), 3.79 (s, 3H), 7.00–7.03 (m, 3H), 7.17 (t, 1H, J = 7.5 Hz); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 21.3, 27.8, 29.7, 32.1, 33.9, 36.0, 108.5, 125.5, 127.4, 128.4, 129.3, 138.1, 139.3, 148.5, 150.9, 151.5, 154.5; EI-HRMS *m/z*: calcd for $C_{17}H_{20}N_4O_2S$, 344.1307, found 344.1259; Purity (HPLC): 99%.

4.2.12. 8-{[2-(3-Methoxyphenyl)ethyl]sulfanyl}caffeine (1l)

The title compound was prepared from 2-(3-methoxyphenyl)ethane-1-thiol in a yield of 41%: mp 126.6–127.5 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 3.01 (t, 2H, J = 7.5 Hz), 3.36 (s, 3H), 3.48 (t, 2H, J = 7.5 Hz), 3.55 (s, 3H), 3.76 (s, 3H), 3.78 (s, 3H), 6.74 (m, 2H), 6.79 (d, 1H, J = 7.5 Hz), 7.20 (t, 1H, J = 7.5 Hz); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.8, 29.7, 32.1, 33.8, 36.1, 55.1, 108.5, 111.6, 114.6, 120.9, 129.5, 140.9, 148.5, 150.8, 151.5, 154.5, 159.7; EI-HRMS *m/z*: calcd for $C_{17}H_{20}N_4O_3S$, 360.1256, found 360.1255; Purity (HPLC): 97%.

4.2.13. 8-[(Phenylpropyl)sulfanyl]caffeine (3a)

The title compound was prepared from 3-phenylpropane-1-thiol in a yield of 21%: mp 76.4–78.3 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 2.07 (qn, 2H, J = 7.5 Hz), 2.75 (t, 2H, J = 7.5 Hz), 3.24 (t, 2H, J = 7.5 Hz), 3.36 (s, 3H), 3.50 (s, 3H), 3.81 (s, 3H), 7.16–7.19 (m, 3H), 7.24–7.28 (m, 2H); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.8, 29.6, 31.1, 32.0, 32.1, 34.5, 108.5, 126.1, 128.4, 128.4, 140.7, 148.4, 151.0, 151.5, 154.5; EI-HRMS m/z: calcd for $C_{17}H_{20}N_4O_2S$, 344.1307, found 344.1308; Purity (HPLC): 99%.

4.2.14. 8-{[3-(4-Chlorophenyl)propyl]sulfanyl}caffeine (3b)

The title compound was prepared from 3-(4-chlorophenyl)propane-1-thiol in a yield of 27%: mp 95.5–98.0 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 2.04 (qn, 2H, J = 7.5 Hz), 2.72 (t, 2H, J = 7.5 Hz), 3.22 (t, 2H, J = 7.5 Hz), 3.36 (s, 3H), 3.50 (s, 3H), 3.81 (s, 3H), 7.09 (d, 2H, J = 8.7 Hz), 7.22 (d, 2H, J = 8.3 Hz); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.8, 29.6, 31.0, 31.7, 32.1, 33.8, 108.5, 128.5, 129.7, 131.9, 139.1, 148.4, 150.8, 151.5, 154.5; EI-HRMS m/z: calcd for $C_{17}H_{19}ClN_4O_2S$, 378.0917, found 378.0903; Purity (HPLC): 94%.

4.2.15. 8-{[3-(3-Chlorophenyl)propyl]sulfanyl}caffeine (3c)

The title compound was prepared from 3-(3-chlorophenyl)propane-1-thiol in a yield of 24%: mp 87.7–89.3 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 2.07 (qn, 2H, J = 7.5 Hz), 2.73 (t, 2H, J = 7.5 Hz), 3.23 (t, 2H, J = 7.5 Hz), 3.36 (s, 3H), 3.50 (s, 3H), 3.81 (s, 3H), 7.04 (d, 1H, J = 7.5 Hz), 7.15 (m, 2H), 7.19 (m, 1H); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.8, 29.7, 30.8, 31.7, 32.1, 34.2, 108.5, 126.3, 126.6, 128.5, 129.7, 134.2, 142.7, 148.4, 150.8, 151.5, 154.5; EI-HRMS m/z: calcd for $C_{17}H_{19}ClN_4O_2S$, 378.0917, found 378.0902; Purity (HPLC): 98%.

4.2.16. 8-[(3-Chlorobenzyl)sulfanyl]caffeine (4a)

The title compound was prepared from (3-chlorophenyl)methanethiol in a yield of 51%: mp 156.5–158.1 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 3.35 (s, 3H), 3.56 (s, 3H), 3.73 (s, 3H), 4.39 (s, 2H), 7.21 (m, 3H), 7.38 (s, 1H); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.8, 29.7, 32.2, 36.3, 108.8, 127.1, 128.0, 129.3, 129.9, 134.3, 138.7, 148.3, 149.6, 151.4, 154.5; EI-HRMS m/z: calcd for $C_{15}H_{15}ClN_4O_2S$, 350.0604, found 350.0600; Purity (HPLC): 99%.

4.2.17. 8-[(3-Bromobenzyl)sulfanyl]caffeine (4b)

The title compound was prepared from (3-bromophenyl)methanethiol in a yield of 46%: mp 143.9–145.7 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 3.35 (s, 3H), 3.56 (s, 3H), 3.73 (s, 3H), 4.38 (s, 2H), 7.14 (t, 1H, J = 7.9 Hz), 7.26 (d, 1H, J = 7.5 Hz), 7.37 (d, 1H, J = 8.3 Hz), 7.54 (s, 1H); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.8, 29.7, 32.2, 36.3, 108.8, 122.4, 127.5, 130.1, 130.9, 132.2, 139.0, 148.3, 149.5, 151.4, 154.5; EI-HRMS m/z: calcd for $C_{15}H_{15}BrN_4O_2S$, 394.0099, found 394.0113; Purity (HPLC): 99%.

4.3. Synthesis of 8-sulfinylcaffeine analogues (2a–d)

The appropriate 8-sulfanylcaffeine analogue (4 mmol) was dissolved in a mixture of glacial acetic acid (8 mL) and acetic anhydride (2 mL). The reaction was stirred at room temperature and 2 mL of a 30% hydrogen peroxide solution was added dropwise. The reaction was stirred for 4 h and 25 mL of distilled water was added. The product, which precipitated from the reaction mixture, was collected by filtration and purified by recrystallization from ethyl acetate.[31] TLC of the product was conducted using silica gel sheets (Merck) and ethylacetate/n-hexane (60:30) as mobile phase (*Rf* ~ 0.23).

4.3.2. 8-{[2-(4-Chlorophenyl)ethyl]sulfinyl}caffeine (2a)

The title compound was prepared from 8-{[2-(4-chlorophenyl)ethyl]sulfanyl}caffeine in a yield of 72%: mp 155 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) *δ* 3.12 (m, 2H, CH_2), 3.37 (s, 3H, NCH_3), 3.50 (m, 1H, $O{=}SCH_2$), 3.51 (s, 3H, NCH_3); 3.73 (m, 1H, $O{=}SCH_2$), 4.20 (s, 3H, NCH_3), 7.15 (d, 2H, J = 8.3 Hz, ArH); 7.23 (d, 2H, J = 8.3 Hz, ArH); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) *δ* 27.5, 28.1, 29.8, 33.3, 53.7, 110.0, 128.9, 129.9, 132.8, 136.2, 147.1, 149.1, 151.2, 155.0; APCI-HRMS *m/z*: calcd for $C_{16}H_{18}ClN_4O_3S$ 381.0788, found 381.0789; Purity (HPLC): 92%.

4.3.3. 8-{[2-(4-Bromophenyl)ethyl]sulfinyl}caffeine (2b)

The title compound was prepared from 8-{[2-(4-bromophenyl)ethyl]sulfanyl}caffeine in a yield of 45%: mp 166 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 3.11 (t, 2H, J = 7.5 Hz, CH_2), 3.37 (s, 3H, NCH_3), 3.50 (m, 1H, O=SCH_2), 3.51 (s, 3H, NCH_3); 3.74 (m, 1H, O=SCH_2), 4.20 (s, 3H, NCH_3), 7.09 (d, 2H, J = 7.9 Hz, ArH); 7.38 (d, 2H, J = 7.5 Hz, ArH); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.5, 28.1, 29.8, 33.3, 53.6, 110.0, 120.8, 130.3, 131.8, 136.7, 147.1, 149.1, 151.2, 155.0; EI-HRMS *m*/*z*: calcd for $C_{16}H_{17}BrN_4O_3S$ 424.0205, found 424.0200; Purity (HPLC): 96%.

4.3.4. 8-{[2-(4-Fluorophenyl)ethyl]sulfinyl}caffeine (2c)

The title compound was prepared from 8-{[2-(4-fluorophenyl)ethyl]sulfanyl}caffeine in a yield of 38.8%: mp 126 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 3.13 (m, 2H, CH_2), 3.37(s, 3H, NCH_3), 3.50 (m, 1H, O=SCH_2), 3.53 (m, 3H, NCH_3), 3.74 (m, 1H, O=SCH_2), 4.21 (s, 3H, NCH_3), 6.98 (t, 2H, J = 6.4 Hz, ArH); 7.18 (m, 2H, ArH); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.4, 28.1, 29.8, 33.3, 54.0, 110.1, 115.6, 130.1, 133.5, 147.2, 149.3, 151.2, 155.0, 161.0, 162.6; EI-HRMS *m*/*z*: calcd for $C_{16}H_{17}FN_4O_3S$ 364.1006, found 364.0995; Purity (HPLC): 96%.

4.3.5. 8-(Benzylsulfinyl)caffeine (2d)

The title compound was prepared from 8-(benzylsulfanyl)caffeine[30] in a yield of 42%: mp 165.6–169.7 ℃ (ethanol). ^{1}H NMR (Bruker Avance III 600, $CDCl_3$) δ 3.20 (s, 3H, NCH_3), 3.44 (s, 3H, NCH_3), 3.58 (s, 3H, NCH_3), 4.60 (m, 2H, O=SCH_2), 7.15–7.17 (m, 2H, ArH), 7.30–7.35 (m, 3H, ArH); ^{13}C NMR (Bruker Avance III 600, $CDCl_3$) δ 27.7, 29.6, 32.2, 59.3, 108.8, 128.5, 128.6, 129.2, 130.5, 146.9, 149.4, 150.8, 154.4; EI-HRMS m/z: calcd for $C_{15}H_{16}N_4O_3S$, 332.0943, found 332.0932; Purity (HPLC): 99%.

4.4. Synthesis of mercaptan reagents 6

Thiourea (7 mmol) was dissolved in 13.5 mL ethanol and the appropriate alkylbromide (7 mmol) was added. The mixture was stirred and heated under reflux for 2 hours after which the reaction became homogenous. The reaction was allowed to cool to room temperature and the ethanol solvent was removed under reduced pressure. A solution of NaOH (10.5 mmol) in 8.75 mL water was added to the residue, and the reaction mixture was heated under reflux (120 ℃) for 2 hours. The reaction was subsequently cooled to room temperature and aqueous H_2SO_4 (15%)

was added. The mercaptan separated as oil droplets and was extracted to diethylether (30 mL). The organic phase was washed twice with water, dried over anhydrous Na_2SO_4 and removed under reduced pressure. This yielded the desired mercaptans which were used without further purification for the synthesis of **1**, **3** and **4**.

4.5. Inhibition of human MAO

Microsomes prepared from insect cells, which contain recombinant human MAO-A and –B, were obtained from Sigma-Aldrich. Potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl 20.2 mM) was used for all the enzyme reactions and dilutions. The final volume of the enzyme reactions was 500 µL, and each reaction contained kynuramine (45 µM for MAO-A and 30 µM for MAO-B), various concentrations of the test inhibitors (0–100 µM) and 4% DMSO as co-solvent. After the reactions were started with the addition of MAO-A or –B (0.0075 mg protein/mL), they were incubated in a water bath at 37 ºC for 20 min. The reactions were subsequently terminated by the addition of 400 µL NaOH (2 N). A volume of 1000 µL water was added to each reaction, the reactions were centrifuged at 16,000 *g* (10 min) and the concentrations of the MAO generated 4-hydroxyquinoline in the supernatants were measured by fluorescence spectrophotometry (λ_{ex} = 310 nm; λ_{em} = 400 nm).[29,30,41] For the concentration calculations, linear calibration curves were constructed by measuring the fluorescence of samples containing known amounts of 4-hydroxyquinoline (0.047–1.56 µM) dissolved in 500 µL potassium phosphate buffer. To each calibration sample, 400 µL NaOH (2 N) and 1000 µL water were added. The MAO catalytic rates were calculated from the endpoint concentration of 4-hydroxyquinoline (nM) in the supernatants, the incubation time (20 min) and the enzyme concentration (0.0075 mg protein/mL), and were expressed as nmol 4-hydroxyquinoline formed/min.mg protein. For each inhibitor evaluated, the appropriate control samples were analyzed in order to confirm that the test inhibitors do not fluoresce or quench the fluorescence of 4-hydroxyquinoline under the assay conditions. From the enzyme rate data, sigmoidal dose–response curves (plots of the initial rate of kynuramine oxidation versus the logarithm of inhibitor concentration) were constructed. For the construction of each sigmoidal curve, at least 6 different inhibitor concentrations were employed, and the inhibitor concentrations covered at least 3 orders of magnitude. IC_{50} values were estimated from the sigmoidal curves with the aid of the Prism 5 software package (GraphPad), employing the one site competition model. IC_{50} values were determined in triplicate and are expressed as mean ± standard deviation (SD).

4.6. Time-dependency of MAO-A and –B inhibition

The time-dependency of the inhibition of MAO-A and –B by a selected inhibitor, compound **1b**, was investigated. Compound **1b** was firstly incubated with recombinant human MAO-A and –B (0.03 mg/mL) for different periods of time (0, 15, 30, 60 min) at 37 ºC. The buffer used for this purpose was potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl), the reaction volume was 250 µL and the concentration of **1b** was equal to twofold the measured IC_{50} value for the inhibition of MAO-A (16.92 µM) and MAO-B (0.04 µM), respectively. Kynuramine, dissolved in potassium phosphate buffer, was subsequently added to these reactions at a volume of 250 µL. The final volume of the reactions was 500 µL, the MAO enzyme concentrations were 0.015 mg/mL and concentrations of **1b** were equal to the IC_{50} values for the inhibition of MAO-A and –B, respectively. The concentrations of kynuramine were 45 µM and 30 µM for MAO-A and –B, respectively. The reactions were subsequently incubated at 37 ℃ for a further 15 min and terminated with the addition of 400 µL NaOH (2 N). A volume of 1000 µL distilled water was added to each reaction and the rates of formation the MAO generated of 4-hydroxyquinoline were measured by fluorescence spectrophotometry as described above. All measurements were carried out in triplicate and are expressed as mean ± SD.[42] One-way analyses of variances (ANOVA) with Tukey's post hoc test was used to determine if statistical differences exist between the means of the MAO catalytic rates recorded at each time point.

4.7. Recovery of enzyme activity after dilution

Compound **1b** [IC_{50}(MAO-B) = 0.02 µM] or **1j** [IC_{50}(MAO-B) = 0.017 µM] at 10 × IC_{50} and 100 × IC_{50} were preincubated with recombinant human MAO-B (0.75 mg/mL) for 30 min at 37 ºC. The reaction solvent was potassium phosphate buffer (pH 7.4, 100 mM, made isotonic with KCl). DMSO (4%) served as co-solvent in all preincubations. A control reaction was also carried out in the absence of inhibitor. The reactions were subsequently diluted 100-fold with the addition of kynuramine to yield final concentrations of kynuramine equal to 30 µM, and of MAO-B equal to 0.0075 mg/mL. The final concentrations of **1a** and **1j** were 0.1 × IC_{50} and 1 × IC_{50}. The reactions were incubated for a further 20 min at 37 ℃, terminated and the residual rates of 4-hydroxyquinoline formation were measured as described above.[43] For comparison, (R)-deprenyl (IC_{50} = 0.079 µM) was similarly preincubated with MAO-B at 10 × IC_{50} and diluted 100-fold with the addition of kynuramine to yield a final concentration of (R)-deprenyl equal to 0.1 × IC_{50}.[43]

4.8. The construction of Lineweaver-Burk plots

The mode of MAO-B inhibition was investigated with the aid of a set consisting of four Lineweaver–Burk plots. The first plot was constructed in the absence of inhibitor while the remaining three plots were constructed in the presence of different concentrations of an inhibitor. For this study, compound **1b** was selected as representative inhibitor at the following concentrations: 0.005 µM, 0.01 µM and 0.02 µM. Kynuramine at concentrations of 15–90 µM served as substrate and recombinant human MAO-B was used at a concentration of 0.015 mg/mL. The rates of formation of the MAO-B generated 4-hydroxyquinoline were measured by fluorescence spectrophotometry as described above. Linear regression analysis was performed using Prism 5.[42]

4.9. Kinetic parameters

Kinetic parameters that were used in the calculations in this study are the K_m values for the oxidation of kynuramine by MAO-A and –B of 16.1 µM and 22.7 µM, respectively,[28] and a k_{cat} value of 300 min^{-1} for the oxidation of benzylamine by human MAO-B.[44] The calculation of K_i values according to the equation by Morrison was carried out with the Prism 5 software package.[38] For this purpose the concentration of MAO-B used in the IC_{50} determinations (0.0075 mg protein/mL) was estimated at 0.001 µM ($V_{max} = k_{cat} \times [E_0]$) using benzylamine as substrate.[45]

4.10. Shake flask method for LogP determination

n-Octanol (analytical reagent from Sigma-Aldrich) and distilled water were mutually saturated. In a 5 mL glass vessel, 2 mL of each of the n-octanol and water phases were placed followed by the analyte to yield a final analyte concentration of 1 mM. The vessels were shaken by hand for 5 min and centrifuged at 4,000 *g* for 10 min. The n-octanol phase was diluted 20-fold into neat n-octanol and the absorbance of the resulting solution was recorded at an absorbance maximum of 292 nm. The concentrations of the analytes in the n-octanol phase were determined by employing the molar extinction coefficients recorded for each analyte in n-octanol: **1a**, 17600 M^{-1}; **1c**, 18100 M^{-1}; **1e**, 13600 M^{-1}; **1f**, 16500 M^{-1}; **1g**, 17800 M^{-1}; **1j**, 17700 M^{-1}; **3a**, 19200 M^{-1}; **3b**, 19700 M^{-1}; **3c**, 18400 M^{-1}. Without further dilution, the concentration of the analyte in the water phase was determined by HPLC analysis. For this purpose, a Venusil XBP C18 column (4.60 × 150 mm, 5 µm) was used and the mobile phase consisted of 75% acetonitrile and 25% MilliQ water at a flow rate of 1 mL/min. A volume of 20 µL of the water

phases were injected into the HPLC system and the eluent was monitored at a wavelength of 292 nm. To quantify the analytes, linear calibration curves were constructed by similarly analyzing known amounts of the analytes (0.05–10 μM) in 50% acetonitrile. These studies were carried out in triplicate for each analyte and the LogP values are expressed as mean ± SD.

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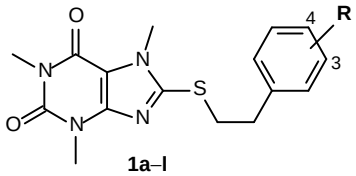
Table 1. The IC_{50} values for the inhibition of recombinant human MAO-A and –B by compounds **1a**–**l**.



1a–**l**

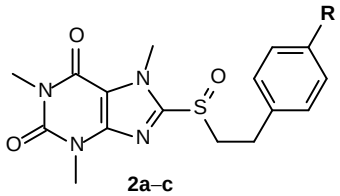
		IC_{50} (μM)[a]		
	R	MAO-A	MAO-B	SI[b]
1a	H	18.7 ± 0.31	0.271 ± 0.006	69
1b	4-Cl	8.46 ± 0.98	0.020 ± 0.005	423
1c	4-Br	108 ± 18.2	0.019 ± 0.003	5684
1d	4-F	14.7 ± 0.94	0.071 ± 0.012	207
1e	4-CF_3	No inh[c]	0.019 ± 0.003	–
1f	4-CH_3	125 ± 9.12	0.072 ± 0.012	1736
1g	4-OCH_3	168 ± 9.12	0.023 ± 0.003	7304
1h	3-Cl	5.66 ± 0.187	0.043 ± 0.0085	132
1i	3-Br	5.70 ± 1.87	0.040 ± 0.0017	143
1j	3-CF_3	141 ± 26.8	0.017 ± 0.007	8294
1k	3-CH_3	12.0 ± 2.39	0.051 ± 0.005	235
1l	3-OCH_3	48.1 ± 5.93	0.125 ± 0.021	385

[a] All values are expressed as the mean ± SD of triplicate determinations.

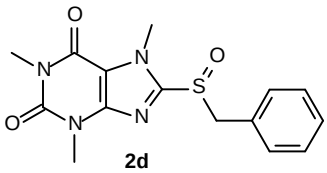
[b] The selectivity index is the selectivity for the MAO-B isoform and is given as the ratio of IC_{50}(MAO-A)/IC_{50}(MAO-B).

[c] No inhibition observed at a maximal tested concentration of 100 μM.

Table 2. The IC_{50} values for the inhibition of recombinant human MAO-A and –B by compounds **2a**–**d**.



2a–**c**



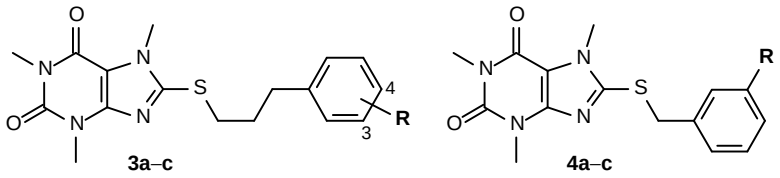
2d

		IC_{50} (μM)[a]		
	R	MAO-A	MAO-B	SI[b]
2a	Cl	203 ± 14.4	0.781 ± 0.048	260
2b	Br	57.3± 12.8	0.471 ± 0.057	122
2c	F	239 ± 17.2	3.50 ± 0.083	68
2d	H	250 ± 18.1	131 ± 33.4	2

[a] All values are expressed as the mean ± SD of triplicate determinations.

[b] The selectivity index is the selectivity for the MAO-B isoform and is given as the ratio of IC_{50}(MAO-A)/IC_{50}(MAO-B).

Table 3. The IC_{50} values for the inhibition of recombinant human MAO-A and –B by compounds **3a**–**c** and **4a**–**c**.



		IC_{50} (μM)[a]		
	R	MAO-A	MAO-B	SI[b]
3a	H	6.48 ± 0.802	0.500 ± 0.041	13
3b	4-Cl	0.708 ± 0.124	0.061 ± 0.004	12
3c	3-Cl	3.53 ± 0.381	0.062 ± 0.008	57
4a	Cl	6.43 ± 1.55	0.227 ± 0.024	28
4b	Br	37.9 ± 1.39	0.199 ± 0.039	190
4c	H	8.22[c]	1.86[c]	4.4

[a] All values are expressed as the mean ± SD of triplicate determinations.

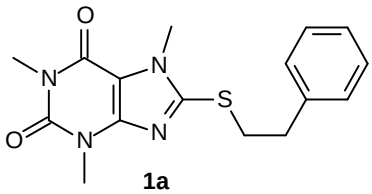
[b] The selectivity index is the selectivity for the MAO-B isoform and is given as the ratio of IC_{50}(MAO-A)/IC_{50}(MAO-B).

[c] Taken form reference.[30]

Table 4. The LogP values of selected compounds as estimated by the shake flask method.

	LogP value[a]		LogP value[a]
1a	3.22 ± 0.008	**1j**	3.14 ± 0.019
1c	2.98 ± 0.029	**3a**	3.30 ± 0.030
1e	3.21 ± 0.024	**3b**	3.59 ± 0.059
1f	3.15 ± 0.008	**3c**	3.76 ± 0.051
1g	2.55 ± 0.026		

[a] All values are expressed as the mean ± SD of triplicate determinations.



1a

Figure 1. The structure of 8-[(phenylethyl)sulfanyl]caffeine (**1a**).

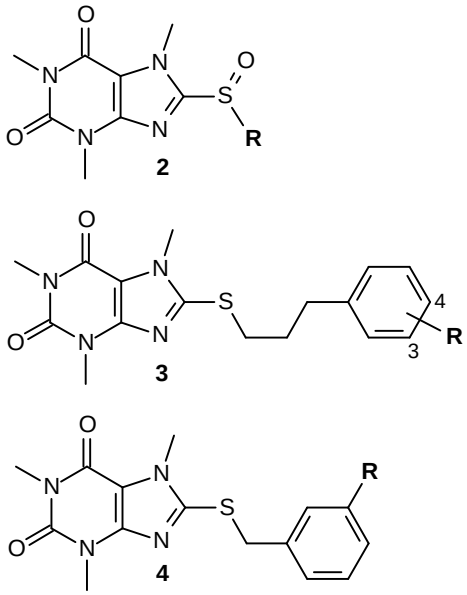


Figure 2. The general structures of the 8-sulfinylcaffeine analogues (**2**), 8-[(phenylpropyl)sulfanyl]caffeine analogues (**3**) and 8-(benzylsulfanyl)caffeine analogues (**4**) which were examined in this study.

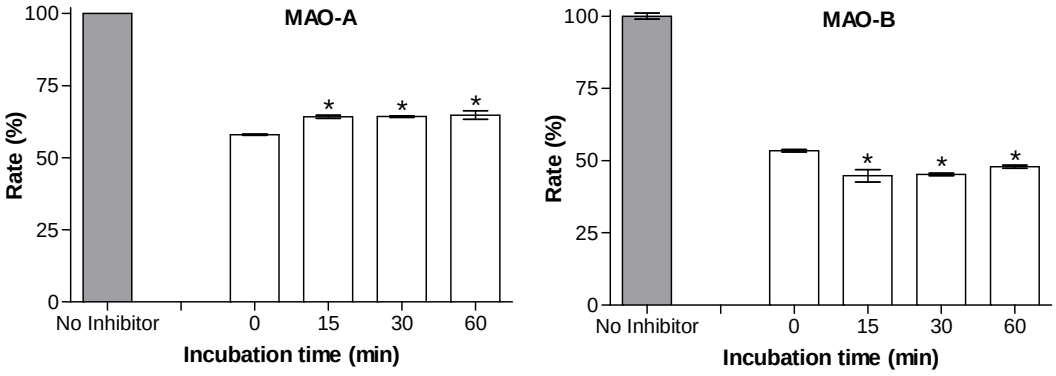


Figure 3. The time-dependency of inhibition of recombinant human MAO-A and –B by inhibitor **1b**. The MAO enzymes were preincubated for various periods of time (0–60 min) in the presence of **1b** at concentrations of 16.92 µM and 0.04 µM, respectively. These concentrations are equal to twofold the recorded IC_{50} values for the inhibition of MAO-A and –B, respectively. The preincubation mixtures were diluted twofold and the residual MAO catalytic rates were measured. *Statistically significantly different from the mean at t = 0.

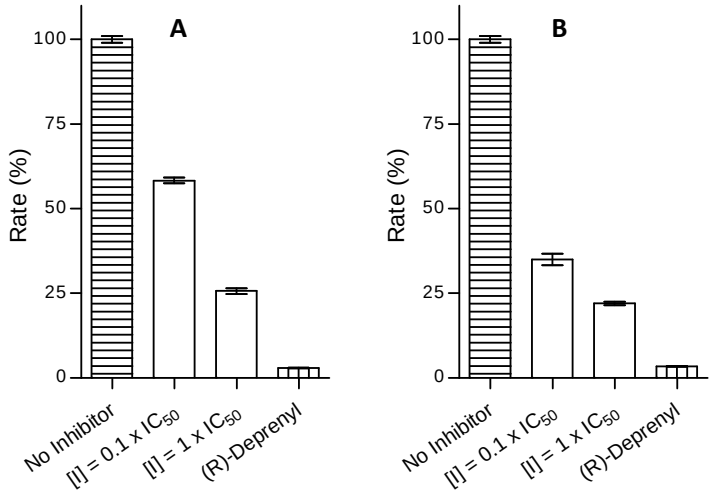


Figure 4. Reversibility of inhibition of MAO-B by **1b** (panel A) and **1j** (panel B). The enzyme was preincubated with **1b** or **1j** at 10 × IC_{50} and 100 × IC_{50} for 30 min and then diluted to 0.1 × IC_{50} and 1 × IC_{50}, respectively. As control, MAO-B was also preincubated with (R)-deprenyl at 10 × IC_{50} and subsequently diluted to 0.1 × IC_{50}. The residual enzyme activities were subsequently measured.

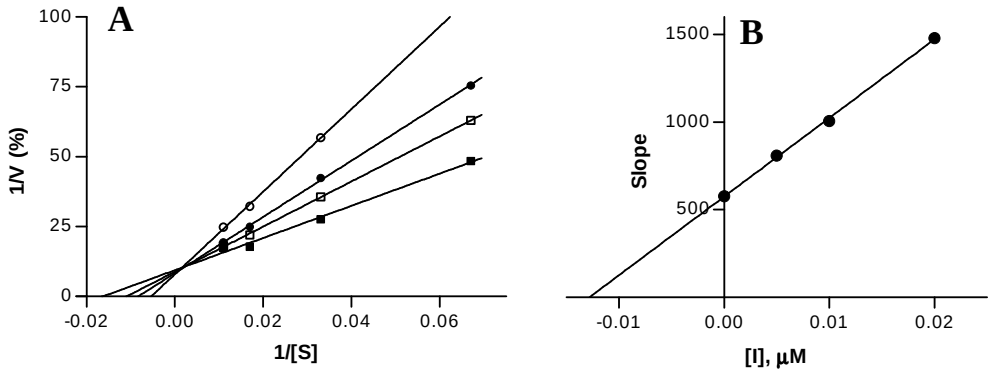
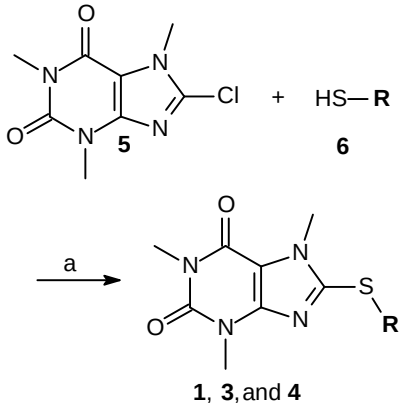
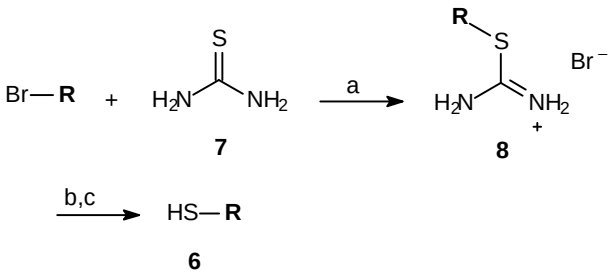


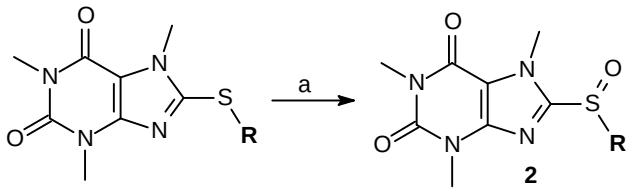
Figure 5. Lineweaver-Burk plots of the oxidation of kynuramine by recombinant human MAO-B (Panel A). The plots were constructed in the absence (filled squares) and presence of various concentrations of **1b**. The concentrations of **1b** employed were 0.005 µM (open squares), 0.01 µM (filled circles) and 0.02 µM (open circles), respectively. A replot of the slopes of the Lineweaver-Burk plots versus inhibitor concentration (Panel B).



Scheme 1. Synthetic pathway to 8-sulfanylcaffeine analogues **1**, **3** and **4**. Reagents and conditions: (a) NaOH, H_2O/ethanol, reflux.



Scheme 2. Synthetic pathway to the mercaptans (**6**) which were required for the synthesis of 8-sulfanylcaffeine analogues. Reagents and conditions: (a) ethanol, reflux; (b) NaOH, reflux; (c) H_2SO_4.



Scheme 3. Synthetic pathway to 8-sulfinylcaffeine analogues **2**. Reagents and conditions: (a) H_2O_2, glacial acetic acid, acetic anhydride, rt.