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BY
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AALBORG UNIVERSITY
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by

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Preface

This thesis describes the research work carried out at the Faculty of Health and Medical Sciences, Department of Drug Design and Pharmacology of the University of Copenhagen between March 2018 and February 2020, with short stays at Enamine Ltd. facilities (Kiev, Ukraine). The work was part of the *SAFER* project (*S*elective *A*gonists *F*or the 5-HT_{2A} S*e*rotonin *R*eceptor), funded by the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement No. 765657. The experimental work was performed under the supervision of Jesper Langgaard Kristensen and external supervision of David E. Nichols from the Department of Medicinal Chemistry and Molecular Pharmacology of Purdue University in West Lafayette (Indiana, USA). Due to numerous incongruencies between EU and Ukrainian regulations concerning laboratory safety, the participation to this project was interrupted prior to the completion of the standard three-year period expected by a Ph.D. program. The attached papers and this dissertation were finalized after termination of the employment at the University of Copenhagen.

This thesis is based on the submitted scientific manuscripts and a published paper, that are listed below. Part of their content is explained and enriched with further details in this dissertation. As needed for assessment, signed co-authors declarations are made available to the assessment committee.

I would like to express my gratitude to my Ph.D. supervisor, Jesper Langgaard Kristensen, to have given me the opportunity to be part of the SAFER project and develop my skills as a researcher. I appreciate the support demonstrated and the effort to find alternative solutions to the project's issues.

I would also like to thank Kristian Agmund Haanes for his interest in the project, which led to testing *in vivo* some of the synthesized drug candidates.

Moreover, I thank the other Ph.D. fellows for sharing two adventurous years of work, new experiences, and laughter. Special thanks to Emil Märcher-Rørsted and Simon Jademyr for the good time spent in the laboratory together.

Finally, I definitely thank my family for all the love and support demonstrated over the years.

ABSTRACT

The 5-HT_{2A} receptor is the main excitatory serotonergic receptor in the human brain. While numerous receptor antagonists, or inverse agonists, have been extensively used to treat cognitive deficiencies and migraine, its agonists still represent a challenge in terms of therapeutic application. The reason lies in the fact that the 5-HT_{2A} receptor can signal via different effector proteins, such as $G\alpha_q$ and β-arrestin2, upon activation by an agonist. Psychedelics, such as lysergic acid diethylamide (LSD) and psilocybin, are typically 5-HT_{2A} receptor agonists, able to fully activate it and trigger the different signalling pathways generated by the activation of both these specific effector proteins $G\alpha_q$ and β-arrestin2. The intracellular transductional signalling cascades triggered by both these proteins lead to diverse physiological consequences, including antidepressant and hallucinogenic effects. Conspicuous *in vitro* and *in vivo* investigation has been carried out to determine how and why the recruitment of these proteins is linked to therapeutic and non-therapeutic effects of 5-HT_{2A} receptor agonists. Finally, it was established that structural changes in the receptor conformation cause the activation of both effector proteins in variable degree. The development of *ad hoc* pharmacological assays, able to separately monitor the recruitment of each protein, demonstrated that known agonists activate both, and that solely $G\alpha_q$ activation seems to be exclusively responsible for the hallucinogenic effect of psychedelics. This finding led to the hypothesis that silencing $G\alpha_q$ recruitment, while activating the 5-HT_{2A} receptor, might lead to retaining antidepressant and other therapeutic effects, excluding any hallucinogenic distortion of reality. The ability of a drug to behave as a receptor agonist, while preferentially triggering one signalling pathway over another, is called *functional selectivity* or *biased agonism*. Hence, this project focused on the attempt to design and synthesize the first functionally selective 5-HT_{2A} agonists, able to exclusively recruit the β-arrestin2 protein effector, thought to be responsible for the introspective and antidepressant effects of known agonists, such as psilocybin. To achieve the project goal, previous knowledge and the most recent findings on this topic were combined and probed to select and design diverse structures, belonging to the substituted phenethylamine class. The chosen compounds were synthesized, whereas they were either novel or unavailable in our laboratory sample collection, and subsequently tested *in vitro* to assess their potential functionally selective behavior. A few of the synthesized compounds were found able to preferentially activate β-arrestin recruitment when compared to LSD, being the first β-arrestin-biased 5-HT_{2A} receptor agonists reported to date. Moreover, these

compounds of interest included new structurally constrained scaffolds that were tested *in vitro* as racemic mixtures. The resulting pharmacological data demonstrated their functionally selective behavior and, most importantly, that inducing a higher degree of structural rigidity to the drug candidates might have a positive impact on the 5-HT_{2A} receptor conformation, upon binding. The beneficial conformational effect ultimately translates in the preferential activation of one of the two effector proteins, and in the possibility of exploiting biased agonism to produce safer drug candidates, while refining their potential therapeutic properties.

ABSTRAKT

5-HT2A-receptoren er den vigtigste excitatoriske serotonerge receptor i den menneskelige hjerne. Mens talrige receptorantagonister eller omvendte agonister i vid udstrækning er blevet brugt til at behandle kognitive mangler og migræne, repræsenterer dets agonister stadig en udfordring med hensyn til terapeutisk anvendelse. Årsagen ligger i, at 5-HT2A-receptoren kan signalere via forskellige effektorproteiner, såsom Gαq og β-arrestin2, ved aktivering af en agonist. Psykedeliske stoffer, såsom lysergsyrediethylamid (LSD) og psilocybin, er typisk 5-HT2A-receptoragonister, som er i stand til at aktivere og udløse de forskellige signalveje, der genereres ved aktiveringen af begge disse specifikke effektorproteiner Gαq og β-arrestin2. De intracellulære transduktionelle signalkaskader udløst af begge disse proteiner fører til forskellige fysiologiske konsekvenser, herunder antidepressive og hallucinogene virkninger. Iøjnefaldende er det at in vitro- og in vivo-undersøgelser er blevet udført for at bestemme, hvordan og hvorfor rekrutteringen af disse proteiner er forbundet med terapeutiske og ikke-terapeutiske virkninger af 5-HT2A-receptoragonister. Endelig blev det fastslået, at strukturelle ændringer i receptorkonformationen forårsager aktivering af begge effektorproteiner i variabel grad. Udviklingen af ad hoc farmakologiske assays, der er i stand til separat at overvåge rekrutteringen af hvert protein, viste, at kendte agonister aktiverer begge dele, og at udelukkende Gαq-aktivering ser ud til at være udelukkende ansvarlig for den hallucinogene virkning af psykedelika. Dette førte til hypotesen, at dæmpning af Gαq-rekruttering, samtidig med at 5-HT2A-receptoren aktiveres, kan føre til bibeholdelse af antidepressive og andre terapeutiske virkninger, og udelukke enhver hallucinogen forvrængning af virkeligheden. Et lægemiddels evne til at opføre sig som en receptoragonist, mens det fortrinsvis udløser en signalvej frem for en anden, kaldes funktionel selektivitet eller forudindtaget agonisme. Derfor fokuserede dette projekt på at designe og syntetisere de første funktionelt selektive 5-HT2A-agonister, i stand til udelukkende at rekruttere β-arrestin2-proteineffektoren, der menes at være ansvarlig for de introspektive og antidepressive virkninger af kendte agonister, såsom psilocybin. For at nå projektets mål blev tidligere viden og de seneste resultater om dette emne kombineret og undersøgt for at udvælge og designe forskellige strukturer, der tilhører den substituerede phenethylamin-klasse. De valgte forbindelser blev syntetiseret, hvorimod de enten var nye eller utilgængelige i vores laboratorieprøvesamling, og efterfølgende testet in vitro for at vurdere deres potentielle funktionelt selektive adfærd. Nogle få af de syntetiserede forbindelser blev fundet i stand til fortrinsvis at aktivere β-arrestin-rekruttering, og de er de første β-arrestin-selektive 5-HT2A-receptoragonister kendt til dato, når det sammenlignes med LSD. Desuden inkluderede disse forbindelser af interesse nye strukturelt begrænsede molekyler, der blev testet in vitro som racemiske blandinger. De resulterende farmakologiske data demonstrerede deres funktionelt selektive adfærd og, vigtigst af alt, at induktion af en højere grad af strukturel rigiditet til lægemiddelkandidaterne kan have en positiv indvirkning på 5-HT2A-

receptorkonformationen ved binding. Den gavnlige konformationelle effekt omsættes i sidste ende i den foretrukne aktivering af et af de to effektorproteiner og i muligheden for at udnytte forudindtaget agonisme til at producere sikrere lægemiddelkandidater, mens de forfiner deres potentielle terapeutiske egenskaber.

List of publications

1. **Discovery of β-Arrestin-Biased 25CN-NBOH-Derived 5-HT2A Receptor Agonists** - DOI: 10.1021/acs.jmedchem.2c00702

Christian B. M. Poulie[1,†], Eline Pottie[2,†], Icaro A. Simon[1], Kasper Harpsøe[1], Laura D'Andrea[1], Igor V. Komarov[3], David E. Gloriam[1], Anders A. Jensen[1], Christophe P. Stove[2,*], Jesper L. Kristensen[1,*]

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[3]Enamine Ltd., Kyiv 02094, Ukraine

†These authors contributed equally to this work.

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2. **Structure-activity assessment and in-depth analysis of biased agonism in a set of phenethylamine 5-HT2AR agonists** - (submitted and under revision, *Neuropharmacology*, *Elsevier*)

Eline Pottie[1,#], Christian B.M. Poulie[2,#], Icaro A. Simon[2], Kasper Harpsøe[2], Laura D'Andrea[2], Igor V. Komarov,[3] David E. Gloriam[2], Anders A. Jensen[2], Jesper L. Kristensen[2,*], Christophe P. Stove[1,*]

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[3]Enamine Ltd., Kyiv 02094, Ukraine

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3. **Facile One-pot Reduction of Nitrostyrenes to Phenethylamines using Sodium Borohydride and Copper(II) chloride** - (submitted to *Beilstein J. Org. Chem.*)

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Abbreviations

2C-*X*: general formula for 2,5-dimethoxyphenethylamine with variable substitution in 4-position
2C-B: 2,5-dimethoxy-4-bromophenylethylamine
2C-I: 2,5-dimethoxy-4-iodophenylethylamine
2C-CN: 2,5-dimethoxy-4-cyanophenylethylamine
2C-N: 2,5-dimethoxy-4-nitrophenylethylamine
2C-T: 2,5-dimethoxy-4-(methylthio)phenylethylamine
2C-T-2: 2,5-dimethoxy-4-(ethylthio)phenylethylamine
2C-T-7: 2,5-dimethoxy-4-(*n*-propylthio)phenylethylamine
2C-TFM: 2,5-dimethoxy-4-trifluoromethylphenylethylamine
5-HIAA: 5-hydroxyindoleacetic acid
5-HT: serotonin
5-HT_{2A}: a type of serotonin receptor in the brain
5-HT_{2A}R: serotonin 2A receptor
5-HTTP: 5-hydroxytriptophan
AA: arachidonic acid
AADC: aromatic amino acid decarboxylase
AC: adenylyl cyclase
AcOH: acetic acid or glacial acetic acid
ad hoc: specific for or purposely/specifically for (original Latin meaning)
βarr2: functional assay for measuring β-arrestin 2 recruitment
Boc: tert-butoxycarbonyl
BOL-148: 2-bromolysergic acid diethylamide
cAMP: 3'-5'-cyclic adenosine monophosphate
CIMBI: Center for Integrated Molecular Brain Imaging
CNS: central nervous system
DCM: dichloromethane
DEAD: Diethyl azodicarboxylate
DETA: Diethylenetriamine
DIBAL-H: diisobutyl aluminum hydride
Dioxane: 1,4-dioxane
DIPEA: diisopropylethylamine
DMF: dimethylformamide
DMT: N,N-dimethyltryptamine
DNP: dinitrophenylhydrazine
DNs: 2,4-dinitrobenzenesulfonyl chloride
DOB: 2,5-dimethoxy-4-bromoamphetamine
DOCN: 2,5-dimethoxy-4-cyanoamphetamine
DOI: 2,5-dimethoxy-4-iodoamphetamine
DOM: 2,5-dimethoxy-4-methylamphetamine
DOT: 1-(2,5-dimethoxy-4-(methylthio)phenyl)propan-2-amine
DOT-7: 1-(2,5-dimethoxy-4-(propylthio)phenyl)propan-2-amine
DO*X*: general formula for 2,5-dimethoxyamphetamine with variable substitution in 4-position

E_{max}: efficacy, maximum response on a dose or concentration-response curve for a certain drug
EC_{50}: potency, half maximal effective concentration of a certain drug
ECL*X*: extracellular loops
ED_{50}: half maximal effective dose of a certain drug
EDA: ethylenediamine
EDDA: Ethylenediammonium diacetate
EDG: Electron Donating Group
EtOH: ethanol
ERK: extracellular-signal-regulated kinase pathway
EWG: Electron Withdrawing Group
FDA: U.S. Food and Drug Administration
G protein: guanine nucleotide-binding proteins
GABA: neurotransmitter gamma-aminobutyric acid
GI tract: gastrointestinal tract
GPCR: G protein-coupled receptor
$Gx/G_x/Gx_x$: G protein subunits
HPLC: High Performance Liquid Chromatography
ICL*X*: intracellular loops
IDO: indoleamine-2,3-dioxygenase
IP_3: inositol 1,4,5-trisphosphate
i-PrOH: 2-propanol
Kyn: kynurenine
LC-MS: Liquid Chromatography–Mass Spectrometry
LSD: lysergic acid diethylamide
MAO: monoamine oxidase
*m*CPBA: *meta*-chloroperoxybenzoic acid
MeOH: methanol
$MeNO_2$: nitromethane
$EtNO_2$: nitroethane
mini$G\alpha_q$: functional assay for measuring $G\alpha_q$ recruitment
MEM: 2,5-dimethoxy-4-ethoxyamphetamine
MPM: 2,5-dimethoxy-4-propoxyamphetamine
NBPEA: N-benzyl phenethylamine
NBF: N-(2-fluorobenzyl)
NBMD: N-(benzo[d][1,3]dioxol-4-ylmethyl)
NBOH: N-(2-hydroxybenzyl)
NBOMe: N-(2-methoxybenzyl)
NMR: Nuclear Magnetic Resonance
Ns: 2-nitrobenzenesulfonyl chloride
OCD: obsessive-compulsive disorder
PKA: protein kinase
PKC: protein kinase
PLA_2: phospholipase A_2
PLC: phospholipase C
pNs: 4-nitrobenzenesulfonyl chloride

Ser159^{3x36}: serine residue 159 and its specific location in a residue contained in TM3
SERT: serotonin reuptake transporter
TEA: triethylamine
TFA: trifluoroacetic acid or trifluoroacetate
THF: tetrahydrofuran
TLC: Thin Layer Chromatography
TMA-2: 2,4,5-trimethoxyamphetamine
TM*X*: transmembrane domains
TPH2: tryptophan hydroxylase 2
Trp: tryptophan
UPLC-MS: Ultra Performance Liquid Chromatography – Mass Spectrometry
VMAT2: vesicular monoamine transporter 2

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1. SEROTONIN AND ITS RECEPTORS

1.1. SEROTONIN

Serotonin, or 5-hydroxytryptamine, *5-HT*, is a pivotal biogenic monoamine and one of the most ancient signalling molecules, being of immense importance for its numerous biological functions.[1] (Figure 1)

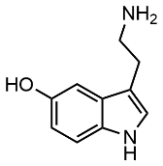


Figure 1: The structure of serotonin (5-HT). Its chemical scaffold displays a basic terminal amino group separated from an aromatic core by a two-carbon aliphatic chain, as typical of tryptamine molecules, with a hydroxide in 5-position on the benzene ring.[2]

Its biological action, as a hormone, a neurotransmitter, and a mitogen, is found to be omnipresent in animal physiology. Particularly as a neurotransmitter, 5-HT is highly present in both the periphery and central nervous system (CNS) of mammals, distributed at varying concentrations in different regions of the brain.[1] In neuronal mammalian cells, serotonin is biosynthetically produced in two essential steps: the hydroxylation of the benzene ring in the essential amino acid tryptophan by the enzyme tryptophan hydroxylase 2 (TPH2), leading to 5-hydroxytryptophan (5-HTP) as product, and the decarboxylation of the side chain of the latter by another enzyme, the aromatic amino acid decarboxylase (AADC).[2] (Figure 2)

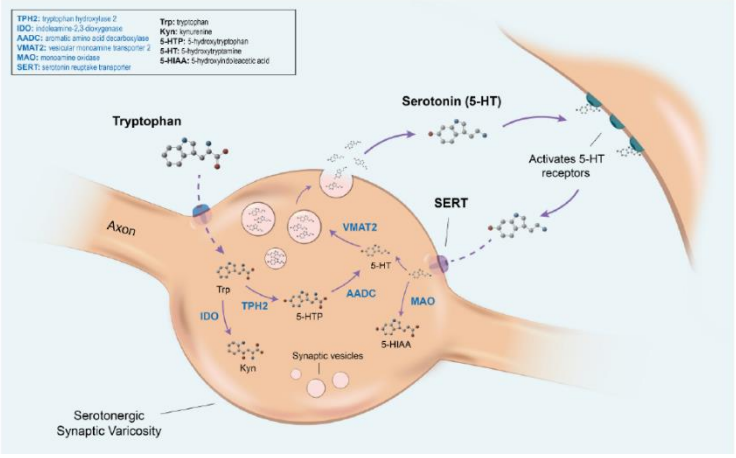


Figure 2: Biosynthesis of serotonin, its transport, and 5-HT receptors activation. An amino acid transporter allows the tryptophan (Trp) to enter the cell, where it is mostly converted to 5-hydroxytryptophan (5-HTP)

by tryptophan hydroxylase 2 (TPH2) and 5-HTP further transformed into serotonin by aromatic L-amino acid decarboxylase (AADC). Tryptophan can otherwise be converted into kynurenine (Kyn) by indoleamine-2,3-dioxygenase enzyme (IDO) metabolism. The vesicular monoamine transporter 2 (VMAT2) whose role is to transport monoamines, moves serotonin molecules in the cytosol to vesicles. The latter, once stimulated, merge into the plasma membrane and release serotonin into the synaptic cleft. Once released, 5-HT can activate post-synaptic 5-HT receptors on the targeted cell and, thereafter, transported back into the presynaptic neuron by the serotonin reuptake transporter (SERT). At this point, it can either be embedded into vesicles and released again into the synaptic cleft or transformed by the monoamine oxidase (MAO) into the metabolite 5-hydroxyindoleacetic acid (5-HIAA). Figure adapted by Del Colle et al.[3]

1.2. GPCRS AND THE 5-HT RECEPTOR FAMILY

As the largest family of membrane proteins present in human genome, G protein-coupled receptors (GPCRs) play a key-role by mediating signaling between the extracellular and the intracellular sides of cell membranes through multiple peptides, hormones, and neurotransmitters. To perform this mediation, GPCRs are shaped as a single polypeptide distributed across the membrane and having the N-terminus protruding in the extracellular space, and the C-terminus in the inner area of the cell. In addition, these proteins share seven hydrophobic transmembrane domains (TM1-TM7) connected through three extracellular loops (ECL1-ECL3), and three intracellular loops (ICL1-ICL3).

To date, ca. 800 G protein coupled receptors have been recognized in humans: where around half of them has been linked to sensory functions, such as taste, olfaction, and light perception regulation, the other half has been associated with non-sensory functions, while its ability and extent of signal transduction can be manipulated by use of specifically designed ligands. These ligands, which may be very different in size from small molecules to large proteins, are typically targeted for designing *ad hoc* drugs that might beneficially alter the mediation of selected signalling pathways of interest for potential medical application.[4] Thanks to this useful feature and their broad distribution in mammalians, GPCRs ligands represent about one third of all the drugs FDA-approved to date.[5] Once a ligand, acting as an agonist, binds to a GPCR, the initiated interaction between the heterotrimeric G protein and the receptor causes the Gα (or $G\beta_{\gamma}$) subunits to dissociate, resulting in a G protein-mediated signaling cascade.[6, 7] (Figure 3)

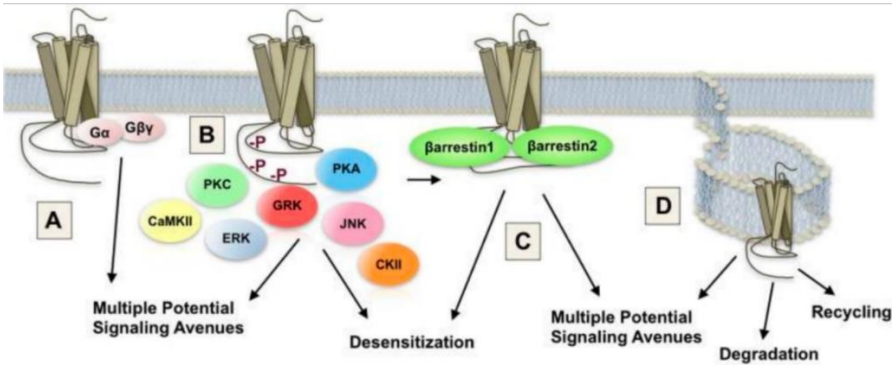


Figure 3: A GPCR, such as the serotonergic receptor targeted in this study, can either activate A) G proteins, B) kinases, C) ß-arrestins, or D) internalize to degrade or recycle. Furthermore, the complexity is increased by the fact that there are 16 G proteins classified into four classes, each with a distinct response, and all types of signalling protein can have multiple downstream effects.[8] (by kind permission of Raehal *et al.*)[8a]

Endogenous serotonin levels are regulated through serotonin receptors, which are divided in 15 subtypes. (Scheme 1) Beside the 5-HT_3 receptor as a ligand-gated ion channel, fourteen of them are guanine nucleotide-binding protein-coupled receptors and, therefore, GPCRs.

Receptor subtype	Localization	G-protein coupling	Signalling linkage	Functions
5-HT_{1A}	**CNS**: raphe nuclei, cortex, hippocampus **GI tract**	$G_{i/o}$	↓AC, cAMP, and K^+ channels Stimulates ERK	Neuronal hyperpolarization (CNS); Release of mediators (GI tract)
5-HT_{1B}	**CNS**: subiculum, globus pallidus, substantia nigra	$G_{i/o}$	↓AC and cAMP Stimulates ERK	Inhibition of neurotransmitter release (CNS)
5-HT_{1D}	**CNS**: globus pallidus, substantia nigra **Intracranial vessels**	$G_{i/o}$	↓AC and cAMP	Inhibition of neurotransmitter release (CNS); Contraction of vascular smooth muscle
5-HT_{1E}	**CNS**: cortex, striatum	$G_{i/o}$	↓AC and cAMP	Hypothesized to be involved in olfactory regulation, memory deficit-related diseases (CNS)[6c, d]
5-HT_{1F}	**CNS**: brain and periphery	$G_{i/o}$	↓AC and cAMP	Integration of sensormotor information related to limbic functions
5-HT_{2A}	**CNS**: prefrontal cortex, amygdala, hippocampus, striatum **GI tract**: smooth muscle **Platelets**	G_q	↑PLC, PKC, and PLA_2 Stimulates ERK ↑IP_3	Neuronal excitation (CNS); smooth muscle contraction and platelet aggregation (Periphery); Involvment in the neurochemical and behavioral effects of psychostimulants, hallucinogens, and antipsychotics
5-HT_{2B}	**GI tract**: stomach fundus	G_q	↑PLC and PKC Stimulates ERK ↑IP_3	GI mobility and increase in response of longitudinal smooth muscle (Periphery)
5-HT_{2C}	**CNS**: hypothalamus **Chroid plexus**	G_q	↑PLC, PKC, and PLA_2 Stimulates ERK ↑IP_3	Cerebrospinal fluid secretion and regulation of emotional states (CNS)
5-HT_3[a]	**CNS**: solitary tract, area postrema **GI tract**: parasympathetic nerves	-	Cations	Depolarization and modulation of other neurotransmitters release (Peripheral and central neurons); Pain transmissionan motility (GI tract)[6e]
5-HT_4	**CNS**: hippocampus **GI tract**	G_s	↑AC, PKA, and cAMP	Neuronal excitation related to memory and cognitive functions, and neurotransmitter relase (CNS); GI tract smooth muscle contraction and tachycardia (Periphery)
5-HT_{5A}	**CNS**: hippocampus	$G_{i/o}$	↓AC and cAMP	Regulation of sensory perception, learning, memory, and affection (CNS)
5-HT_{5B}[b]	**CNS**: hippocampus, bulbus olfactorius	$G_{i/o}$	↓cAMP	Regulation of neurodevelopment and other CNS-related functions in rodents[6g]
5-HT_6	**CNS**: hippocampus, striatum, nucleus accumbens	G_s	↑AC, PKA, and cAMP	Neuronal excitation and regulation of affection (CNS)
5-HT_7	**CNS**: hypothalamus, hippocampus **GI tract**	G_s	↑AC, PKA, and cAMP	Neuronal excitation and regulation of affection (CNS); GI tract muscle relaxation (Periphery)

Scheme 1: Summary of the serotonin receptors and their main features, such as their localization and main role.[6]

[a]5-HT_3 is the only serotonin receptor characterized as ligand-gated ion channel.[6e]
[b]5-HT_{5b} gene is only found in rodents.[6f,g]
G_q and $G_{i/o}$ are subunits of the Gα subunit.
↑: activation/increase; ↓: inhibition/decrease; *GI tract*: gastrointestinal tract; *AC*: adenylyl cyclase; *cAMP*: 3'-5'-cyclic adenosine monophosphate; *IP_3*: inositol 1,4,5-trisphosphate; *PLC*: phosphatidylinositol-specific phospholipase C; *PLA_2*: phospholipase A_2; *PKA*: protein kinase A; *PKC*: protein kinase C; *ERK*: extracellular-signal-regulated kinase pathway.

Since the first classification in 1957, 5-HT receptors have been thoroughly studied and investigated, resulting in the current revised classification.[9, 10] The 5-HT_{2A} was the first receptor subtype of this family to be officially identified, being named *D receptor* in 1957.[9] During the 80's, 5-HT_{2A}R had already been extensively investigated, despite being still called *5-HT_2*, and its distribution in the brain as well as its connection with CNS and neuroendocrine systems were identified and profusely reported.[11]

The 5-HT_{2B} receptor was originally classified as a "5-HT_1-like" receptor and renamed *5-HT_{2F}* in 1992 by Kursar *et al.*, due to the link between its activation and fundic smooth muscle contraction in rats.[11b, 12]

Similarly, the 5-HT_{2C} receptor was at first mistakenly identified as 5-HT_{1C} in 1984. The uncertainties related to the 5-HT_2 receptors subtypes were finally clarified in 1994 when the *VII. International Union of Pharmacology Classification of Receptors for 5-Hydroxytryptamine (Serotonin)* was published by Hoyer *et al.* and the current 2A, 2B and 2C receptors classification was made official.[10] The latter classification groups the 5-HT receptors in seven main families, according to their structural features and transductional signalling functions. These families are subdivided further in various subtypes, that exert similar functions, while being differently located in the body, as illustrated in Scheme 1.

1.3. THE 5-HT_{2A} RECEPTOR AND ITS CONNECTIONS WITH NEUROPSYCHIATRY

In humans, the 5-HT_{2A} receptor subtype is distributed in high concentration in the postsynaptic serotonergic neurons of the prefrontal cortex and claustrum, which is connected to the visual cortex, as well as in some areas of the limbic system, such as amygdala, hippocampus, thalamus, and in the basal ganglia, especially in the striatum. (Figure 4) Moreover, this receptor subtype is found on GABAergic interneurons and pyramidal projection neurons.[7, 11]

It was the 1947 when the first scientific investigations of the effects of lysergic acid diethylamide (*LSD*) in human behavior began at the Psychiatric Clinic at the University of Zurich, while Sandoz Laboratories started distributing it to psychiatrists and psychologist to test its psychosis-inducing potential.[14, 2] The outcome of these studies and several others, highlighting important structural similarities between LSD and serotonin, led to finally discover the pivotal connection between the mental effects of this drug and an alteration of the regular serotonergic functions in the brain.[15] This newly established link between the brain chemistry and abnormal behavior resulted in the beginning of modern neuropharmacology.[2]

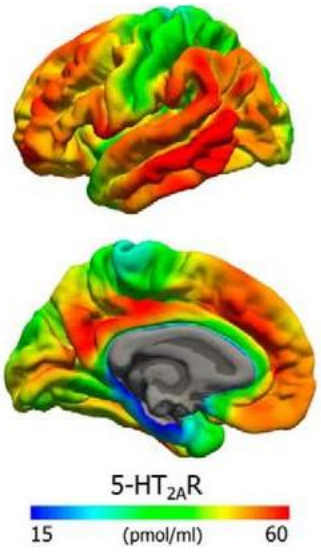


Figure 4 - Map of the average density (B_{max}) of the 5-HT_{2A} receptors in the human brain. Adapted from Beliveau et al. [13]

However, the identification of the specific receptor responsible for psychotic behavior seemed to be far to be achieved. The tight bond between the 5-HT_{2A} receptor, cognitive processing, and psychosis, given by its remarkable expression in brain regions directly associated with them, became evident when, in the 1980s', the hallucination-relieving action on patients treated with the 5-HT_{2A}R antagonist *clozapine*, used in psychiatry as an atypical antipsychotic, was first reported.[16] This early finding was further confirmed by subsequent studies involving other atypical antipsychotics such as *risperidone*, *olanzapine*, *sertindole*, and *quetiapine*, all receptor antagonists.[17] Eventually, it was later demonstrated that the hallucinations experienced by patients with acute schizophrenia were directly correlated to a 5-HT_{2A} receptor dysfunction in the apical dendrites of the pyramidal cells.[18]

Over the last decades, a growing number of in-depth studies managed to firmly link the activation of this receptor subtype to the relief of severe symptoms caused by treatment-resistant major depression, last-stage cancer-induced anxiety, obsessive-compulsive disorder (*OCD*), and drugs addiction, particularly when conventional approaches failed. The reason seems to lie in the still mysterious ability of 5-HT_{2A}R agonists to interrupt the loop of negative thoughts that is common to all these issues. This beneficial interruption donates to the patient immediate relief since the very first administration of the drug, when in a controlled environment where parallel psychotherapeutic guidance is provided.[19] Consequently, the 5-HT_{2A} receptor gained considerable attention by the scientific community over the years, due to its pivotal role in several important cognitive functions, and involvement in the physiological

modulation and response exerted by diverse biogenic amines, beyond serotonin itself, such as dopamine, psychedelics, and numerous drugs, including antidepressants and antipsychotics.[20]

1.4. DOWNSTREAM SIGNALLING PATHWAYS

One of the key-concepts behind designing new ligands is considering that all the 5-HT receptors family, as examples of G protein coupled receptors, triggers a number of different signaling pathways upon activation. When referring to $G\alpha_q$-protein activation, for instance, these pathways include cyclic adenylate cyclase and cAMP, phospholipase C (PLC) and the phosphatidylinositide cycle, and phospholipase A_2 (PLA_2) and the arachidonic acid (AA) cascade. The latter is shared by all the 5-HT_2 receptors, which activate PLA_2 causing the release of AA.[21] (Figure 5)

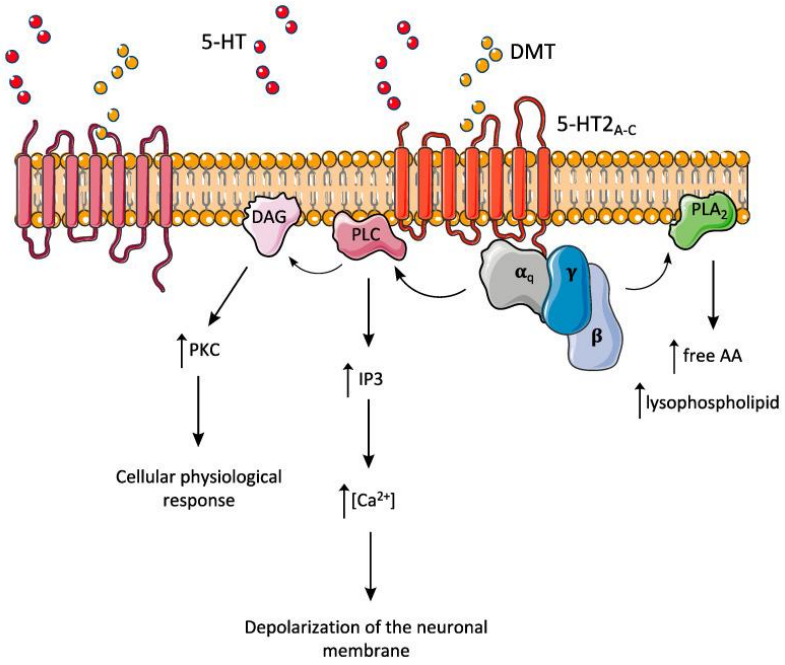


Figure 5: Some of the intracellular signalling pathways triggered by N,N-dimethyltryptamine (*DMT*, a psychedelic used as a known 5-HT_2 agonist example) or 5-HT upon activation of 5-HT_{2A-C} receptors. Hallucinogens bound to these receptors activate G_q (**$G\alpha_q$**) downstream signalling pathway that ultimately mobilizes the phosphatidylinositol-specific phospholipases C (PLC) and A_2 (PLA_2). PLC hydrolyses the lipids of the phosphatidylinositol membrane, causing the release of inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 leads to the intracellular increase in calcium, which causes depolarization in the neuronal membranes. On the other hand, DAG, remaining bound to the membrane, activates PKC as a second messenger, mediating the cellular physiological response. PLA_2, upon stimulation, hydrolyzes

phospholipids containing arachidonic acid (AA), causing the intracellular release of the latter and lysophospholipids. The herein illustration takes into account only part of the several different possible downstream transductional signalling cascades that can be provoked upon 5-HT_2 receptors activation (Figure 3). (By kind permission of Brito-da-Costa *et al.*)[22]

However, some 5-HT receptors agonists demonstrated to overall activate the receptor and, yet, to show a preference towards the recruitment of specific effector proteins over others. This preferential recruitment causes the transductional intracellular signalling cascades originated by these specific effector proteins to be initiated, and the other downstream pathways related to other proteins to be silenced. This ability takes shape into the fundamental concept of *functional selectivity*, or *biased agonism*, a notion that attracted the attention of countless researchers over the last two decades, as a base to develop safer and more effective next generation drugs.

1.4.1. FUNCTIONAL SELECTIVITY

The phenomenon called "functional selectivity" regarding GPCRs refers to the selective or preferential triggering of a specific signaling transductional pathway over others, via interaction of the receptor with one or more signalling proteins, as described earlier. The importance of a thoroughly designed drug molecule lies on the individual ability of a ligand to stabilize a unique receptor conformation, that regulates specific sets of signalling pathways. Therefore, agonists of the same receptor subtype can stimulate very different intracellular responses.[23]

To recognize the extent of potential bias that a ligand, or drug, might display, binding assays do not seem to be suitable for the scope, as they give an exclusive indication of affinity for the receptor under investigation, though no information about receptor activation nor downstream intracellular response are given. Indeed, diverse functional assays have been designed and used to measure the actual downstream transductional signal response at specific points of the protein-specific biochemical cascade. Specifically, functional GPCR assays are designed according to which G protein mediated second messenger needs to be monitored, and its measured concentration finally indicates the extent of activation of that specific pathway, while a certain ligand is bound into the receptor binding pocket.[24] Specifically for the 5-HT_{2A}R, the receptor ability to signal through members of both $G\alpha_q$, among other G proteins, as well as through β-arrestin2-mediated pathways, determines the possibility that a specific ligand might cause the preferential activation of only one intracellular transduction cascade, acting as a $G\alpha_q$- or β-arrestin-biased 5-HT_{2A} agonist.[25] (Figure 6) Mechanistically, upon agonist binding, the 5-HT_{2A} receptor is phosphorylated by GPCR kinases (GRKs, Figure 3), while the β-arrestin recruitment occurs. At this

point, the G protein signalling pathway is interrupted, and another set of events, such as desensitization and internalization, arise.[24]

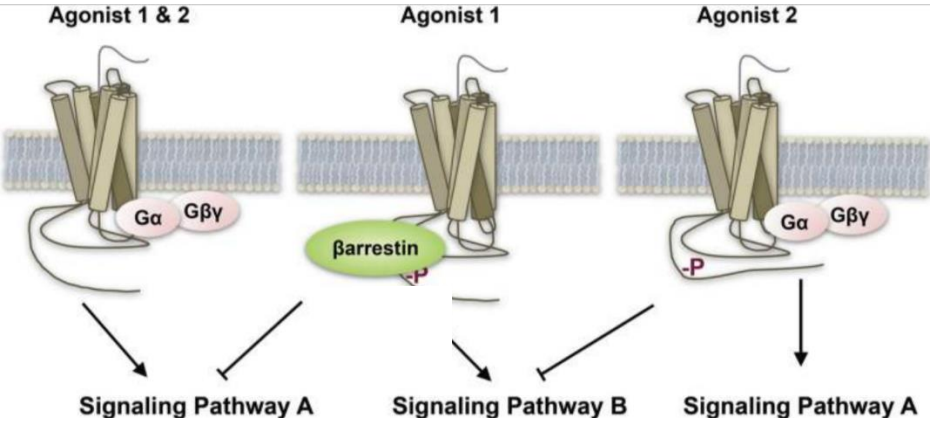


Figure 6: Representation of the behavior of an unbiased (Agonist 1) and a $G\alpha_q$-biased ligand (Agonist 2), upon GPCR activation. Both agonists can trigger the Signaling Pathway A by recruiting the $G\alpha_q$-protein. However, only Agonist 1 provokes β-arrestin recruitment, resulting in the activation of the Signaling Pathway B. As Agonist 1 can activate both transductional signalling pathways, while Agonist 2 cannot, the latter is a $G\alpha_q$-biased receptor agonist.[8] (Image adapted from Raehal *et al.*)[8a]

In-depth pharmacological studies remarkably demonstrated that the activation of the 5-HT_{2A} downstream $G\alpha_q$ protein-dependent pathway has a key-role in the hallucinogenic and behavioral effects of psychedelics and hallucinogen-inducing drugs. (As shown in Figure 5, where DMT is used to activate the $G\alpha_q$-related downstream pathway)

Consistently, the administration of 2,5-dimethoxy-4-iodoamphetamine (*DOI*, another potent 5-HT_{2A}R agonist) to $G\alpha_q$-knocked out mice reduced their head-twitch response* by over 50%, while not triggering any anxiolytic-like induced behavior, typically observed in non-mutant mice treated with the same dosage of DOI.[26] Similar studies employing LSD and *lisuride*, confirmed that psychedelic drugs trigger hallucinations, and therefore head-twitch-induced behavior, in a β-arrestin-2 independent manner, clearly indicating, in accordance with previous results, that the hallucinogenic effect might be exclusively correlated to the G_q/11- and G_i/o G proteins expression.[27] However, the physiological mechanism behind G_q activation and β-arrestin2 recruitment by serotonergic psychedelics is still unclear.

To date, numerous known psychedelics and other 5-HT_{2A} receptor agonists have been analyzed on both $G\alpha_q$ and β-arrestin2 functional assays to assess their bias profiles, and one single partial βarr2-biased agonist, using 5-HT as a reference, has been reported.[25, 28] Nevertheless, no agonists with significant preference towards a specific G protein-mediated signalling pathway have been reported prior to the work presented in this thesis. Design, synthesis, and testing of 5-HT_{2A} receptor agonists displaying

functional selectivity towards β-arrestin2 over $G\alpha_q$ activation might play a crucial role in the *ad hoc* invention of non-hallucinogenic drugs with potential long-term therapeutic properties.[5]

***** *Head-twitch response (HTR)*: the *HTR* is a rapid side-to-side rotational movement of rats and mice heads resulting from the administration of hallucinogens and other 5-HT_{2A} agonists. This parameter has been used as a standard behavioral assay to evaluate the 2A receptor activation and the extent of hallucinations induced.[29]

2. DEVELOPMENT OF SELECTIVE 5-HT$_{2A}$ AGONISTS

2.1 - PSYCHEDELICS AS 5-HT$_{2A}$ AGONISTS

Prior to attempting the development of a functionally selective agonist, it is obviously essential to start from a scaffold already displaying selectivity towards the 5-HT$_{2A}$ receptor over any other receptor family and subtype. Due to the previously mentioned high 5-HT$_{2A}$R distribution in the brain, drugs able to interfere with the normal biochemical functions of this receptor, such as LSD, have been broadly studied and used as tools with notable psychopharmacological and/or CNS-related properties.[30] For instance, 2-bromolysergic acid diethylamide (*BOL-148*) has been characterized as an LSD-derived neutral antagonist for the treatment of cluster headaches, and several inverse agonists, such as clozapine and risperidone, have been extensively used as atypical antipsychotics.[31] Molecules acting as 5-HT$_{2A}$ receptor agonists can be structurally categorized into three separate groups, according to their main backbone framework pattern: the ergolines, like LSD, the tryptamines, such as serotonin and the psychedelic *psilocybin,* and the phenethylamines, like *mescaline*.[30, 32] (Figure 7)

Figure 7: Overview of the structures of three 5-HT$_{2A}$R agonists representatives: lysergic acid diethyl--amide (LSD), 3-[2-(dimethylamino)ethyl]-1H-indol-4-yl dihydrogen phosphate (psilocybin), and 3,4,5-trimethoxyphenethylamine (mescaline). The orange dashed frame highlights the ergolines backbone. The tryptamine backbone present in psilocybin appears in green, resulting also embedded in the ergoline scaffold. The main phenethylamine framework is highlighted in grey.

Despite derivatives of these three classes might mainly interact with the 5-HT$_{2A}$ receptor, they usually display agonism towards several other receptors, showing variable degree of selectivity towards multiple 5-HT receptors subtypes.[33] The ergoline class finds its origins in the *ergot fungus*, as being the first discovered species able to produce ergot alkaloids. Nonetheless, ergolines were also later found in several other plants and fungi. This class of compounds possess several properties of pharmacological interest, by being able to bind different monoamine and non-serotonergic receptors.[34] Indeed, apart from migraine, ergoline analogues are

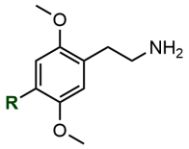
clinically used for Parkinson's disease treatment and within the gynecological medical area.[35]

The family of tryptamines display a natural higher affinity for the 5-HT receptors, as they are analogues to the serotonin scaffold, typically lacking subtype selectivity. As 5-HT activates, indeed, all the 5-HT receptors, some main representatives of classic psychedelics belonging to this class, such as psilocybin and N,N-dimethyltryptamine (*DMT*), were proved to activate the 5-HT_{1A} receptor, as well as the whole 5-HT_2 family.[36]

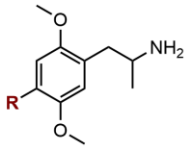
The third class of psychedelics, represented by substituted phenethylamines, finds its origins in mescaline, a natural occurring hallucinogenic protoalkaloid found in different species of cacti, such as *Lophophora williamsii*, better known as *peyote*. The consumption of peyote is one of the oldest medicinal and entheogenic treatments adopted in human history, dating back to over 5500 years ago, when native Americans in Southern Texas and Mexico used to consume it during religious rituals.[37]

2.2 – DEVELOPMENT OF 5-HT_{2A} SELECTIVE PHENYLALKYLAMINES

One of the pioneers of psychedelic phenylalkylamines structural modification and research was Alexander Shulgin, an American chemist who prepared and tested numerous and varied mescaline-inspired analogues, constituting the *2C-X* and *DOX* family. (Scheme 2) One of the most relevant outcomes of his studies was determining the effects of diverse substituents on the phenylalkylamine scaffold and framing the structural features of those with therapeutic potential.[38]



2C-*X*



DO*X*

R	Abbreviation	
Br	2C-B	1.1
Me	2C-D	1.2
SMe	2C-T	1.3
I	2C-I	1.4
Et	2C-E	1.5
CN	2C-CN	1.6
Cl	2C-C	1.7
NO_2	2C-N	1.8
SEt	2C-T2	1.9
SPr	2C-T7	1.10
Pr	2C-P	1.11
CF_3	2C-TFM	1.12
H	2C-H	1.13

R	Abbreviation	
Me	DOM	2.1
Et	DOEt	2.2
Pr	DOPr	2.3
Bu	DOBu	2.4
Br	DOB	2.5
I	DOI	2.6
Cl	DOC	2.7
NO_2	DON	2.8
*i*Pr	DOIp	2.9
EtF	DOEf	2.10
CN	DOCN	2.11
CF_3	DOTFM	2.12
H	DMA	2.13

Scheme 2: The most relevant examples of psychoactive synthetic phenylalkylamines, and their commonly used abbreviated names.

However, all the synthetic psychedelics prepared over the decades preceding early 90's could not be tested to evaluate their receptor subtype predilection, as the identification of the 5-HT_2 family members was still ongoing.[10] Thereafter, the phenylalkylamines in Scheme 2 and several other 5-HT_{2A} agonists, including tryptamines and ergolines examples, were tested *in vitro* to assess their receptor subtype binding affinities and were proved not to display any significant selectivity for individual subtypes.[39] At the same time, studies on the 5-HT_2 family highlighted that the latter shares a high degree of orthosteric sites conservation, and that the 2A and 2C species, in particular, seemed to share even more similarities, causing the agonists' binding affinities for these two subtypes to be extremely close.[2]

Data collected by pharmacological testing on some DO*X* as amphetamine analogues examples, such as 2,5-dimethoxy-4-methylamphetamine (*DOM*, 2.1), 2,5-dimethoxy-4-bromoamphetamine (*DOB*, 2.5), and DOI (2.6), indicated an improved selectivity towards the 5-HT_2 receptor family, while, nonetheless, lacking significant selectivity towards the individual 5-HT_{2A}R subtypes.[40] At first, 2,5-dimethoxy-4-cyanoamphetamine (*DOCN*, 2.11) seemed to stand out due to its 22-fold selectivity for 5-HT_{2A}. Despite its binding affinity towards the receptor subtype resulted to be nearly 100-fold lower than DOB, DOCN was the first reported moderately selective 5-HT_{2A} agonist.[41]

Following studies on the 4-position by use of 4-alkyl and 4-alkylphenyl substituted DO*X* analogues reached inconsistent results, by demonstrating that alkyl substituents longer than butyl caused the compound to switch from agonist to receptor antagonist, and, at the same time, identifying 4-alkylphenyl compounds as agonists.[42] These apparent incongruencies were further explored by several subsequent studies, focusing on the importance of the 4-position.[43] At the same time, as a follow-up to previous work published by Nichols group over 1980s, a number of constrained phenylalkylamines were synthesized and tested, in order to explore the effects induced by conformational constraint, locking the two methoxy groups in more rigid structures.[44] This investigation led to the discovery of one of the most potent 5-HT_{2A} agonists thus far, 1-(4-bromofuro[2,3-f][1]benzofuran-8-yl)propan-2-amine, also known as *Bromo-DragonFLY* (2.15).[45a] (Figure 8)

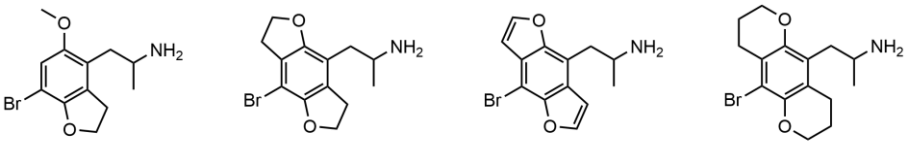


Figure 8: Some examples of conformationally restricted analogues: 1-(5-methoxy-7-bromo-2,3-dihydrobenzofuran-4-yl)-2-aminopropane (2.13),[46a] 1-(8-bromo-2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b‘]difuran-4-yl)-2-aminopropane (2.14),[46b] bromo-DragonFLY (2.15),[45a] and 10-bromo-5-(2-aminopropyl)-2,3,4,7,8,9-hexahydro [1,2b:4,5b′]dipyran (2.16)[46c].

2.3 – DEVELOPMENT OF 5-HT_{2A} SELECTIVE PHENETHYLAMINES

Thereupon, further research studies started showing relevant findings on the effects of the compound selectivity by substitution on the amino group. While alkylation of the amino moiety with simple alkyl groups was proved to be detrimental for the 5-HT_{2A}R activity, benzylation seemed to largely increase binding affinity and agonist potency at the 5-HT_{2A}R.[45b, 47]

The latter finding played a major role in the investigation of new potent and selective agonists. Based on previous studies conducted by Glennon’s group in 1995, Heim synthesized a few phenethylamines from 2,5-dimethoxy-4-iodophenylethylamine (*2C-I*, 1.4) and 2,5-dimethoxy-4-bromophenylethylamine (*2C-B*, 1.1) by addition of a 2-methoxybenzyl group on the amino moiety.[47a, 48] (Figure 9, see 1.41 and 1.11) *In vivo* testing results on rats’ arteries demonstrated that the N-substitution increased the agonists potency of over 100-fold, paving the way to a new class of potent 5-HT_{2A}R agonists, renamed *NBOMes*.

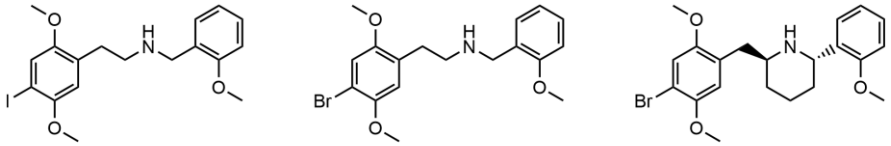


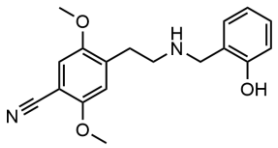
Figure 9: The potent 5-HT_{2A} agonists, 4-iodo-2,5-dimethoxy-N-(2-methoxybenzyl)phenethylamine (25I-NBOMe, 1.41), 4-bromo-2,5-dimethoxy-N-(2-methoxybenzyl)phenethylamine (25B-NBOMe, 1.1), synthesized by Heim, and (±)-trans-2-(4-bromo-2,5-dimethoxybenzyl)-6-(2-methoxyphenyl)piperidine (DMBMPP, 2.16), the first compound displaying noteworthy selectivity towards the 2A subtype over 120-fold higher than the 2C recombinant, published by Nichols' group in 2012.[48, 47b]

In 2006, Nichols and his group reported new fascinating insights after fully characterizing several N-benzyl phenylethylamines (*NBPEAs*), such as some of Heim's NBOMes representatives including 4-iodo-2,5-dimethoxy-N-(2-methoxybenzyl)phenethylamine (*25I-NBOMe*, 1.41) and 4-bromo-2,5-dimethoxy-N-(2-methoxybenzyl)phenethylamine (*25B-NBOMe*, 1.11).[49] Their studies, indeed, concluded that NBPEAs are a novel class of high affinity, potent, and modestly selective 5-HT_{2A}R agonists, particularly when carrying 2-methoxy or 2-hydroxy substitution on the N-benzyl moiety.

This phenomenon seems to be particularly evident by comparison of the binding affinities of 25I-NBOMe and 25B-NBOMe with their precursors, the simple phenethylamines 2C-I and 2C-B, going from a K_i of 0.62 nM and 6 nM to 0.087 nM and 0.19 nM for the 5-HT_{2A} receptor, respectively. This latter result brought Nichols' group to design, synthesize, and test in 2012 a few conformationally constrained structures based on the 25B-NBOMe scaffold, for ease of synthesis and its 30-fold improved affinity upon 2-methoxybenzylation.[50] The interest into testing rigid structures, related to their aim of developing better homology models for the 5-HT_{2A}R studies, led them to finally prepare and test one of the most selective 5-HT_{2A} agonists thus far: (±)-trans-2-(4-Bromo-2,5-dimethoxybenzyl)-6-(2-methoxyphenyl)piperidine or *DMBMPP* (2.16, Figure 9).[47b] DMBMPP displayed a high binding affinity equal to 2.5 nM and an astonishing 124-fold selectivity over 5-HT_{2C}, suggesting that conformational restraint might be beneficial to develop further receptor agonists.

Over the same period, Martin Hansen, from Jesper Langgaard Kristensen's group, designed, prepared, and tested 48 NBPEA analogues to establish the effects on receptor binding affinity and functional activity at 5-HT_{2A} and 5-HT_{2C} receptors. His work was published in 2014, introducing structural-activity relationships of several known and novel NBPEA analogues.[45c] The choice of focusing on substituted N-benzyl phenethylamines structures originated from relevant data from previous studies, determining that NBPEAs proved to be remarkably selective for the 5-HT_2

receptors family over a variety of other neuroreceptors.[50] Thanks to a broad screening of several ligands, Hansen was able to determine that the N-substituted derivative of 2,5-dimethoxy-4-cyanophenylethylamine (*2C-CN*, 1.6), as 4-cyano-2,5-dimethoxy-N-(2-hydroxybenzyl)phenethylamine or *25CN-NBOH* (1.61), was a 5-HT_{2A} agonist with high binding affinity (K_i = 1.3 nM) and, more interestingly, 100-fold selectivity towards 5-HT_{2A} over 5-HT_{2C}. (Figure 10)



25CN-NBOH
1.61

Figure 10: The structure of 4-cyano-2,5-dimethoxy-N-(2-hydroxybenzyl)phenethylamine or *25CN-NBOH*, a new selective 5-HT_{2A} agonist, reported by Hansen *et al.* in 2014.[45c]

3. PROJECT WORK AND INVESTIGATION: DESIGN AND SYNTHESIS OF 5-HT$_{2A}$R BIASED AGONISTS

Based on Jesper Kristensen's group previous outcomes, NBPEAs seemed the perfect starting point for investigating new structures displaying functional selectivity.
In order to determine the degree of bias that a ligand possessed, two *in vitro* assays were developed *ad hoc* by Anders A. Jensen and his group at the Department of Molecular and Cellular Pharmacology of the University of Copenhagen to quantify the extent of both G_q and β-arrestin (*miniG*α_q and *βarr2*) recruitment upon agonist-5-HT$_{2A}$R binding. However, their results could not be eventually used, due to numerous internal issues leading the project to crumble. Nonetheless, at a later stage and under Jensen's directions, Eline Pottie and Christophe P. Stove developed and tested the two *in vitro* assays at their facilities at the Department of Bioanalysis of Ghent University in Belgium.[5, 51] Similarly to Hansen's ligands screening, first a library of compounds had to be created and tested in order to design and synthesize novel scaffolds of potential interest. By combining relevant outcomes of previous studies, 2C-*X*, DO*X*, phenethylamines deprived of methoxy groups, flexible and rigid NBPEAs were successfully synthesized, some of which underwent *in vitro* and *in vivo* testing.

3.1 – SYNTHESIS OF 2,5-DIMETHOXY-4-NITROPHENYLETHYLAMINE (2C-N) AND ITS DERIVATIVES

As previously mentioned in the paragraphs 1.3.2 and 1.4, the term *functional selectivity* is associated with the ability of a ligand to stabilize a receptor in a unique conformation that triggers preferential downstream signalling pathways over others, which are also activated by functionally non-selective compounds. One of the most relevant studies performed on this matter and specifically targeting the 5-HT$_{2A}$ receptor is represented by the work of Moya *et al.*, who reported to have finally identified a functional selective 5-HT$_{2A}$ subtype agonist.[52] Their research focused on screening 2C-*X* and DO*X* representatives as known partial-to-full 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors agonists, in order to investigate and prove the concept of functional selectivity.
A typical pharmacological characterization of compounds leans on *binding affinity*, determined by the strength with which a ligand binds to a receptor, and *intrinsic efficacy*, indicating the ligand's ability to affect the receptor behavior with regard to its downstream intracellular signalling response. Moya *et al.* highlighted that most of the pharmacological *in vitro* characterization data on 2C-*X* and DO*X* analogues reported prior to their study was exclusively based on PLC-mediated responses, therefore not considering other additional effector pathways such as PLA_2 and AA

release, as intrinsic efficacy can vary across different cell types.[40, 41, 30, 53] (Figure 5 – paragraph 1.4)

The idea of screening specifically 2C-*X* and DO*X* derivatives was thus connected to an interest into investigating the pharmacological data previously reported showing that, while 2C-*X* and DOX binding affinities for 5-HT_{2A} were similar, functional models indicated that 2C-*X* intrinsic efficacies were lower than those expressed by their α-methylated analogs, as a result of PLC-mediated responses analysis only. By simultaneous detection and consequent comparison of PLC and, also, PLA_2 responses in Chinese hamster ovary-K1 cells expressing human 5-HT_{2A} or 5-HT_{2C}, Moya and his group identified 2,5-dimethoxy-4-nitrophenylethylamine (*2C-N*, 1,8, as a functionally selective 5-HT_{2A} agonist as PLA_2-biased.[52] (Figure 11)

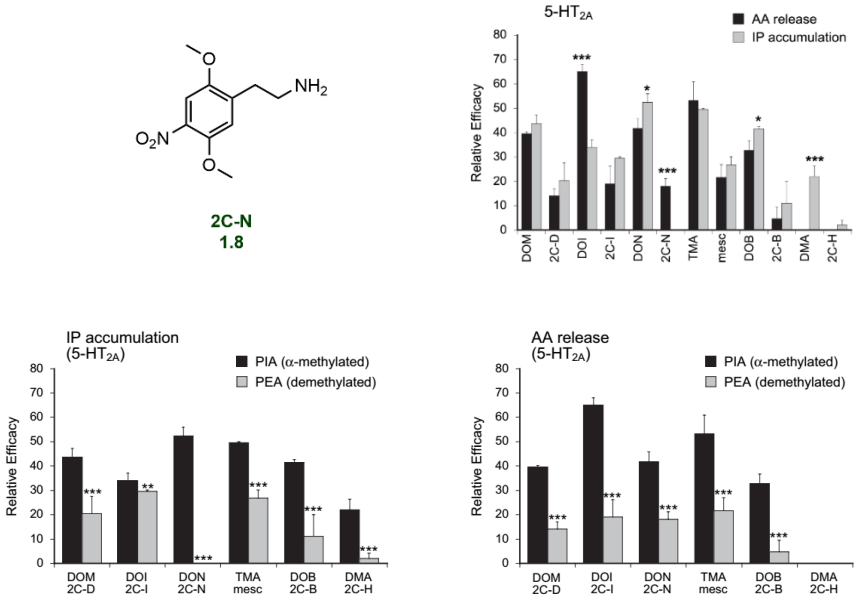
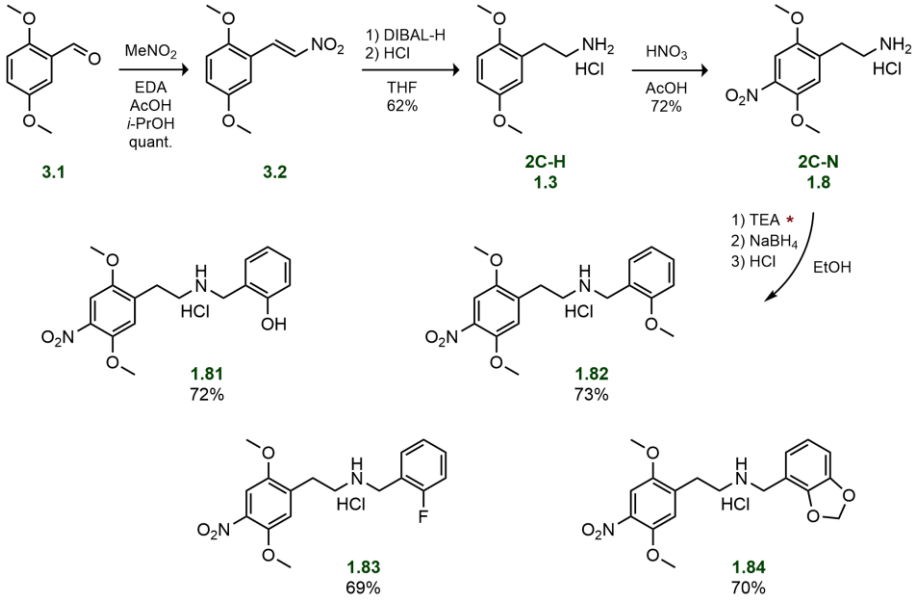


Figure 11: The structure of 2C-N (1.8) and the relative efficacies comparison of 2C-*X* and DO*X* analogues (here called *PEA* and *PIA* respectively) associated with PLC-IP and PLA_2-AA responses of the 5-HT_{2A} receptor, showing that all the analogues tested provoke PLC and PLA_2 activation, apart from 2C-N, which does not trigger IP accumulation and, therefore, no or negligible PLC activation. (Top) The other two graphs display the effects of α-methylation of the 2C-*X* analogues primary amine on their efficacies for PLC-IP and PLA_2-AA responses. (Bottom) Particularly, the contrast between 2C-N and its amphetamine analogue, *DON*, results make clear how the α-methylation erases any biased behavior showed by its demethylated equivalent. (By kind permission of Moya *et al.*)[52]

In light of these fascinating results, 2C-N and its NBPEAs derivatives seemed to be the perfect start to investigate biased agonism towards the 5-HT_{2A} receptor.

The synthesis of the nitro products was performed according to previously described procedures.[54, 55, 56] 2,5-Dimethoxybenzaldehyde (3.1) was conveniently converted to β-nitrostyrene (3.2) via base-catalyzed Henry condensation, by use of

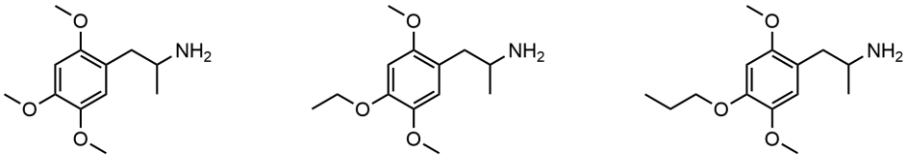
ethylenediamine and glacial acetic acid to produce *in situ* ethylenediammonium diacetate (EDDA), acting as effective Henry reaction catalyst.[56] The conversion to β-nitrostyrene 3.2 was followed by reduction to 2,5-dimethoxyphenyletylamine (*2C-H*, 1.13, Scheme 2) using diisobutyl aluminum hydride (DIBAL-H) and precipitation as hydrochloride (1.13).[54a,c, 55] Standard nitration of 2C-H salt delivered the first phenethylamine of a long series as 2,5-dimethoxy-4-nitrophenylethylamine hydrochloride (2C-N, 1.8). The simplest way to prepare the final NBPEAs analogues is reductive amination, beginning with the condensation with the desired aldehyde and ending with the reduction of the newly formed imine, as a Schiff base, by use of $NaBH_4$.[54b] (Scheme 3)



Scheme 3: Scheme summarizing the synthetic pathway from 2,5-dimethoxybenzaldehyde (3.1) to 2C-N (1.8) and its NBPEAs salt derivatives. 2C-N was condensed with (*****) 2-salicylaldehyde, 2-methoxybenzaldehyde, 2-fluorobenzaldehyde, and 2,3-(methylenedioxy)benzaldehyde by use of triethylamine (TEA) and $NaBH_4$ in dry EtOH, to deliver 4-nitro-2,5-dimethoxy-N-(2-hydroxybenzyl)phenethylamine hydrochloride (*25N-NBOH*, 1.81), 4-nitro-2,5-dimethoxy-N-(2-methoxybenzyl)phenethylamine hydrochloride (*25N-NBOMe*, 1.82), 4-nitro-2,5-dimethoxy-N-(2-fluorobenzyl)phenethylamine hydrochloride (*25N-NBF*, 1.83), and N-(benzo[d][1,3]dioxol-4-ylmethyl)-2-(2,5-dimethoxy-4-nitrophenyl)ethan-1-amine hydrochloride (*25N-NBMD*, 1.84), respectively, after precipitation with hydrogen chloride.
AcOH: glacial acetic acid; DIBAL-H: diisobutyl aluminum hydride; EDA: ethylenediamine; i-PrOH: 2-propanol; TEA: triethylamine; THF: tetrahydrofuran.

3.2 – SYNTHESIS OF 2,5-DIMETHOXY-4-PROPOXYAMPHETAMINE (MPM)

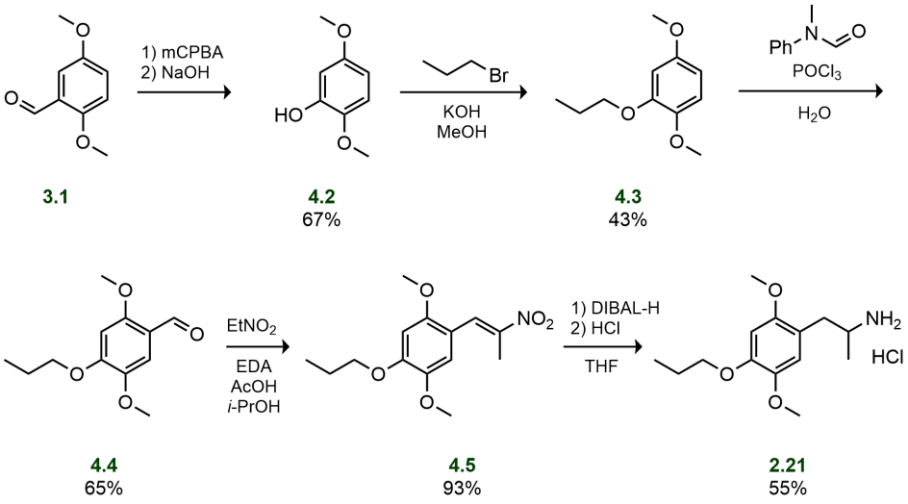
One of the first experimental challenges lied in the preparation of 2,5-dimethoxy-4-propoxyamphetamine (*MPM*, 2.21).
The compound was commissioned and tested by Adam L. Halberstadt at the Department of Psychiatry of the University of California (San Diego) to evaluate and characterize the behavioral response to hallucinogens in rodents.[57] Halberstadt noticed that, despite the broad experimental characterization carried out prior to his work on head-twitch response in *in vivo* models reacting to administration of psychedelics, only a minor part of it specifically involved mescaline, 2C-*X*, and DO*X* derivatives. MPM was compared to its direct alkoxy analogues, 2,4,5-trimethoxyamphetamine (*TMA-2*, 2.18) and 2,5-dimethoxy-4-ethoxyamphetamine (*MEM*, 2.19). Their potencies relationships appeared to be very close to and consistent with the considerations achieved by Shulgin, who reported the effects of hallucinogens on humans in 1978.[58] (Figure 12)



TMA-2 2.18 **MEM 2.19** **MPM 2.21**

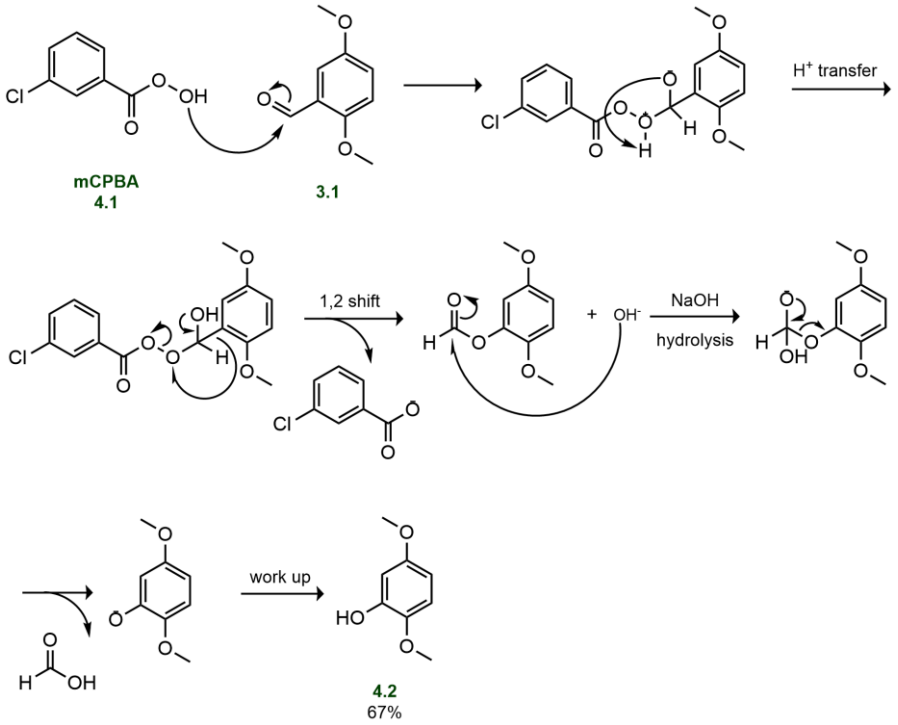
Figure 12: Structure of 2,4,5-trimethoxyamphetamine (TMA-2, 2.18), 2,5-dimethoxy-4-ethoxyamphetamine (MEM, 2.19), and 2,5-dimethoxy-4-propoxyamphetamine (MPM, 2.21), some of the amphetamine derivatives tested in his study.[57]

As TMA-2 induced head-twitch response with an ED_{50} = 12.4 μmol/kg, while MEM and MPM led to ED_{50} values equal to 13.6 and 12.1 μmol/kg, Halberstadt demonstrated that extending the methoxy group in the 4-position of TMA-2 to ethoxide and propoxide did not affect their potencies. MPM was mostly synthesized by use of previously published methods.[38a, 54, 56] (Scheme 4)



Scheme 4: Reaction scheme summarizing the steps for the synthesis of MPM (2.21).

However, an alternative convenient method was investigated and, thus, successfully employed for the first step to convert 2,5-dimethoxybenzaldehyde (3.1) to the corresponding phenol analogue (4.2).[59] This procedure was based on Dakin oxidation reaction, now modified by de Silva *et al.* as a prompt and effective solvent-free procedure for the oxidation of aromatic aldehydes to their phenol derivatives.[60] Dakin reaction, similarly to the Baeyer–Villiger oxidation, consisted originally in the oxidation of hydroxybenzaldehydes to phenols by use of hydrogen peroxide as the oxidating agent in basic conditions and involved lengthy reaction time and work-up. Once the peroxide or peracetic acid, the latter used by Shulgin, is substituted with another oxidant, *meta*-chloroperoxybenzoic acid (*mCPBA*), the solid-state reaction reached completion in less than five minutes, by simple grinding 3.1 and the oxidant together in a mortar. The remarkable amount of heat generated ignited the reagents in a pyrotechnic reaction. As the spontaneous extinguishing of the fire was found to be an indication of completion of the reaction, addition of 10% NaOH aqueous solution, pH correction, and extraction quickly delivered 4.2 in 67% yield. (Scheme 5)



Scheme 5: Mechanism of the Dakin solvent-free oxidation carried out to convert 2,5-dimethoxybenzaldehyde (3.1) to 2,5-dimethoxyphenol (4.2) by use of *meta*-chloroperoxybenzoic acid (*m*CPBA, 4.1) and NaOH.

Classic alkylation in basic conditions was carried out to generate 4-*n*-propoxy-2,5-dimethoxybenzene (4.3). Vilsmeier–Haack reaction was thus performed on the latter to formylate the benzene ring in *para*-position to the propoxy moiety.[38a, 61] Prior to the formylation, a Vilsmeier reagent was prepared by use of phosphoryl chloride and N-methylformanilide as the formyl carrier. Once the aldehyde functionality was in place, MPM (2.21) was finally isolated as hydrochloride salt by following the same reaction steps used for the synthesis of 2C-N (1.8), described in the previous paragraph.

3.3 – SYNTHESIS OF OTHER PHENETHYLAMINES AND THEIR DERIVATIVES

As previously mentioned, diverse derivatives were synthesized to create a library of compounds to test *in vitro*, to investigate biased agonism, and *in vivo*, to explore other

parameters, such as vasodilation, for still ongoing studies. Four phenethylamine scaffolds were prepared. (Figure 13)

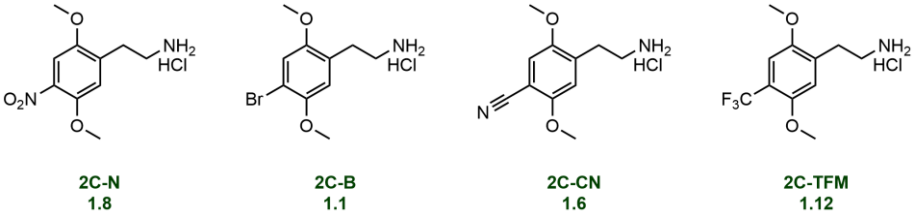
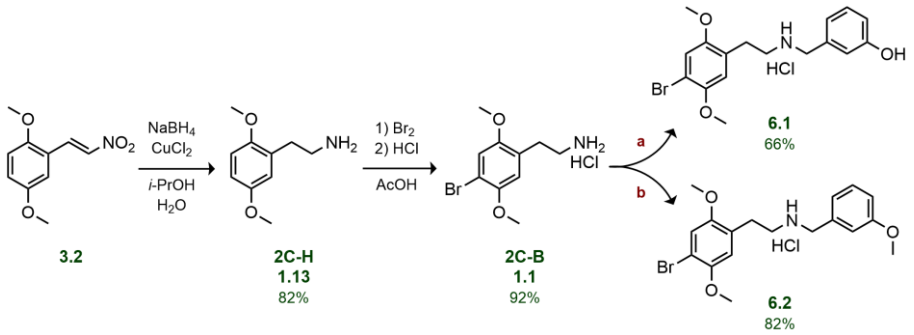


Figure 13: The four phenethylamines synthesized: 2,5-dimethoxy-4-nitrophenylethylamine (2C-N, 1.8), 2,5-dimethoxy-4-bromophenylethylamine (2C-B, 1.1), 2,5-dimethoxy-4-cyanophenylethylamine (2C-CN, 1.6), and 2,5-dimethoxy-4-trifluoromethylphenylethylamine (2C-TFM, 1.12).

3.3.1 – SYNTHESIS OF 2,5-DIMETHOXY-4-BROMOPHENYLETHYLAMINE (2C-B) AND ITS DERIVATIVES

The synthesis of 2,5-dimethoxy-4-bromophenylethylamine hydrochloride (2C-B, 1.1) and its derivatives started with the two-step preparation of 2,5-dimethoxyphenylethylamine (2C-H, 1.13) from 2,5-dimethoxybenzaldehyde as previously mentioned to prepare the nitro derivatives (see paragraph 3.1). However, in this case 2C-H (1.13) was obtained by use of a different reductive method, described in detail in paragraph 3.4, and was used as a free base to undergo classic bromination with bromine in acetic acid, and subsequent precipitation as a salt to deliver 2C-B (1.1). (Scheme 6)

Similarly to previously described reductive amination procedures, the bromo compound was condensed with 3-hydroxybenzaldehyde and 3-methoxybenzaldehyde to produce the desired NBPEA derivatives with substitution on the second ring shifted to the neighboring carbon in 3-position.



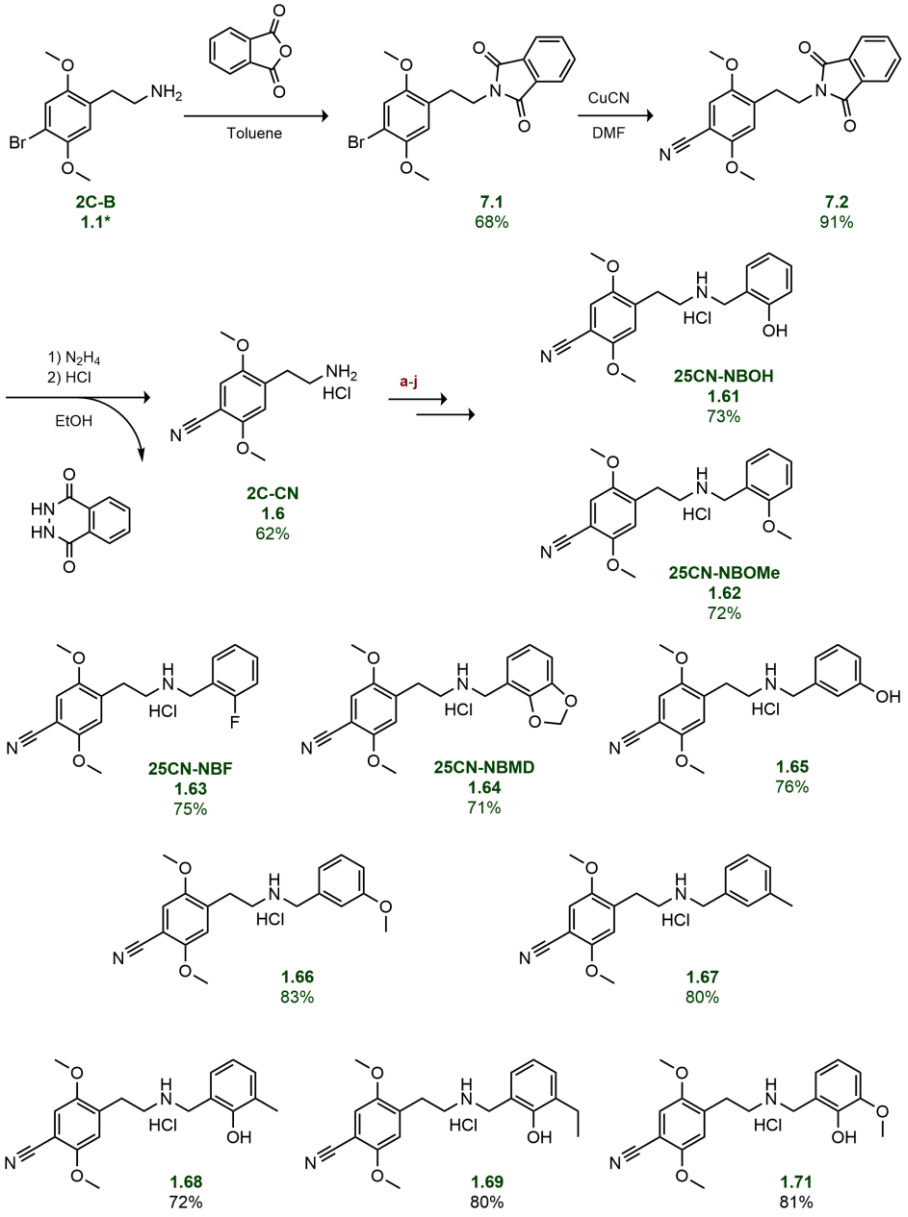
Scheme 6: Reaction scheme for the synthesis of 2C-B hydrochloride (1.1) and its two NBPEA derivatives. **a** and **b**: 2C-B was condensed with 3-hydroxybenzaldehyde and 3-methoxybenzaldehyde by use of TEA and $NaBH_4$ in dry EtOH to deliver 4-bromo-2,5-dimethoxy-N-(3-hydroxybenzyl)phenethylamine hydrochloride (6.1) and 4-bromo-2,5-dimethoxy-N-(3-methoxybenzyl)phenethylamine hydrochloride (6.2), respectively.

The choice of exploring the effects of substitution in *meta*-position on the second ring, instead of the classic *ortho*-position, originated by preliminary *in vitro* testing results on similar scaffolds that highlighted a discrepancy between the potencies of the latter and their ortho-substituted analogues on the 5-HT_{2A} receptor. These results, indeed, demonstrated that substitution in 3-position seems to induce functional selectivity with preference towards β-Arrestin recruitment over G_q activation.
These preliminary data were finally confirmed by the further analyses performed by Christophe P. Stove and his group at Ghent University, whose conclusions led to a manuscript attached at the end of this thesis.[5] (Publication I, Appendix)

3.3.2 – SYNTHESIS OF 2,5-DIMETHOXY-4-CYANOPHENYLETHYLAMINE (2C-CN) AND ITS DERIVATIVES

The synthesis of 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) was carried out according to previously described methods and started with the protection of the terminal amino group of 2C-B (1.1*), previously free based, by use of phthalic anhydride (7.1).[62] (Scheme 7)
Once the amine was protected, the bromide could be replaced via classic Rosenmund–von Braun reaction by use of cuprous cyanide and heat, to produce the protected 4-cyano analogue (7.2).[63] Final deprotection by use of hydrazine and precipitation with hydrogen chloride led to 2C-CN (1.6), which was further used to produce different N-substituted scaffolds via classic reductive amination with triethylamine and sodium borohydride (1.61 - 1.71). As for 2C-B, a number of 2C-CN NBPEA derivatives were prepared with the intention of both investigating any possible display of biased agonism and exploring the effects of diverse substitution patterns on 2- and 3-

positions on the second ring. For this reason, beside known 2-substituted NBPEA structures, such as 25CN-NBOH (1.61), both 3-substituted (1.65 – 1.67) and 2,3-susbtituted (1.68 – 1.71) N-benzylated analogues were synthesized for comparison.



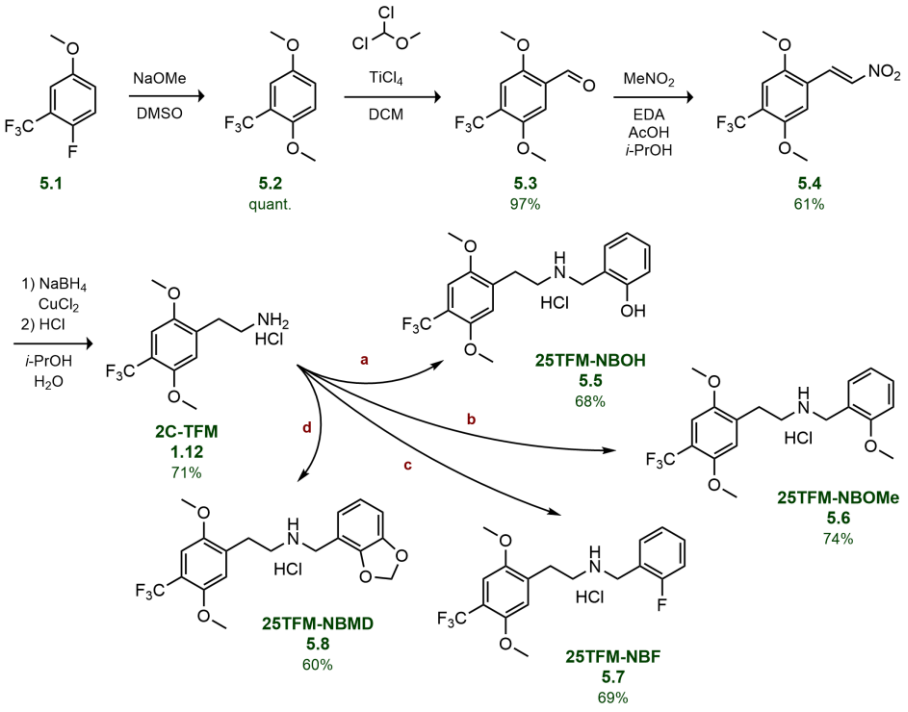
Scheme 7: Reaction scheme for the synthesis of 2C-CN (1.6) and its NBPEA derivatives.
a-**j**: 2C-CN was condensed by use of TEA and, secondly, $NaBH_4$ in dry EtOH with **a**) 2-salicylaldehyde, **b**) 2-methoxybenzaldehyde, **c**) 2-fluorobenzaldehyde, **d**) 2,3-(methylenedioxy)benzaldehyde, **e**) 3-

hydroxybenzaldehyde, **f**) 3-methoxybenzaldehyde, **g**) 3-methylbenzaldehyde, **h**) 2-hydroxy-3-methylbenzaldehyde, **i**) 2-hydroxy-3-ethylbenzaldehyde, and **j**) 2-hydroxy-3-methoxybenzaldehyde. The products were precipitated with HCl to deliver 4-cyano-2,5-dimethoxy-N-(2-hydroxybenzyl)phenethylamine hydrochloride (25CN-NBOH, 1.61), 4-cyano-2,5-dimethoxy-N-(2-methoxybenzyl)phenethylamine hydrochloride (25CN-NBOMe, 1.62), 4-cyano-2,5-dimethoxy-N-(2-fluorobenzyl)phenethylamine hydrochloride (25CN-NBF, 1.63), N-(benzo[d][1,3]dioxol-4-ylmethyl)-2-(2,5-dimethoxy-4-cyanophenyl)ethan-1-amine hydrochloride (25CN-NBMD, 1.64), 4-cyano-2,5-dimethoxy-N-(3-hydroxybenzyl)phenethylamine hydrochloride (1.65), 4-cyano-2,5-dimethoxy-N-(3-methoxybenzyl)phenethylamine hydrochloride (1.66), 4-cyano-2,5-dimethoxy-N-(3-methylbenzyl)phenethylamine hydrochloride (1.67), 2-(4-cyano-2,5-dimethoxyphenyl)-N-(2-hydroxy-3-methylbenzyl)ethanamine hydrochloride (1.68), 2-(4-cyano-2,5-dimethoxyphenyl)-N-(2-hydroxy-3-ethylbenzyl)ethanamine hydrochloride (1.69), and 2-(4-cyano-2,5-dimethoxyphenyl)-N-(2-hydroxy-3-methoxybenzyl)ethanamine hydrochloride (1.71), respectively.

3.3.3 – SYNTHESIS OF 2,5-DIMETHOXY-4-TRIFLUOROMETHYLPHENYLETHYLAMINE (2C-TFM) AND ITS DERIVATIVES

Based on relevant pharmacological data collected by Nichols and his group, 2,5-dimethoxy-4-trifluoromethylphenylethylamine (*2C-TFM*, 1.12) was one of the scaffolds of greatest interest for the development of novel 5-HT_{2A} receptor agonists potentially displaying functional selectivity, along with 2C-N, 2C-B, and 2C-CN analogues.[64] The reason resides in their high potencies towards the 5-HT_{2A} receptor measured via *in vitro* and, particularly, *in vivo* testing, when 2C-TFM officially entered in the family of 5-HT_{2A}R potent agonists in 1994.

The synthetic pathway chosen started with an expensive, but more convenient, starting material, 1-fluoro-4-methoxy-2-(trifluoromethyl)benzene (5.1), which underwent nucleophilic substitution on the fluoride, promptly displaced by the methoxide of NaOMe to deliver 1,4-dimethoxy-2-(trifluoromethyl)benzene (5.2) in quantitative yields.[65] In this case, Rieche formylation was used to introduce the aldehyde functionality on the benzene ring by use of $TiCl_4$ as Lewis acid and dichloromethyl methyl ether, and the resulting 4-trifluoromethyl-2,5-dimethoxybenzaldehyde (5.3) was then converted to 2C-TFM via Henry condensation and copper-catalyzed reduction of its nitrostyrene derivative (5.4), as previously done to prepare 2C-H (1.13).[66] Finally, reductive amination was performed to produce the four trifluoromethylated NBPEA analogues (5.5 – 5.8) illustrated in Scheme 8.

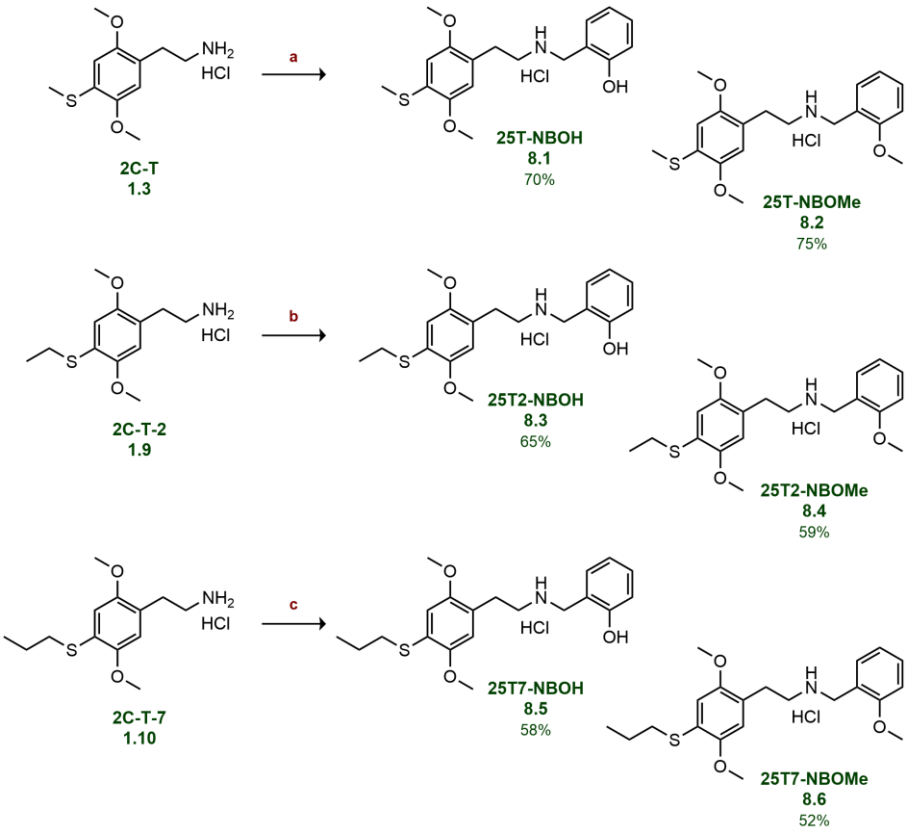


Scheme 8: Reaction scheme for the synthesis of 2C-TFM hydrochloride (1.12) and its NBPEA derivatives. **a**,**b**,**c**, and **d**: 2C-TFM was condensed with 2-salicylaldehyde, 2-methoxybenzaldehyde, 2-fluorobenzaldehyde, and 2,3-(methylenedioxy)benzaldehyde by use of triethylamine (TEA) and, secondly, $NaBH_4$ in dry EtOH, and the N-benzyl products were then precipitated with hydrogen chloride to deliver 25TFM-NBOH hydrochloride (5.5), 25TFM-NBOMe hydrochloride (5.6), 25TFM-NBF hydrochloride (5.7), and 25TFM-NBMD hydrochloride (5.8), respectively.

3.3.4 – SYNTHESIS OF SUBSTITUTED N-BENZYL THIO DERIVATIVES

Synthesized for the first time in 1976 as a result of a collaboration between Shulgin and Nichols, alkylthio phenethylamines belong to a well-recognized class of potent 5-HT_{2A} receptor agonists, with high 5-HT_{2A}R and 5-HT_{2C}R affinities and among the highest efficacies towards the 5-HT_{2A} receptor ever registered within the 2C-*X* family.[67, 68, 69] Due to their potent receptor agonism, the *2C-T* family made its unfortunate entrance into the street drugs market in the late '90s.[70] For the purpose of this project, the three simplest alkylthio phenethylamine structures were chosen as representatives of the thio- class: 2,5-dimethoxy-4-(methylthio)phenylethylamine (*2C-T*, 1.3), 2,5-dimethoxy-4-(ethylthio)phenylethylamine (*2C-T-2*, 1.9), and 2,5-dimethoxy-4-(*n*-propylthio)phenylethylamine (*2C-T-7*, 1.10). (Scheme 9) As the

latter compounds were available, reductive amination by use of the targeted substituted benzyl aldehyde promptly delivered the desired NBOH and NBOMe scaffolds (8.1 – 8.6) in modest to good yields.

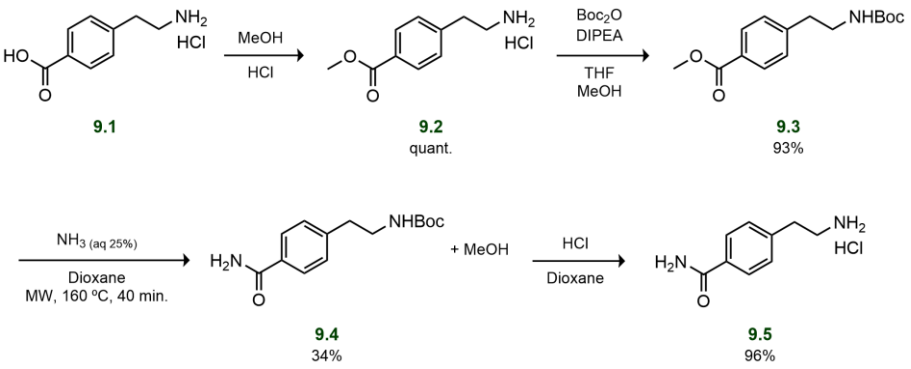


Scheme 9: Reaction scheme for the syntheses of the NBPEA derivatives of 2C-T (1.3), 2C-T-2 (1.9), and 2C-T-7 (1.10).
a: 2C-T (1.3) was condensed with 2-salicylaldehyde and 2-methoxybenzaldehyde by use of triethylamine (TEA) and, secondly, $NaBH_4$ in dry EtOH, and the N-benzyl product was then precipitated with hydrogen chloride to deliver 25T-NBOH hydrochloride (8.1) and 25T-NBOMe hydrochloride (8.2), respectively.
b: 2C-T-2 (1.9) was condensed with 2-salicylaldehyde and 2-methoxybenzaldehyde by use of the procedures described above to synthesize 25T2-NBOH hydrochloride (8.3) and 25T2-NBOMe hydrochloride (8.4), respectively.
c: 2C-T-7 (1.10) was condensed with 2-salicylaldehyde and 2-methoxybenzaldehyde by use of the procedures described above to synthesize 25T7-NBOH hydrochloride (8.5) and 25T7-NBOMe hydrochloride (8.6), respectively.

3.3.5 – SYNTHESIS OF SUPPLEMENTARY COMPOUNDS

Due to the need of analyzing different phenethylamine scaffolds for further computational investigation, a few compounds lacking the two methoxides, typically present in all the 2C-*X* and DO*X* families' members, were prepared. As most of the designed compounds were simple building blocks often commercially available and inexpensive, only three structures needed to be synthesized.

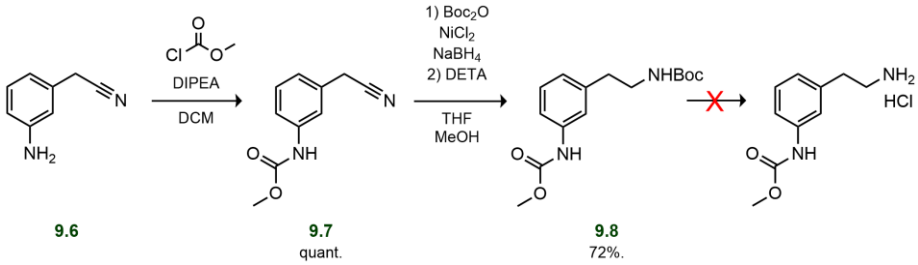
The first targeted compound was methyl 4-(2-aminoethyl)benzoate hydrochloride (9.2), which was prepared from 4-(2-aminoethyl)benzoic acid hydrochloride (9.1) via classic Fischer esterification by refluxing the benzoic acid with methanol and hydrogen chloride overnight. (Scheme 10) After Boc-protection of the amine delivering methyl 4-(2-((*tert*-butoxycarbonyl)amino)ethyl)benzoate (9.3), a few attempts were made to exchange the ester with and amido group via classic aminolysis. First, 9.3 was refluxed overnight with a 25% ammonia aqueous solution in dioxane, leading to minor conversion and recover of the starting material. Secondly, to improve the yields and decrease the reaction time, microwave-assistance was employed, and the reaction was repeated at different temperatures and times. Finally, the desired nucleophilic addition-elimination reaction occurred at 160 ˚C in 40 minutes in low yields to deliver *tert*-butyl (4-carbamoylphenethyl)carbamate (9.4), which was promptly converted to the final 4-(2-aminoethyl)benzamide hydrochloride (9.5) via classic Boc-deprotection and precipitation as a salt.



Scheme 10: Reaction scheme for the synthesis of methyl 4-(2-aminoethyl)benzoate hydrochloride (9.2) and 4-(2-aminoethyl)benzamide hydrochloride (9.5).

As 9.2 and 9.5 were synthesized to test phenethylamine structures bearing an electron density cloud in 4-position, and were functionalized with two EWGs, another compound with EDGs in 4-position was prepared for comparison. The commercially available 2-(3-aminophenyl)acetonitrile (9.6) went through amide coupling by use of

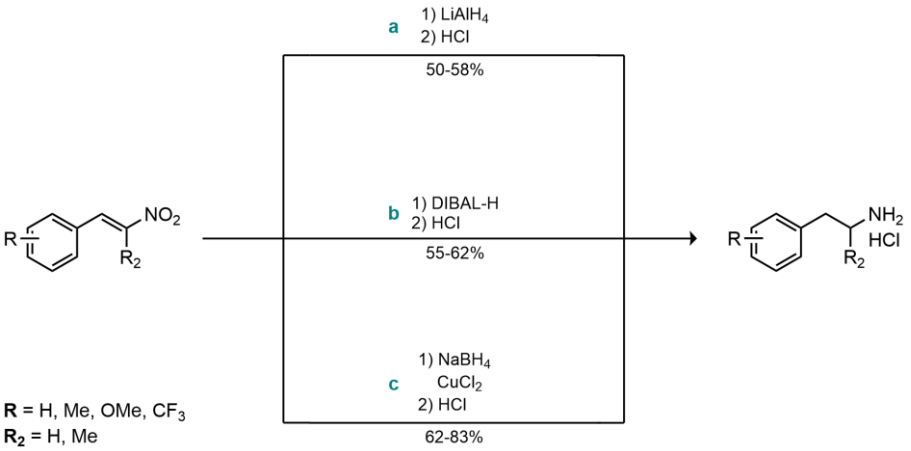
methyl chloroformate, as activated methylformate donor, and N,N-Diisopropylethylamine (DIPEA), as a bulky base not nucleophilic enough to compete with the amine.[71] (Scheme 11) The reaction delivered methyl (3-(cyanomethyl)phenyl)carbamate (9.7) in excellent yields, which was one of the compounds tested *in vitro* thereafter. Mistakenly, at first the freshly synthesized carbamate seemed to be a good candidate to prepare its phenethylamine analogue, but as either treatment of 9.7 with a reducing agent (e.g., $LiAlH_4$) and treatment of 9.8 with an acid would have led to reduction of the carbamate moiety, the phenethylamine analogue was not prepared.



Scheme 11: Reaction scheme for the synthesis of methyl (3-(cyanomethyl)phenyl)carbamate (9.7). Once Boc-protected, the methyl (3-(2-((tert-butoxycarbonyl)amino)ethyl)phenyl)carbamate (9.8) could not be converted to the phenethylamine derivative.
DETA: Diethylenetriamine

3.4 – DEVELOPMENT OF A NEW REDUCTIVE METHOD TO SYNTHESIZE 2C-X ANALOGUES

Classic synthetic routes to reduce 2,5-dimethoxy-β-nitrostyrene (3.2) analogues to their 2-(2,5-dimethoxyphenyl)ethan-1-amine (2C-H, 1.13) derivatives in one step involve the use of metal hydrides, especially $LiAlH_4$. (see paragraphs 3.1 and 3.2) Lithium aluminium hydride as well as DIBAL-H need special precautions to be taken, such as inert atmosphere and anhydrous conditions, and require implementing lengthy separation techniques of the products, usually in modest yields.[72] (Scheme 12)



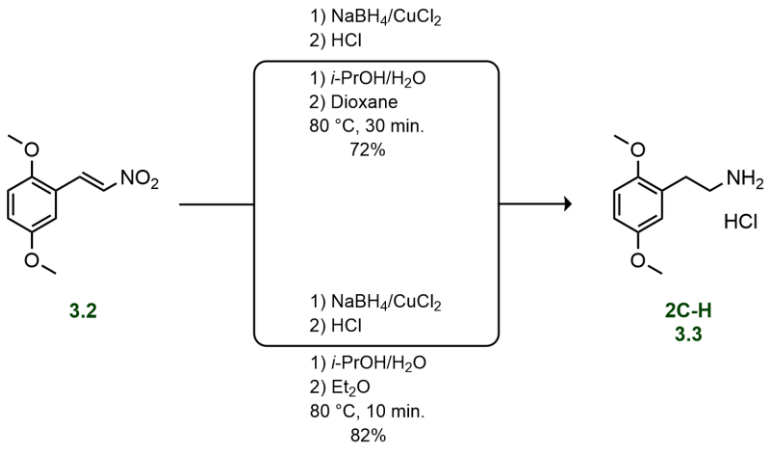
Scheme 12: General overview comparing different methods to achieve reduction of substituted β-nitrostyrenes to aminoalkane derivatives. **a)** Classic route using lithium aluminium hydride, requiring special precaution and cumbersome separation methods, with modest yields;[72] **b)** Another used route requiring a hydride, diisobutylaluminium hydride (DIBAL-H), characterized by the same issues of method **a** and employed in the paragraphs 3.1 and 3.2. **c)** The new route presented in this thesis involving sodium borohydride and copper (II) chloride. Differently from the other methods, this pathway does not require anhydrous conditions nor lengthy separation techniques, is faster and higher yielding.

For this reason, since 1967, sodium borohydride, as an inexpensive, safe, and easy-to-handle reducing agent, has been extensively investigated to accomplish the reduction of β-nitrostyrenes to the corresponding phenethylamines. However, this accomplishment requires in fact both the double bond and the nitro group to be reduced, where $NaBH_4$ per se can convert exclusively alkenes to alkanes but not nitro groups. Several catalysts, therefore, were tested over the last decades in combination to $NaBH_4$ to reduce the nitro moiety as well, though successfully converting nitroarene analogues to their corresponding aromatic amines only.[73]

In 2018, Simon Jademyr was a visiting M.Sc. student from the University of Gothenburg in Jesper Kristensen's group, when he had the idea of testing a new method that could potentially accomplish the conversion of substituted β-nitrostyrenes in milder conditions. Inspired by previous studies reporting the successful conversion of aliphatic nitro compound to amines by use of $NaBH_4$ and $CuSO_4$, he developed an alternative method that involved another copper (II) salt, now $CuCl_2$, to achieve in combination with sodium borohydride the desired conversion of α,β-unsaturated nitroalkenes into aminoalkanes.[74] However, this method was attempted by other students and appeared to be irreproducible, by typically delivering a brown suspension, impossible to purify by acetone washing or other simple separation techniques. To solve this issue, a collaboration started, and I began diving into any detail of this method, to frame its weak points and, possibly, optimize it and

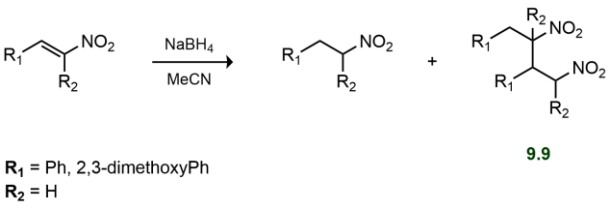
expand it. The research outcome led to a manuscript, attached to this thesis. (Manuscript III, Appendix)

Among the numerous β-nitrostyrenes analogues, 2,5-dimethoxy-β-nitrostyrene (3.2) was chosen as the best representative starting material on which to probe the method, due to its versatility for the preparation of several 2C-*X* structures. (Scheme 13)



Scheme 13: Comparison between the reaction conditions used originally (top) and the optimized setting developed (bottom), herein described. The discovery that the amino product formation is extremely rapid, in fact, led to even shorter reaction times and the suppression of side products formation. Moreover, the achievement of a better phase separation during extraction, and the substitution of acetone washing with HCl precipitation in ether, made the method even faster, higher yielding, and completely reproducible.

Several different orders of addition of the reagents were tested, leading to the conclusion that rapid addition of the reagents, as described in the manuscript attached to this thesis, is crucial in terms of yields and formation of side products. Particularly, it was noticed that stirring the mixture containing the reagents and the reducing agent before copper chloride addition led to reduced yields. This phenomenon might have been already explained by Meyers and Sircar in 1967, when they published their conclusions after investigating the reduction of a few nitroalkenes to their corresponding nitroalkane derivatives by $NaBH_4$ in either ethanolic aqueous solution or acetonitrile.[75] According to their results, stirring borohydride with two of the diverse nitro substrates used in their study delivered, beside the desired products, dimeric structures as side products, which seemed to be the consequence of Michael addition between the α-carbanion of nitroalkane molecules with the unreacted nitroalkene. (Scheme 14)



Scheme 14: Overview of the findings reported by Meyers and Sircar in 1967.[75] Only unsubstituted β-nitrostyrene and 2,3-dimethoxy-β-nitrostyrene displayed the formation of Michael adducts as dimeric structures (9.9).

Furthermore, the reaction was monitored by UPLC-MS (Ultra Performance Liquid Chromatography – Mass Spectrometry) at 15 minutes intervals over 2 hours, and this analysis showed that the product formation is very rapid, differently from what previously supposed, and the product concentration steadily drops overtime. (Figure 14)

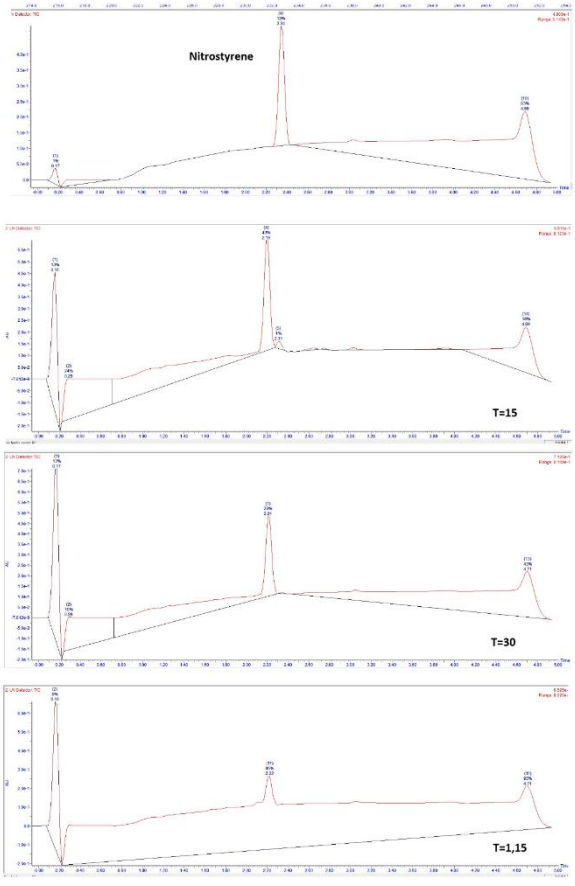


Figure 14: UPLC chromatograms stacked chronologically showing the reaction mixture species formed over 1.15 hours. After 15 minutes, the concentration of the product, 2-(2,5-dimethoxyphenyl)ethan-1-amine hydrochloride (2C-H), eluted between 2.19 and 2.22 minutes, is much higher than after 30 minutes, decreasing further overtime.

Moreover, the reaction was repeated and stopped at different times up to 90 minutes, confirming what was observed by UPLC analysis: after 10 minutes the yield of 3.2 reached its highest concentration, to start dropping progressively thereafter. As it was not possible to carry out the reduction on all the substrates used by Jademyr, it might be possible, nonetheless, to speculate that the actual yields for those compounds might be much

higher than those recorded, as his idea involved 30-minute stirring and did not investigate reaction times. The reaction was tested in other solvents, such as H_2O and methanol, which were found non-optimal for either solubility or extraction issues, and, thus, the 2-propanol/H_2O system seemed to be the most convenient solution to adopt. During extraction, the non-sharp phase separation expected while employing the original method, together with the formation of a jellified phase in between, were easily solved by increasing both the volume and the basicity of the aqueous phase, from 25% to 35% NaOH solution, which guarantees optimal phase separation and quick extraction time, while no jellification occurs. Filtration of the reaction mixture before extraction as well as DETA addition to coordinate any possible catalyst leftover were both proved to be irrelevant on the phase separation clarity.

Furthermore, the purification step by use of acetone was thoroughly investigated. NMR analysis on the acetone washing leftover highlighted the presence of variable amounts of the amino products, indicating that their partial solubility in acetone is one of the diverse reasons why repeating the original method led to inconsistent results. As the modifications applied before the purification step made possible to already extract the aminoalkane product as a free base after concentration of the organic extracts in good degree of purity, precipitation of the products as hydrochloride salts is optional, but quickly achievable by dilution in a large volume of diethyl ether and addition of an excess of HCl solution in ether, followed by filtration and drying.

The improved method was tested on another nitrostyrene representative, *p*-methoxy-β-nitrostyrene (10.1) with consistent results. (Table 1) In Table 1, other substrates reduced by the $NaBH_4/CuCl_2$ system are listed. The system was tested on two nitroarene examples, nitrobenzene (10.2) and *p*-bromo-nitrobenzene (10.3), delivering the corresponding aniline derivatives in excellent yields. Additionally, aromatic esters seem to be also effectively reduced by this method to their alcohol derivatives, as methyl benzoate (10.4) was fully converted to benzyl alcohol (10.41) by overnight stirring.

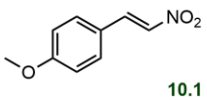
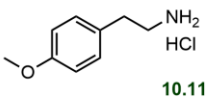
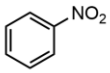
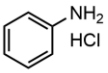
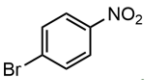
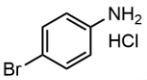
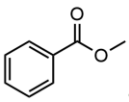
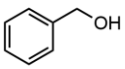
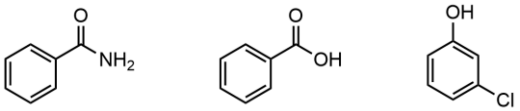
Substrate	Product	Yield (%)	Time
 10.1	 10.11	82	10 min.
 10.2	 10.21	96	18 h
 10.3	 10.31	97	30 min.
 10.4	 10.41	92	18 h

Table 1: Overview of other substrates reduced by use of this method, their products, yields, and reaction times.

Finally, the reduction was tested on additional simple aromatic structures carrying a reducible moiety. (Figure 15) By continuous stirring up to 24 hours, neither benzamide (10.5) nor benzoic acid (10.6) were affected by the reducing system, while the conservation of the chloride atom on 3-chlorophenol (10.7) confirmed that, as for 10.3, $NaBH_4/CuCl_2$ system does not cause dehalogenation of aryl halides.



10.5 10.6 10.7

Figure 15: Structures that were not reduced by the $NaBH_4/CuCl_2$ system.

For more details, the manuscript describing this work and its results is attached to this thesis. (Manuscript III, Appendix)

3.5 – DESIGN AND SYNTHESIS OF STRUCTURALLY CONSTRAINED N-BENZYL PHENETHYLAMINES

Based on previous pharmacological results of Anders A. Jensen about some tested CIMBI radioligands with structural constraint on the N-substitution of 2C-B and 2C-CN derivatives, a total of eight conformationally constrained structures were designed and synthesized.[76] The targeted constraint consisted in the addition of either a 2,3-dihydrobenzofuran or a chroman moiety to the amino nitrogen of the phenethylamines, creating a chiral center on the carbon in α- position to the nitrogen atom. (Figure 16)

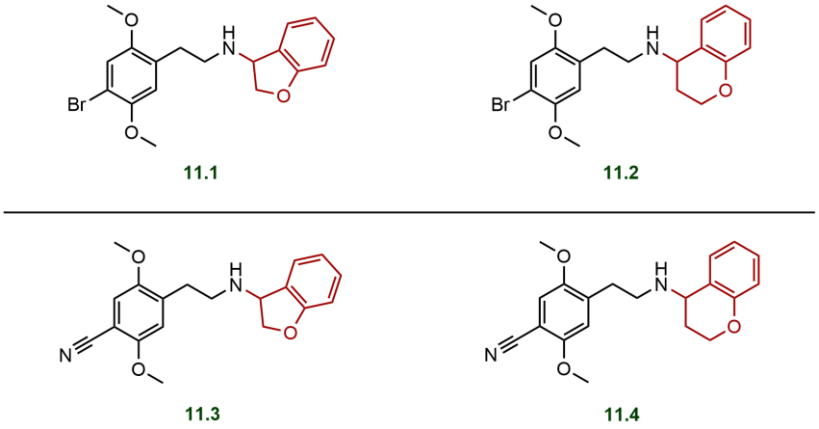
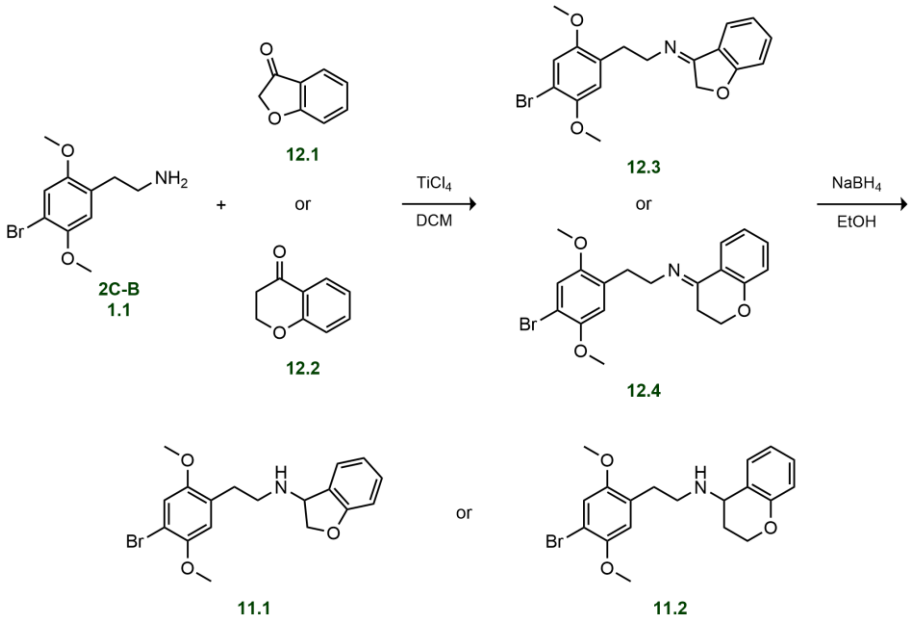


Figure 16: Illustration of the four targeted constrained scaffolds: (±)-N-(4-bromo-2,5-dimethoxyphenethyl)-2,3-dihydrobenzofuran-3-amine (11.1) and (±)-N-(4-bromo-2,5-dimethoxyphenethyl)chroman-4-amine (11.2) as 2C-B derivatives (top), and (±)-4-(2-((2,3-dihydrobenzofuran-3-yl)amino)ethyl)-2,5-dimethoxybenzonitrile (11.3) and (±)-4-(2-(chroman-4-ylamino)ethyl)-2,5-dimethoxybenzonitrile (11.4), as 2C-CN derivatives (bottom). In dark red, 2,3-dihydrobenzofuran and chroman functionalities substituted in 2- and 3- position, respectively, with 2C-B and 2C-CN scaffolds.

The interest towards these compounds emerged by the idea of investigating the effects of rigid structures on the 5-HT_{2A} receptor conformation, in order to test them *in vitro* on the individual intracellular pathway-specific assays, measuring EC_{50} and E_{max} relative to G_q and β-arrestin as happened for all the compounds delivered, and explore structure-activity relationships in the attempt to determine the link between conformational constraint and biological response.

For the condensation of 2C-B and 2C-CN with the substituents of choice, old research journals from previous work carried out in Kristensen's group were investigated. The only attempts to synthesize the compounds illustrated in Scheme 15 involved a two-step pathway using $TiCl_4$ as Lewis acid and triethylamine in dichloromethane, to implement the condensation between the phenethylamine and the ketones of the 2,3-dihydrobenzofuran and the chroman moieties in need to be attached, namely 3-

coumaranone (12.1) and 4-chromanone (12.2), by formation of the corresponding imines (12.3 and 12.4).[77] Subsequently, the imines could have been reduced in a second step by $NaBH_4$, to deliver the targeted bromo- and cyano- constrained derivatives. (Scheme 15)

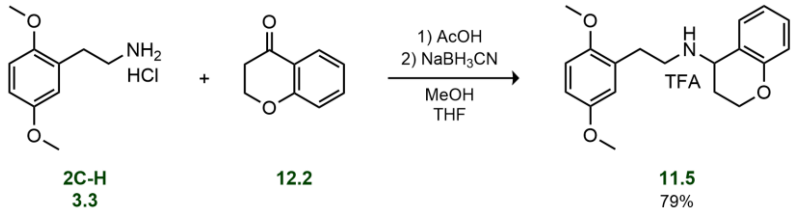


Scheme 15: Scheme of the 2-step synthesis of 11.1 and 11.2 described by previous research studies and tested on 2C-B (1.1). The method involves the formation of a 2C-B/$TiCl_4$ complex prior to addition of the ketone. Once the imine is formed (12.3 and 12.4), $NaBH_4$ should reduce the double bond to deliver the desired products: (±)-N-(4-bromo-2,5-dimethoxyphenethyl)-2,3-dihydrobenzofuran-3-amine (11.1) and (±)-N-(4-bromo-2,5-dimethoxyphenethyl)chroman-4-amine (11.2). However, no imine formation was detected up to 24 hours stirring and the targeted scaffolds could not be prepared by use of this method.

However, testing this route on 2C-B and both the ketones led to a complex mixture of products, where neither unreacted 2C-B nor the imine could be detected. Not surprisingly, addition of the borohydride to the hypothetical imine mixture delivered neither of the targeted bromo compounds, but a complex mixture carrying multiple species. Therefore, titanium tetrachloride seemed to be unsuitable for this synthesis. For this reason, alternative synthetic pathways were explored. Quickly, while studying reducing agents, sodium cyanoborohydride stood out as a compound theoretically able to carry out the targeted conversion, after formation of the imine in acidic conditions. At this point, as the investigation of constrained structures was at its early stages and it was not clear yet how many analogues needed to be prepared, it seemed more reasonable to test the new route first on 2C-H, in order to produce both a general method to synthesize 3-coumaranone and 4-chromanone derivatives being

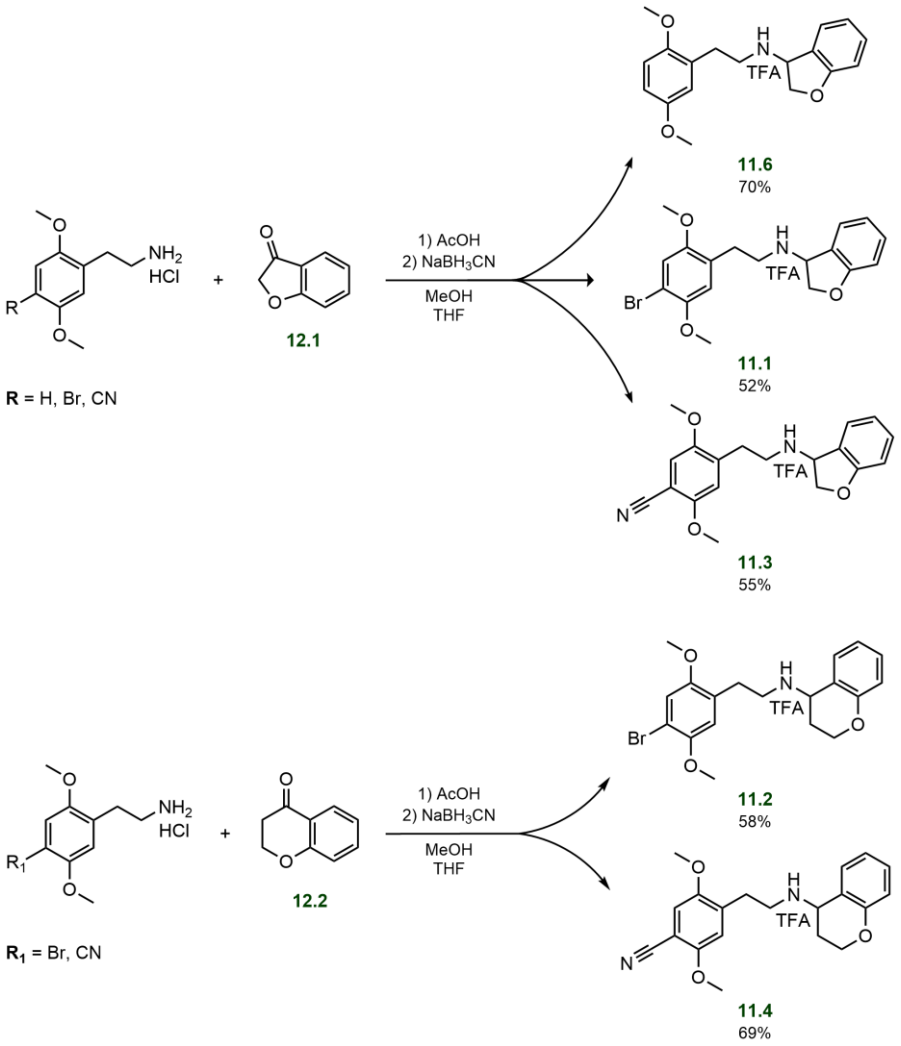
unsubstituted in the 4-position of the phenethylamine, and a reference compound for later stage *in vitro* studies.

First attempts to form the imine by use of glacial acetic acid in methanol/tetrahydrofuran (2:1) solvent system turned out to be immediately successful, reaching full conversion in 30 minutes. Addition of an excess of $NaBH_3CN$ 1.0 M solution in THF and HPLC separation of the crude, delivered (±)-N-(2,5-dimethoxyphenethyl)chroman-4-amine trifluoroacetate (11.5) in good yields. (Scheme 16)



Scheme 16: Scheme illustrating the new developed method, consisting in an initial formation of the imine in acidic conditions and the reduction of the latter by $NaBH_3CN$ to the racemic mixture of the final constrained amine, being (±)-N-(2,5-dimethoxyphenethyl)chroman-4-amine trifluoroacetate (11.5), which is also a novel compound.
TFA: trifluoroacetate.

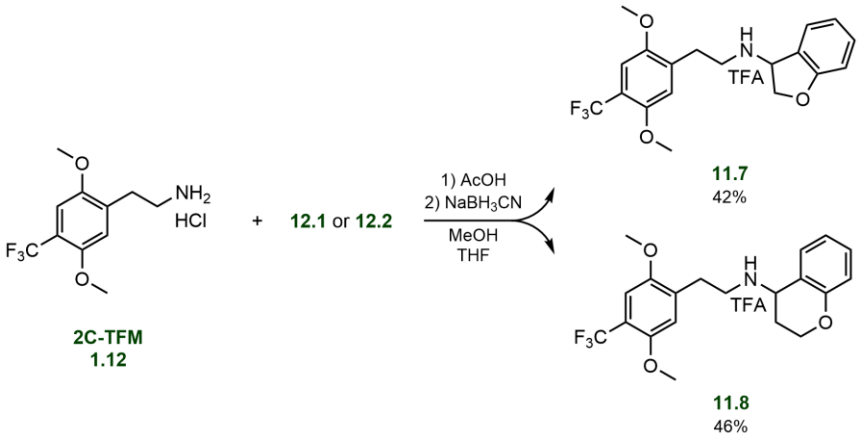
Due to the effectiveness of this method, that guarantees the preparation of the desired constrained structure in good yields by simple addition of the reagents in the same flask, without special precautions nor cumbersome workup, this procedure was employed for the synthesis of all the other conformationally restrained products. (Scheme 17)



Scheme 17: This reductive amination method was satisfactorily used to synthesize all the racemic mixtures of the targeted structurally constrained derivatives. Condensation of 3-coumaranone (12.1) with the phenethylamine hydrochloride of choice, and reduction by $NaBH_3CN$ delivered three products: (±)-N-(2,5-dimethoxyphenethyl)-2,3-dihydrobenzofuran-3-amine trifluoroacetate (11.6), (±)-N-(4-bromo-2,5-dimethoxyphenethyl)-2,3-dihydrobenzofuran-3-amine trifluoroacetate (11.1), and (±)-4-(2-((2,3-dihydrobenzofuran-3-yl)amino)ethyl)-2,5-dimethoxybenzonitrile trifluoroacetate (11.3). (top) Repeating the procedure by use of 4-chromanone (12.2) instead of 12.1, as previously done with 2C-H in Scheme 16, led to the preparation of (±)-N-(4-bromo-2,5-dimethoxyphenethyl)chroman-4-amine trifluoroacetate (11.2) and (±)-4-(2-(chroman-4-ylamino)ethyl)-2,5-dimethoxybenzonitrile trifluoroacetate (11.4). (bottom)

Occasionally, the products were separated by classic column chromatography and later precipitated as hydrochloride salts. However, this procedure led to lower yields, due to the non-ideal separation of the fractions carrying the product, and, therefore, HPLC separation was usually preferred.

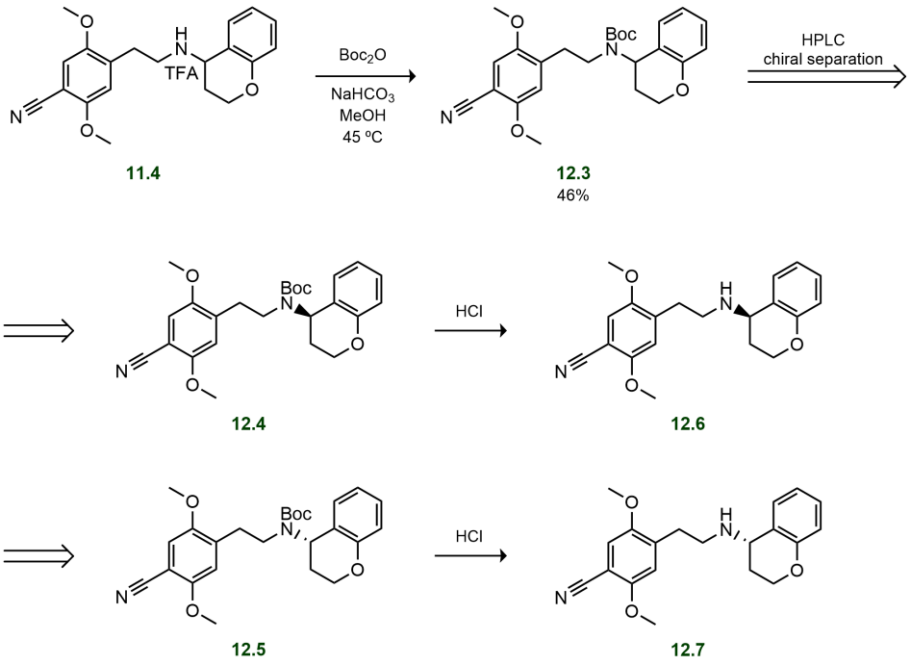
This technique was exploited further to synthesize novel scaffolds of potential interest in the investigation of biased agonism and the effect of structural constraint on the drug behavior towards the 5-HT_{2A} receptor. Due to the remarkable potency of 2C-TFM (1.12) towards this receptor subtype, (±)-N-(2,5-dimethoxy-4-(trifluoromethyl)phenethyl)-2,3-dihydrobenzofuran-3-amine trifluoroacetate (11.7) and (±)-N-(2,5-dimethoxy-4-(trifluoromethyl)phenethyl)chroman-4-amine trifluoroacetate (11.8) were prepared as novel compounds. (Scheme 18)



Scheme 18: The AcOH/$NaBH_3CN$ method was used on 2C-TFM hydrochloride (1.12) as well, delivering (±)-N-(2,5-dimethoxy-4-(trifluoromethyl)phenethyl)-2,3-dihydrobenzofuran-3-amine trifluoroacetate (11.7) and (±)-N-(2,5-dimethoxy-4-(trifluoromethyl)phenethyl)chroman-4-amine trifluoroacetate (11.8) in modest yields. These products were difficult to handle as trifluoroacetate salts, due to the high electrostatic force generated between the product powder and the glassware. For delivery purposes, the hydrochloride salts were preferred.

As all the constrained structures synthesized were racemic mixture, the next step involved the chiral separation of the individual enantiomers. The compound 11.4 was chosen as good candidate for attempting the resolution of its racemic mixture.

First, 11.4 needed to undergo Boc-protection prior to chiral separation. Monitoring the reaction by TLC and UPLC-MS showed only partial conversion of the cyano substrate when stirred with one equivalent of di-*tert*-butyl dicarbonate (Boc_2O) and 1.1 of bicarbonate overnight. As the addition of a further equivalent of them did not lead to full conversion, heating the mixture to 45 ˚C, after adding a third equivalent of dicarbonate and bicarbonate, finally delivered (±)-tert-butyl chroman-4-yl(4-cyano-2,5-dimethoxyphenethyl)carbamate (12.3). (Scheme 19)



11.4 | 12.3 (46%) | 12.4 | 12.6 | 12.5 | 12.7

Scheme 19: Classic Boc-protection was performed to convert (±)-4-(2-(chroman-4-ylamino)ethyl)-2,5-dimethoxybenzonitrile trifluoroacetate (11.4) to (±)-tert-butyl chroman-4-yl(4-cyano-2,5-dimethoxyphenethyl)carbamate (12.3). The latter was chirally separated by HPLC, delivering the Boc-protected enantiomers, namely (+)-tert-butyl chroman-4-yl(4-cyano-2,5-dimethoxyphenethyl)carbamate and (-)-tert-butyl chroman-4-yl(4-cyano-2,5-dimethoxyphenethyl)carbamate, which are conventionally referred to as 12.4 and 12.5. Final deprotection would deliver the targeted separated constrained enantiomeric structures, 12.6 and 12.7.

Surprisingly, enantiomeric resolution was quickly achieved by HPLC using ChiralPak® AD-H column, packed with amylose tris(3,5-dimethylphenylcarbamate) coated silica gel and heptane:2-propanol:methanol (98:1:1) solvent system as mobile phase. (Figure 17)

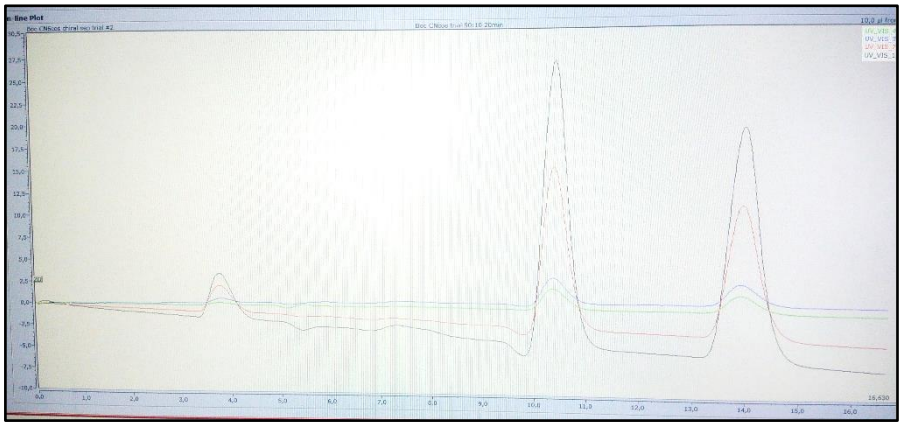
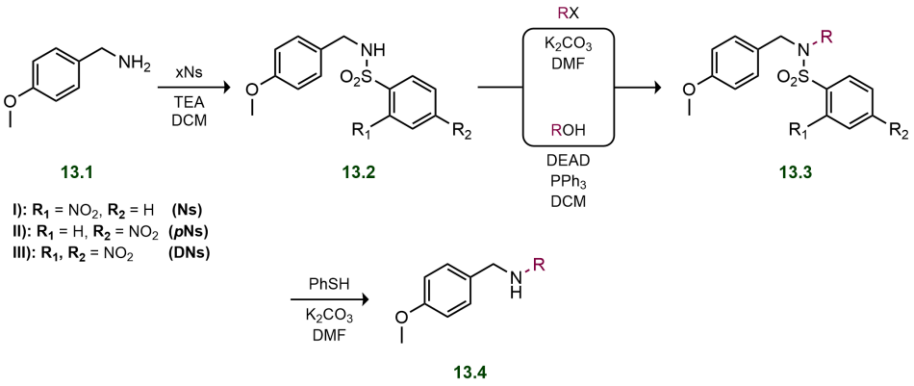


Figure 17: Picture of the chromatogram of the chiral separation of (±)-tert-butyl chroman-4-yl(4-cyano-2,5-dimethoxyphenethyl) carbamate (12.3) into its separate enantiomeric forms, by injection of 10 µl of racemic mixture.

Unfortunately, as the project was suddenly interrupted, deprotection of the enantiomers, followed by absolute configuration determination and characterization, could not be performed. Nonetheless, the synthetic method for the preparation of constrained 2C-*X* derivatives, herein described, is solid, reproducible, rapid, and effective. As some of the racemic mixtures were tested *in vitro*, proving to be the first β-arrestin biased 5-HT_{2A} receptor agonists ever synthesized, which will be discussed in the next section, these scaffolds might open the way for future research on this topic. Further studies, indeed, might finally investigate the extent of functional selectivity of each individual enantiomer and some species that could not be tested, such as 11.4, 11.7, and 11.8. Another possible follow-up study might involve testing on β-arrestin and G_q assays an anti-Parkinson's drug, *lisuride*, which belongs to the class of ergolines. Lisuride, despite sharing the ability of activating numerous receptor subtypes, including 5-HT_{2A}, with another ergoline analogue, LSD, does not cause hallucinations to arise.[78] Therefore, it might be possible to hypothesize that lisuride might exhibit a preference towards β-arrestin recruitment over $G\alpha_q$, upon 5-HT_{2A} receptor binding.

3.5.1 – ALTERNATIVE ENANTIOSELECTIVE SYNTHESIS OF STRUCTURALLY CONSTRAINED N-BENZYL PHENETHYLAMINES

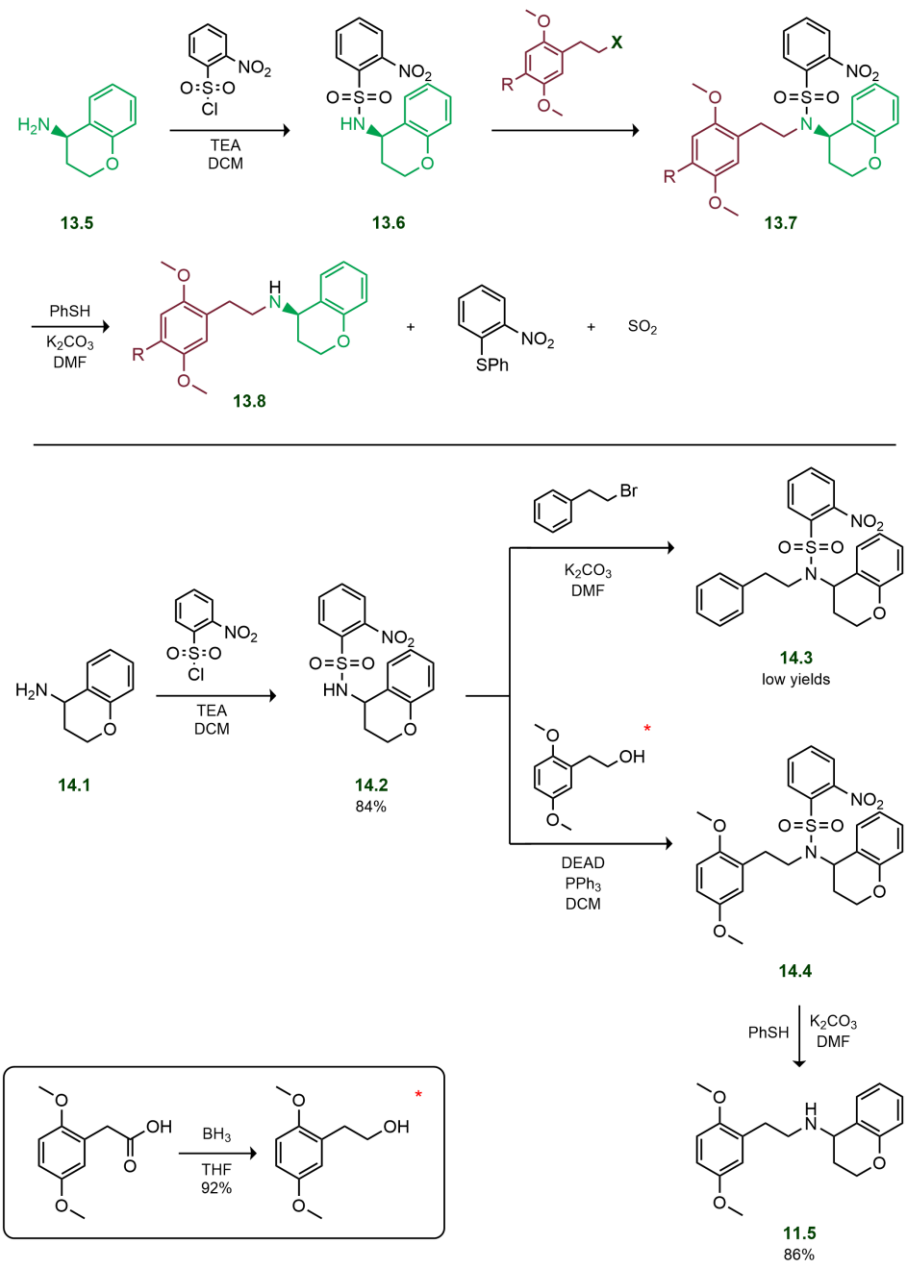
Alternative procedures to enantioselectively synthesize the targeted constrained enantiomer with predictable absolute configuration were explored. The Fukuyama method, which takes advantage of nosyl-protection, seemed appropriate to this purpose and, therefore, was investigated.[79] (Scheme 20)



Scheme 20: Overview of the Fukuyama method, where a primary amine (13.1) undergoes nosyl-protection in basic conditions, and the protected resulting amine (13.2) is alkylated by use of either conventional alkylation with a halide (RX) (top) or Mitsunobu conditions employing an alcohol (ROH) (bottom) as alkyl donor.[80] The alkylated protected amine (13.3) is easily deprotected to the corresponding mono-substituted amino product (13.4). *xNs* can be **I)**: 2-nitrobenzenesulfonyl chloride (Ns), **II)**: 4-nitrobenzenesulfonyl chloride (*p*Ns), or **III)**: 2,4-dinitrobenzenesulfonyl chloride (DNs).

Fukuyama and his coworkers developed a method to mono-alkylate amines by first protecting and activating them with nitrobenzenesulfonyl chloride (nosyl group or *Ns*) to produce the corresponding nitrobenzenesulfonamides that will undergo alkylation. In his reports, (4-methoxyphenyl)methanamine (13.1) was employed as amino substrate, that was converted to N-(4-methoxybenzyl)benzenesulfonamide (13.2) containing one or two nitro groups in *ortho* and/or *para* position to the sulfur atom on the nosyl moiety. The sulfonamide was N-alkylated via either classic alkylation in basic conditions with the proper alkyl halide, or via Mitsunobu reaction, where the amine reacts with an alcohol in the presence of diethyl azodicarboxylate (*DEAD*) and triphenylphosphine.[80] The resulting N,N-disubstituted sulfonamide (13.3) was deprotected by aromatic nucleophilic substitution with thiophenol in mild basic conditions, to deliver the mono-alkylated secondary amine, 1-(4-methoxyphenyl)-N-alkylmethanamine (13.4).

As the final goal was to create a method where controlling the stereochemistry of the molecule since the beginning would lead to the direct preparation of 12.4, 12.5, and their analogues, it was hypothesized that, by employing an enantiomer of the amino version of 2,3-dihydrobenzofuran and chromane, with known absolute configuration, it would be possible to exploit the Fukuyama method to achieve this goal. (Scheme 21)



Scheme 21: Top: Reaction scheme illustrating the idea of exploiting the Fukuyama method to enantioselectively synthesize separated stereoisomers with pre-established spatial configuration. (R)-chroman-4-amine (13.5) was used as an example of pure enantiomer, converted by nosyl-protection to (R)-N-(chroman-4-yl)-2-nitrobenzenesulfonamide (13.6). The latter is alkylated to a properly substituted (R)-N-(chroman-4-yl)-N-(2,5-dimethoxyphenethyl)-2-nitrobenzenesulfonamide derivative (13.7), that can be

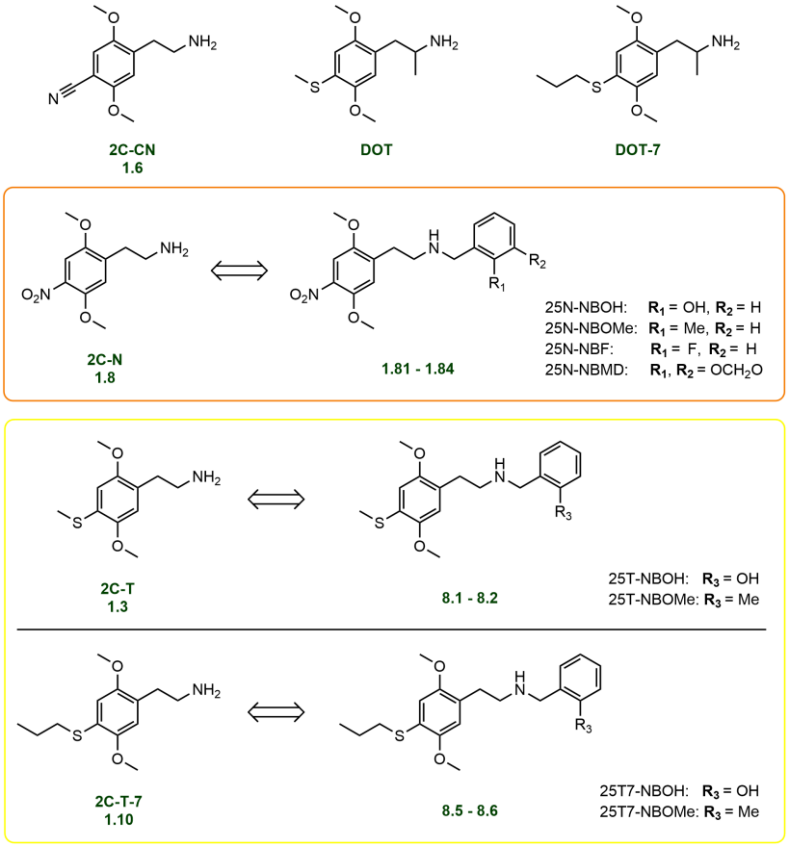
eventually converted to the final product (13.8) by deprotection under basic conditions. Bottom: Overview of the experimental route used to test the feasibility of this new approach. After nosyl-protection, Mitsunobu reaction with 2-(2,5-dimethoxyphenyl)ethan-1-ol (*) seemed to be the best choice as conventional alkylation with 2-phenylethyl bromide the product in low yields.
2-(2,5-dimethoxyphenyl)ethan-1-ol (*) was obtained by reduction of 2-(2,5-dimethoxyphenyl)acetic acid with borane in THF, from 0 ˚C to rt over 8 hours.[81]

Before using one of the two enantiomers of chroman-4-amine, the method was tested on its racemate in hydrochloride form, which was free based to chroman-4-amine (14.1) before use. The latter was reacted with 2-nitrobenzenesulfonyl chloride to deliver the nosyl-protected N-(chroman-4-yl)-2-nitrobenzenesulfonamide (14.2) in good yields.
Classic alkylation using (2-bromoethyl)benzene was carried out, leading to low yields and cumbersome separation of the final alkylated product. Therefore, Mitsunobu conditions were employed to successfully generate the N-(chroman-4-yl)-N-(2,5-dimethoxyphenethyl)-2-nitrobenzenesulfonamide (14.4) by reaction of the sulfonamide with 2-(2,5-dimethoxyphenyl)ethan-1-ol, freshly prepared. However, this step was performed by Christian Klindt Hvass, a bachelor student under my supervision in 2019, and the corresponding experimental data, such as yields and NMR spectra, could not be retrieved. The alkylated tertiary amine was, thus, deprotected to deliver (±)-N-(2,5-dimethoxyphenethyl)chroman-4-amine (11.5) via conventional nosyl group deprotection procedure. Eventually, this approach demonstrated to be a feasible alternative for the synthesis of 11.5 and, possibly, its separated chiral structures.

4. PHARMACOLOGICAL INVESTIGATION OF SYNTHESIZED COMPOUNDS

4.1 - MANUSCRIPT II

One of the two manuscripts resulting from the experimental investigation of biased 5-HT_{2A} receptor agonists involved the investigation of structure-activity relationships of some synthesized compounds according to their the *in vitro* screening results.[51] The compounds analyzed included 2C-CN (1.6), 2C-N (1.8), 2C-T (1.3), and 2C-T-7 (1.10), as 2C-*X* representatives, 1-(2,5-dimethoxy-4-(methylthio)phenyl)propan-2-amine (*DOT*) and 1-(2,5-dimethoxy-4-(propylthio)phenyl)propan-2-amine (*DOT-7*), as DO*X* analogues and, therefore, representing the amphetamine class, in addition to some N-benzyl substituted nitro and thio derivatives. (Scheme 22) For the pharmacological characterization, Eline Pottie developed and used the two functional assays previously mentioned, namely *β-arrestin2* (βarr2) and *miniG*α_q.



Scheme 22: Illustration of the synthesized compounds that were tested on βarr2 and miniGα_q assays by Eline Pottie and her co-workers. 2,5-dimethoxy-4-methylthioamphetamine (DOT) and 2,5-Dimethoxy-4-*n*-propylthioamphetamine (DOT-7) were tested, in parallel to LSD and serotonin, as reference compounds. 2C-CN (1.6), 2C-N (1.8), 2C-T (1.3), 2C-T-7 (1.10), together with all the four nitro NBPEA derivatives (1.81 – 1.84), and the NBOHs and NBOMes of 1.3 and 1.10, were tested in this study.

Monitoring the listed compounds while inducing either β-arrestin or Gα_q recruitment at the 5-HT_{2A} receptor showed that all the analyzed compounds are receptor agonists, with potencies in the nanomolar order, and, sometimes, even picomolar (EC_{50}s between 0.7 nM and 170 nM in the βarr2 assay, and between 1.7 nM and 500 nM in the miniGα_q recruitment assay), and efficacies with E_{max} values between 71 and 180 % (βarr2), and 86 – 190 % (miniGα_q), once compared to LSD as a reference agonist. In this analysis, 25N-NBOH (1.81) exhibited the lowest values, being the most potent agonist tested.

Computational studies highlighted that increased lipophilicity in 4- position of the 2C-*X* structures may be linked to a consistent increase in the compounds' efficacies, and, at the same time, an increased preference towards miniGα_q activation. Furthermore, molecular docking analysis suggested that a receptor conformation favorable to miniGα_q recruitment might be stabilized by insertion of the lipophilic functional group in 4-position into a hydrophobic subpocket between two specific transmembranes. Considering that the estimation of bias could be calculated by use of different methods, various approaches were employed to assess biased agonism, quantitatively and qualitatively. One of these methods involved measuring the concentration-response in the β-arrestin2 (βarr2) and miniGα_q functional assays for each compound. (Figure 18)

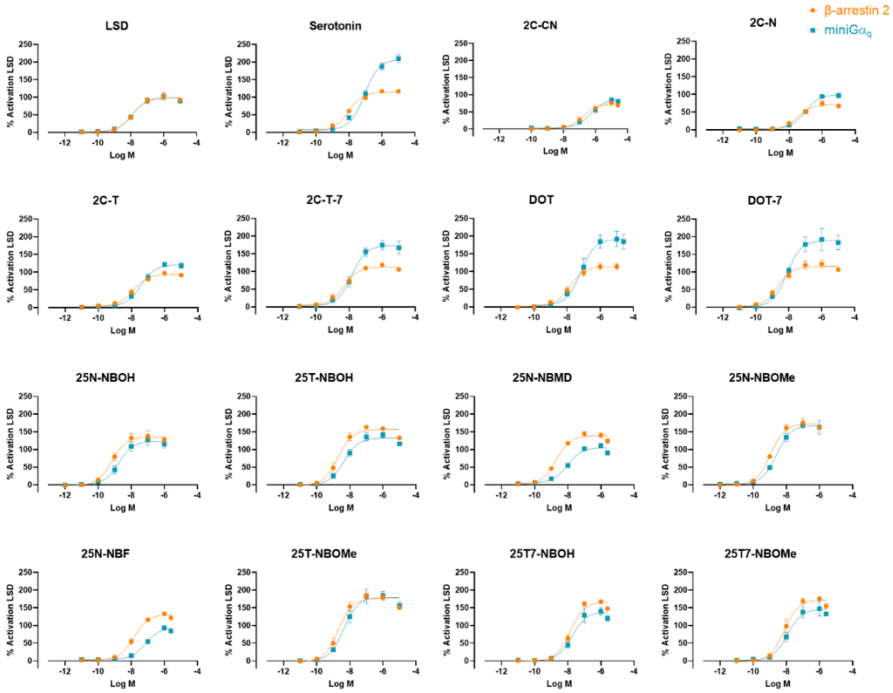


Figure 18: Averaged concentration-response curves for each compound in the βarrestin (orange) and miniGα_q (blue) recruitment assays.

The combination of data illustrated in Figure 18 with quantitative and qualitative bias factors calculation led to the possibility of ranking the compounds, according to the extent of preference exhibited towards each effector protein activation. (Figure 19)

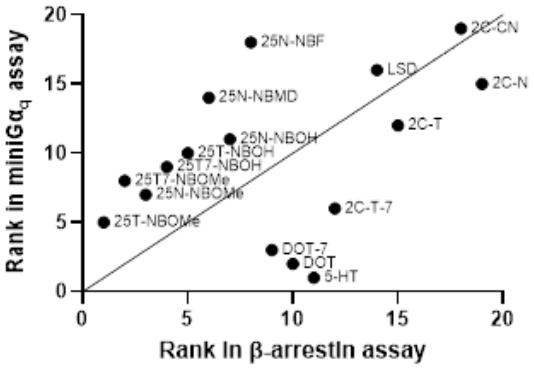


Figure 19: Ranking of the compounds according to their efficacies relative to both assays. The x-axis represents the ranking scale according to the β-arrestin assay efficacies, while the y-axis refers to the miniGα_q assay efficacies. The compounds appearing above the diagonal line display relative preference towards β-arrestin recruitment, while those below the line towards miniGα_q. The more distant the compound is from the diagonal line, the bigger the extent of bias. DOT and DOT-7 displayed values consistent with previous studies, together with preference towards miniGα_q over β-arr2 recruitment. LSD and serotonin (5-HT) were included as references.

As a result, all the four 2C-*X* analogues were found to be either unbiased or biased, in variable extent, towards miniGα_q activation in comparison to LSD, substance known to be unbiased, as confirmed by the data shown in Figure 18. 2C-T-7 was the phenethylamine analogue displaying the greatest preference towards miniGα_q recruitment, which supports the hypothesis that increasing lipophilicity of the 4-position on the phenethylamine leads to higher preference for this pathway. Moreover, all the NBPEA derivatives showed consistently a preference towards the recruitment of β-arr2 over miniGα_q, where 25N-NBF (1.83) and 25N-NBMD (1.84) displayed the highest bias factors in comparison to LSD.
For more details, the manuscript is attached to this thesis. (Manuscript II, Appendix)

4.2 - PUBLICATION I

A parallel study was carried out to pharmacologically characterize a different set of structures. The latter focused on diverse N-substituted 2C-B and 2C-CN analogues, including both the five-membered constrained racemates, 11.1 and 11.3, and the brominated six-membered analogue, 11.2. (Figure 20)

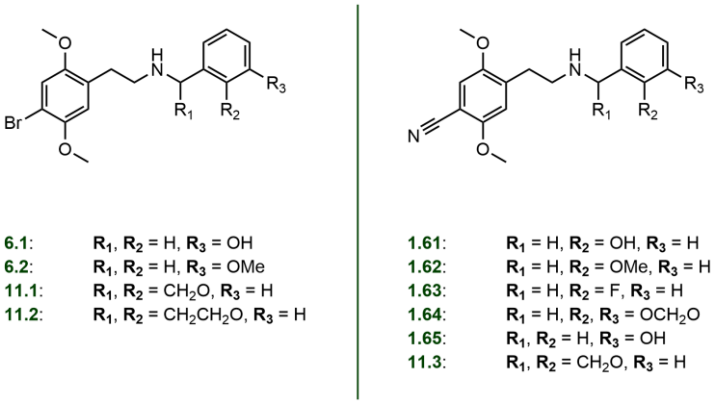


Figure 20: Overview of the bromo and cyano derivatives analyzed in this study.

The compounds were screened using LSD as the reference agonist for efficacies and β-factor calculations, while serotonin was employed as a positive control. The choice of selecting these types of structures, which were also functionally characterized in the β-arrestin2 (βarr2) and miniGα_q recruitment assays, was based on the high selectivity displayed by 25CN-NBOH (1.61), already discussed in the paragraph 2.3. Furthermore, as the latter has the ability of interacting with the 5-HT$_{2A}$R serine residue 159^{3x36} (*Ser159*, where 3x36 indicates its location within TM3) via two different moieties, most of the tested compounds lack this interaction and, therefore, seemed to

be ideal to explore the importance of this residue regarding functional selectivity.[82] For this reason, the agonist concentration-response curves of the analyzed compounds were measured on both the wild-type 5-HT_{2A} and the S159A mutated receptors.

The *in vitro* screening results, combined with computational studies, pointed out that the impossibility of establishing an interaction between the hydroxyl oxygen of all the compounds, except for 1.61, 1.62, and 1.64, and $Ser159^{3x36}$ seemed to cause a decrease in both potencies and efficacies of these substrates in both assays. However, the G_q-mediated signaling pathway was remarkably more affected, causing some compounds to show a significant preference for β-arrestin activation. Beside the lack of oxygen – $Ser159^{3x36}$ interaction, the ability of the same compounds to create multiple interactions with other amino acidic residues seemed to be crucial in terms of 5-HT_{2A}R activation and β-arrestin recruitment.

This work concluded that 4-bromo-2,5-dimethoxy-N-(3-hydroxybenzyl)phenethylamine (6.1), 4-bromo-2,5-dimethoxy-N-(3-methoxybenzyl)phenethylamine (6.2) 4-cyano-2,5-dimethoxy-N-(3-hydroxybenzyl)phenethylamine (1.65), and (±)-4-(2-((2,3-dihydrobenzofuran-3-yl)amino)ethyl)-2,5-dimethoxybenzonitrile (11.3) are the first notably β-arr2-biased 5-HT_{2A} receptor agonists, when LSD is used as reference.[5] (Figure 21)

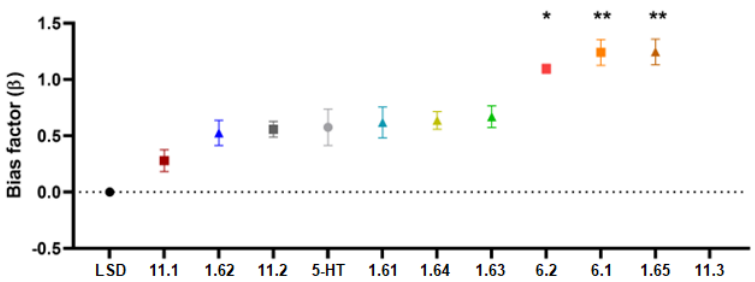


Figure 21: Visual representation of the calculated bias factors (β), showing that the values obtained for the compounds 6.2, 6.1, 1.65, and 11.3 were significantly different from those related to the other analyzed structures. The value for 11.3 was omitted as it could not be calculated. LSD was used as a reference.[5]

In particular, 6.1 displayed potency and efficacy values equal to 11.1 nM and 112 %, respectively, for β-arrestin recruitment, and 48.8 nM and 28 % for mini$G\alpha_q$ activation, when compared to LSD. The compound 11.3 displayed potency and efficacy values of 108 nM and 82.9 %, respectively, in the βarr2 assay, and 631 nM and 18 % for mini$G\alpha_q$ recruitment. The reported values indicate that 4-bromo-2,5-dimethoxy-N-(3-hydroxybenzyl)phenethylamine (6.1) and (±)-4-(2-((2,3-dihydrobenzofuran-3-yl)amino)ethyl)-2,5-dimethoxybenzonitrile (11.3) are biased receptor agonists of particular interest, perhaps paving the way for the development of medical tools that

might allow us to exploit functionally selective compounds for new and effective neuropsychiatric and psychotherapeutic treatments.
For more details, the paper describing this work and its results are attached to this thesis. (Publication I, Appendix)

CONCLUSIONS AND PERSPECTIVES

There is accumulating clinical evidence that the 5-HT_{2A} receptor agonists might relieve the symptoms induced by treatment resistant depression, addiction, OCD, and anxiety in last-stage cancer patients.

However, their therapeutic mode of action is currently poorly understood since agonists of this receptor subtype generally lack selectivity, and/or functional selectivity towards this specific receptor. Consequently, 5-HT_{2A}R agonists can either bind other receptors, lacking selectivity, or recruit different protein effectors which trigger both desired and undesired intracellular transductional pathways.

The final goal of the SAFER project consisted in investigating a varied set of phenethylamine scaffolds and their derivatives as receptor agonists that might potentially exhibit selective and functionally selective behavior towards this receptor subtype. These scaffolds, however, first needed to be thoroughly selected and synthesized. Preliminary pharmacological testing results, progressively obtained thanks to the development of the β-arr2 and mini$G\alpha_q$ functional assays by Anders A. Jensen, provided precious information about any structural modification that could lead us closer to the ambitious project aim. These data allowed me to direct the experimental synthetic work towards those structures presenting a substitution pattern that affected the extent of the effector proteins activation to different degrees. Beside the preliminary *in vitro* data, the final design of the synthesized molecules also derived from the constant consultation with Jesper L. Kristensen, due to his extensive expertise in this field.

In parallel, the reductive amination step, necessary for the preparation of different phenethylamine analogues, was largely explored, improved, and summarized in a manuscript, proposing an alternative, simpler, faster, and higher yielding method than those conventionally used. Furthermore, two different and unprecedented synthetic procedures for the preparation of structurally constrained compounds were developed. The most rapid method was used to prepare the racemic mixtures of diverse rigid structures, and successful enantiomeric separation was achieved via HPLC chiral separation, upon Boc-protection of the amine. Alternatively, a second method was attempted to enantioselectively control the spatial orientation of the product, that was proven to work and made the racemates resolution not needed.

Eventually, a final in vitro screening on a some of the synthesized structures showed that four scaffolds, including one of the conformationally restrained compounds, preferentially activate β-arrestin recruitment over $G\alpha_q$, when compared to the

notoriously non-selective LSD, used as a potent 5-HT_{2A} agonist representative. Therefore, these compounds are the first β-arrestin-biased 5-HT_{2A} agonists reported to date. The limited number of restrained products that were in fact tested directly highlights the need for future pharmacological characterization of all the other scaffolds left behind. Particularly, *in vitro* testing of the novel constrained structures and their individual enantiomers should be implemented. Moreover, designing new NBPEA analogues, with hydroxy and methoxy substitution in 3-position on the second ring, would also be useful to further investigate functional selectivity for the 5-HT_{2A} receptor. Nevertheless, the achievement of synthesizing the first β-arrestin-biased 5-HT_{2A} agonists might be the first of a long series of new findings that might help us understanding how biased agonism works and might be exploited for safer and more effective drugs development. The synthesized biased agonists herein introduced, including novel scaffolds, were tested *in vivo* on pigs' coronary arteries to assess their potential as vasodilating agents and the resulting data are expected to be soon published. Furthermore, the prepared compounds might also undergo *in vivo* testing to further characterize their properties and evaluate their potential as future medical tools for neuropsychiatric and psychotherapeutic application.

EXPERIMENTAL SECTION

All reactions involving dry solvents or sensitive agents were performed under a nitrogen or argon atmosphere and glassware was flame-dried prior to use. Commercially available chemicals were used without further purification. Solvents were dried prior to use with an SG water solvent purification system or dried by standard procedures. NMR spectra were recorded on Bruker Avance 400 MHz and Bruker Avance III HD 600 MHz spectrometer. Residual solvent peaks ($CDCl_3$, Methanol-d_4 and DMSO-d_6) were used as internal standard (7.26, 3.31 and 2.50 ppm for ^1H, and 77.06, 49.15 and 39.51 ppm for ^{13}C, respectively). Thin-layer chromatography (TLC) was performed on aluminum sheets precoated with silica gel 60 F254 (Merck). The TLC plates were visualized by use of UV radiation (254 nm), and for some compounds iodine chamber and/or staining solutions were employed (ninhydrin, DNP). Ninhydrin solution was prepared by mixing 1.5 g ninhydrin, 100 ml ethanol, and 3.0 ml acetic acid. Dinitrophenylhydrazine (DNP) solution was prepared by mixing 12 g of 2,4-dinitrophenylhydrazine, 60 ml of concentrated sulfuric acid, and 80 ml of water in 200mL of ethanol 95% solution. Semi-preparative and preparative HPLC separation was performed on Dionex Ultimate 3000 HPLC system (Thermo) with a Gemini NX C18 (250 × 21 mm) column, Flow rate: 20 ml/min, mobile phase A: H_2O/TFA (100:0.1) (v/v); B: ACN/H_2O/TFA (90:10:0.1) (v/v/v). All the samples were dissolved in H_2O/ACN (1:1).
UPLC-MS analyses were performed on a Waters Acquity H-class UPLC with a Sample Manager FTN and a TUV dual wavelength detector coupled to a QDa single quadrupole analyser using electrospray ionization (ESI). UPLC separation was achieved with a C18 reversed-phase column (Acquity UPLC BEH C18, 2.1 mm × 50 mm, 1.7 µm) operated at 40 °C, using a linear gradient of the binary solvent system of buffer A (milliQ H_2O:MeCN:formic acid, 95:5:0.1 v/v) to buffer B (MeCN:formic acid, 100:0.1 v/v) from 0 to 100% B in 3.5 min, then 1 min at 100%B, Flow rate: 0.8 ml⁄min. Data acquisition was controlled by MassLynx ver. 4.1 and data analysis was done using Waters OpenLynx browser ver. 4.1. The products were dried under high vacuum or freeze-dried using a ScanVac Cool Safe Freeze Drier. The purity of compounds submitted for pharmacological characterization was determined to be >95%, by HPLC analysis.

2-(2,5-Dimethoxyphenyl)nitroethene (3.2)
2,5-dimethoxybenzaldeyde (50 mmol, 8.309 g) were added to 30 ml of 2-propanol and the vessel was heated up to 75°C. Nitromethane (5.6 ml, 103.4 mmol) was added. Glacial acetic acid (2.29 ml, 40 mmol) and ethylenediamine (0.33 ml, 5 mmol) were added with production of smoke. The mixture turned deep yellow as soon as the ethylenediamine was added and deep orange after 20 minutes of stirring. It was stirred under heating for 4 hours. Once lifted the vessel from the oil bath, nucleation occurred.

Any possible volatile component was evaporated under reduced pressure and the orange solid was washed 3 times with cold 2-propanol (4°C) and evaporated under reduced pressure to deliver 2,5-dimethoxy-β-nitrostyrene, as orange needle crystals in quantitative yield.

2,5-dimethoxyphenylethylamine hydrochloride (2C-H, 1.3) – Classic method
A solution of DIBAL-H 1M in dry THF (56.1 ml) in 100 ml of anhydrous THF under argon atmosphere was stirred and heated to reflux. 2,5-dimethoxy-β-nitrostyrene (0.523 g, 2.5 mmol, 3.2), dissolved in dry THF (100 mL), was added dropwise and the mixture was refluxed for 3 hours. Once reached room temperature, the vessel was cooled to -78°C and 2.25 ml of water were added under stirring. After five minutes, the cooling bath was removed and an excess of diethyl ether was added, followed by 2.25 ml of NaOH 15% aqueous solution and 6 ml of water, respectively. The mixture was stirred for 1 hour with large amounts of $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and the product was isolated by column chromatography (EtOAc/MeOH/TEA - 85:10:5). The resulting free base was dissolved in a large volume of diethyl ether and precipitated by dropwise addition of an excess of HCl 2N in diethyl ether, under vigorous stirring. 2,5-Dimethoxyphenylethylamine hydrochloride (2C-H) was delivered upon filtration and drying under reduced pressure as a white solid (0.337 g, 62%).
^{1}H NMR (600 MHz, DMSO) δ 7.78 (br, 2H), 6.92 (d, *J* = 8.9 Hz, 1H), 6.81 (q, *J* = 4.0 Hz, 1H), 6.78 (d, *J* = 3.1 Hz, 1H), 3.75 (s, 3H), 3.70 (s, 3H), 2.97 (t, *J* = 7.8 Hz, 2H), 2.81 (t, *J* = 7.8 Hz, 2H). ^{13}C NMR (151 MHz, DMSO) δ 153.05, 151.25, 126.03, 116.45, 112.18, 111.78, 55.79, 55.32, 38.65, 28.14.

2,5-dimethoxyphenylethylamine hydrochloride (2C-H, 1.3) – New method (Manuscript III)
The 2,5-dimethoxy-β-nitrostyrene (2 mmol, 1 eq., 3.2) was added in small portions to a stirring suspension of $NaBH_4$ (15 mmol, 7.5 eq.) in *i*-PrOH/H_2O (8:4 ml). 0.1 ml of a freshly prepared $CuCl_2$ 2M solution were added dropwise but rapidly to the vessel. The reaction was monitored by TLC (DCM/MeOH/TEA – 98:2:0.04) and refluxed at 80°C for 10 minutes. Once cooled to room temperature, 35% solution of NaOH (10 ml) was added under stirring. The mixture was extracted with i-PrOH (3 x 10 ml), and the organic extracts were combined, thoroughly dried over $MgSO_4$, and filtered. The residue was concentrated under reduced pressure and dissolved in a large amount of diethyl ether. The product was precipitated under stirring with an excess of HCl 2N in diethyl ether solution and the vessel was cooled to 5°C ca. The white solid was filtered, washed with cold diethyl ether, and dried under reduced pressure as 2,5-dimethoxyphenylethylamine hydrochloride (82%).

2,5-dimethoxy-4-nitrophenylethylamine hydrochloride (2C-N, 1.8)
Acetic acid (20 ml) and 2,5-dimethoxyphenylethylamine hydrochloride (5.45 mmol, 1.14 g) were cooled to 0°C under stirring. 70% HNO_3 solution (4 ml) was added dropwise, and the mixture was stirred for 1 hour. The suspension was poured in cold

H_2O (50 ml) and made strongly basic by adding 35% NaOH solution. The reaction mixture was extracted with CH_2Cl_2 (3 x 50 ml), and the organic layers were combined, washed with H_2O (3 x 30 ml) and saturated solution of NaCl (3 x 30 ml), dried over $MgSO_4$, filtered, and evaporated under reduced pressure. HCl in dioxane (4N, 1.24 ml) was added dropwise to the residue while stirring. After 10 minutes, the precipitated hydrochloride salt was isolated by filtration and dried under reduced pressure as an orange solid (1.03 g, 3.9 mmol, 72%).
^{1}H NMR (600 MHz, DMSO) δ 7.98 (br, 3H), 7.51 (s, 1H), 3.89 (s, 3H), 3.83 (s, 3H), 3.04 (t, *J* = 7.1 Hz, 2H), 2.94 (t, *J* = 7.3 Hz, 2H). ^{13}C NMR (151 MHz, DMSO) δ 150.91, 146.62, 138.19, 132.99, 117.31, 107.75, 57.49, 56.83, 38.46, 28.46.

General procedure (A) for the synthesis of secondary amines (NBPEAs)

The aldehyde (1.1 eq.) was added to a suspension of the phenethylamine hydrochloride (1 mmol, 1 eq.) and Et_3N (1 eq.) in EtOH (8 ml). The reaction mixture was stirred until the formation of the imine was complete by TLC (30 minutes – 3 hours). After addition of $NaBH_4$ (2 eq.), the mixture was stirred for 45 minutes and concentrated under reduced pressure. The residue was partitioned in DCM/H_2O (1:1, 30 ml) and the aqueous phase was further extracted with DCM (2 x 15 ml). The organic layers were combined, dried over $NaSO_4$, filtered, and evaporated under reduced pressure. The secondary amine product was purified by column chromatography (DCM/MeOH/TEA, 98.2:0.4) and precipitated by addition of HCl in ether or dioxane (≥1 eq.) under stirring. The solid was filtered, dried under reduced pressure, dissolved in minimum amount of methanol, and diluted in diethyl ether. The product was collected by filtration and dried under reduced pressure.

4-nitro-2,5-dimethoxy-N-(2-hydroxybenzyl)phenethylamine hydrochloride (25N-NBOH, 1.81)

Synthesized from 2,5-dimethoxy-4-nitrophenylethylamine hydrochloride (2C-N) and salicylaldehyde by general procedure A in 72% yield as an orange solid.
^{1}H NMR (600 MHz, DMSO) δ 7.45 (s, 1H), 7.21 (s, 1H), 7.05 (d, *J* = 7.1 Hz, 2H), 6.70 (dt, *J* = 2.0, 7.4 Hz, 1H), 6.67 (q, *J* = 3.3 Hz, 1H), 3.87 (s, 3H), 3.81 (s, 2H), 3.78 (s, 3H), 2.81 (t, *J* = 3.0 Hz, 2H), 2.78 (t, *J* = 2.9 Hz, 2H). ^{13}C NMR (151 MHz, DMSO) δ 157.24, 150.31, 146.35, 136.86, 136.03, 128.51, 127.78, 124.25, 118.39, 116.49, 115.29, 107.09, 56.85, 56.16, 50.31, 47.59, 29.97.

4-nitro-2,5-dimethoxy-N-(2-methoxybenzyl)phenethylamine hydrochloride (25N-NBOMe, 1.82)

Synthesized from 2-(2,5-dimethoxy-4-nitrophenyl)ethan-1-amine hydrochloride and 2-methoxybenzaldehyde by general procedure A in 73% yield as an orange solid. Characterization was in accordance to reported values.[51]

4-nitro-2,5-dimethoxy-N-(2-fluorobenzyl)phenethylamine hydrochloride (25N-NBF, 1.83)

Synthesized from 2-(2,5-dimethoxy-4-nitrophenyl)ethan-1-amine hydrochloride and 2-fluorobenzaldehyde by general procedure A in 69% yield as an orange solid.
^{1}H NMR (600 MHz, DMSO) δ 7.43 (t, *J* = 7.6 Hz, 1H), 7.28 (q, *J* = 6.3 Hz, 1H), 7.14 (m, *J* = 5.8, 1H), 3.86 (s, 3H), 3.79 (s, 3H), 3.76 (s, 2H), 2.79 (t, *J* = 6.2, 2H), 2.75 (t, *J* = 6.6 Hz, 2H). ^{13}C NMR (151 MHz, DMSO) δ 161.20, 150.30, 146.34, 136.76, 136.53, 130.26, 128.39, 124.08, 116.46, 114.92, 114.78, 107.01, 56.85, 56.14, 48.07, 45.57, 30.40.

N-(benzo[d][1,3]dioxol-4-ylmethyl)-2-(2,5-dimethoxy-4-nitrophenyl)ethan-1-amine hydrochloride (25N-NBMD, 1.84)
Synthesized from 2-(2,5-dimethoxy-4-nitrophenyl)ethan-1-amine hydrochloride and 2,3-(methylenedioxy)benzaldehyde by general procedure A in 70% yield as an orange solid.
^{1}H NMR (400 MHz, DMSO) δ 7.44 (s, 1H), 7.21 (s, 1H), 6.83 (m, *J* = 3.3 Hz, 1H), 6.78 (m, *J* = 3.1 Hz, 2H), 5.96 (s, 2H), 3.85 (s, 3H), 3.78 (s, 3H), 3.67 (s, 2H), 2.76 (m, *J* = 5.3 Hz, 4H). ^{13}C NMR (100 MHz, DMSO) δ 150.47, 147.22, 146.21, 137.81, 132.36, 123.25, 121.93, 116.84, 113.07, 109.27, 107.41, 101.33, 57.07, 56.42, 45.56, 43.74, 26.60.

2,5-dimethoxyphenol (4.2)
2,5-Dimethoxybenzaldehyde (1.662 g, 10 mmol, 3.1) and *m*CPBA (2.6 g, 15 mmol, 4.1) were rapidly mixed in a mortar by use of a pestle. The reagents melted and generated a large amount of heat, leading to the production of fumes and a fire, quickly and spontaneously extinguished within a few minutes. A solution of NaOH 20% (100 ml, 0.25 mol) was added and the mixture was thoroughly mixed. The aqueous solution was washed with diethyl ether (3 x 200 ml) and, once adjusted its pH to 6, was extracted with DCM (3 x 300 ml). The organic extracts were combined, dried over $MgSO_4$, filtered, and dried under N_2 steam, to deliver 2,5-dimethoxyphenol (4.2) as a solid (67%)
^{1}H NMR (600 MHz, $CDCl_3$) δ 6.70 (d, *J* = 8.8 Hz, 1H), 6.49 (d, *J* = 2.9 Hz, 1H), 6.30 (dd, *J* = 2.9, 8.8 Hz, 1H), 5.57 (br. s, 1H), 3.77 (s, 3H), 3.67 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 153.57, 145.43, 139.94, 110.47, 103.25, 100.73, 55.57, 54.65.

4-*n*-propoxy-2,5-dimethoxybenzene (4.3)
A solution of KOH (0.450 mg, 8 mmol) in boiling methanol (5 ml) was added to 2,5-dimethoxyphenol (0.848 g, 5.5 mmol, 4.2) in methanol (5 ml) under stirring. After addition of *n*-propyl bromide (0.51 ml, 5.6 mmol), the mixture was refluxed at 70°C for 2 hours. The reaction was quenched by adding 10 ml of NaOH 10% aqueous solution and extracted with DCM (3 x 30 ml). The organic extracts were combined, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to deliver 0.463 g of 4-*n*-propoxy-2,5-dimethoxybenzene (4.3, 43%).

4-*n*-propoxy-2,5-dimethoxybenzaldehyde (4.4)
A solution of $POCl_3$ (1.14 ml, 12 mmol) and N-methylformanilide (1.5 ml, 12 mmol) was stirred and gently heated over 1 hour until formation of a deep red color. 4-*n*-propoxy-2,5-dimethoxybenzene (0.457 mg, 2.33 mmol, 4.3) was added and the

mixture was refluxed for 2 hours. After addition of 100 ml of cold water (0°C), the mixture was kept under vigorous stirring at room temperature overnight. The red solid was collected by filtration, washed with water, and dried under N_2 steam over 3 hours. The crude was recrystallized in boiling methanol and gradually cooled to 2°C, when the crystals could be filtered and washed with cold methanol. The solids were dried *in vacuo* and the leftover traces of N-methylformanilide removed by recrystallization in boiling heptane. The solution was filtered, while still hot to eliminate insoluble particles, cooled to room temperature, and 4-*n*-propoxy-2,5-dimethoxybenzaldehyde (4.4) was finally collected by filtration and dried, as red-orange crystals (0.329 g, 1.47 mmol, 63%).

1-(2,5-dimethoxy-4-(*n*)-propoxyphenyl)-2-nitropropene (4.5)
A solution of 4-*n*-propoxy-2,5-dimethoxybenzaldehyde (0.156 g, 0.697 mmol, 4.4) in 2-propanol (2.3 ml) was refluxed at 75°C until complete dissolution of 4.4. Nitroethane (0.12 ml, 1.4 mmol) was added, followed by glacial acetic acid (0.03 ml, 0.4 mmol) and ethylene diamine (2 drops, ~0.07 mmol), under stirring. The mixture was refluxed for 3 hours, when both TLC staining with DNP and NMR spectroscopic analysis confirmed the complete consumption of the benzaldehyde 4.4. The residue was cooled to room temperature, concentrated under reduced pressure, washed thoroughly with cold 2-propanol (-20°C), and dried in vacuo to deliver 1-(2,5-dimethoxy-4-(n)-propoxyphenyl)-2-nitropropene (4.5) as deep orange needle crystals (0.182 g, 0.648 mmol, 93%).

2,5-dimethoxy-4-propoxyamphetamine hydrochloride (MPM, 2.21)
1-(2,5-Dimethoxy-4-(*n*)-propoxyphenyl)-2-nitropropene (0.123 g, 0.437 mmol, 4.5) in dry THF (4.5 ml) was added dropwise to stirring solution of DIBAL-H 1M in THF (4.5 ml) under argon. After an initial spontaneous increase in temperature, the mixture was refluxed for 2.5 additional hours, under Ar. After this time, the pale-yellow color of the initial mixture disappeared, and the vessel was cooled to room temperature. After addition of 15 ml of dry diethyl ether, the mixture was cooled further to 0°C, and quenched by adding water (0.3 ml), NaOH 15% solution (0.2 ml), and water again (0.6 ml), respectively, under stirring. The reaction mixture was let reach room temperature, dried over Na_2SO_4 (3 g), filtered, and concentrated under reduced pressure. The residue was dissolved in methanol (2 ml) and stirred for 30 minutes after dropwise addition of HCl 4N in dioxane (0.4 ml). The solution was concentrated *in vacuo* and the resulting brown powder was dissolved in 1.7 ml of 2-propanol. Diethyl ether (50 ml) was added, and the mixture was cooled to -20°C. After 30 minutes, the solvent was decanted, and the brown residue dried *in vacuo*. The latter was separated by HPLC, resulting in the MPM TFA salt, that was dissolved in NaOH aqueous solution and extracted as a free base with DCM (3 x 20 ml). The organic extracts were combined, dried over $MgSO_4$, filtered, and concentrated under vacuum. The resulting colorless oil was dissolved in a large amount of diethyl ether and precipitated as hydrochloride salt by HCl 2N in diethyl ether addition. The precipitate was filtered and dried in vacuo to deliver 2,5-dimethoxy-4-propoxyamphetamine hydrochloride (MPM, 2.21) as an off-white powder (0.0696 g, 0.240 mmol, 55%).

^{1}H NMR (600 MHz, DMSO) δ 7.70 (br. s, 3H), 6.76 (s, 1H), 6.67 (s, 1H), 3.94 (q, *J* = 4.0 Hz, 2H), 3.76 (d, *J* = 1.1 Hz, 3H), 3.71 (d, *J* = 1.2 Hz, 3H), 2.78 (q, *J* = 6.4 Hz, 1H), 2.64 (t, *J* = 6.8 Hz, 1H), 1.73 (m, *J* = 6.9 Hz, 2H), 1.09 (d, *J* = 6.5 Hz, 3H), 0.98 (m, *J* = 3.7 Hz, 3H). ^{13}C NMR (151 MHz, DMSO) δ 152.10, 148.60, 143.17, 116.60, 115.81, 99.89, 70.51, 56.99, 56.54, 47.65, 34.87, 22.71, 18.41, 10.97.

2,5-dimethoxyphenylethylamine (2C-H, 1.13)
This compound was synthesized by following the same procedure used for 1.3 (new method). Instead of the final precipitation as hydrochloride salt by HCl addition, the oil was dissolved in a large amount of diethyl ether and cooled to 2°C. The ether phase was decanted and concentrated under reduced pressure to deliver 2,5-dimethoxyphenylethylamine (2C-H, 1.13) as a colorless oil (82%).

2,5-dimethoxy-4-bromophenylethylamine hydrochloride (2C-B, 1.1)
2,5-Dimethoxyphenylethylamine (0.101 g, 0.557 mmol, 1.13) was stirred in 2 ml of glacial acetic acid, when a solution of Br_2 (0.09 g, 0.57 mmol) in 1 ml of glacial acetic acid was added dropwise. Once the spontaneous generation of heat stopped, the mixture was cooled to room temperature and the product was isolated after filtration, washing with glacial acetic acid, and drying *in vacuo*, as hydrobromide salt. The latter was dissolved in NaOH aqueous solution and extracted as a free base with DCM (3 x 50 ml). The organic extracts were combined, dried over $MgSO_4$, filtered, and concentrated under vacuum. The resulting oil was dissolved in a large amount of diethyl ether and precipitated as hydrochloride salt by HCl 2N in diethyl ether addition. The precipitate was filtered and dried in vacuo to deliver 2,5-dimethoxy-4-bromophenylethylamine hydrochloride (2C-B, 1.1) as a white powder (0.152 g, 0.512 mmol, 92%). Characterization was in accordance to reported values.[45b]

4-bromo-2,5-dimethoxy-N-(3-hydroxybenzyl)phenethylamine hydrochloride (6.1)
Synthesized from 2,5-dimethoxy-4-bromophenylethylamine hydrochloride (2C-B, 1.1) and 3-hydroxybenzaldehyde by general procedure A in 66% yield as a white powder. Characterization was in accordance to reported values.[5, 83]

4-bromo-2,5-dimethoxy-N-(3-methoxybenzyl)phenethylamine hydrochloride (6.2)
Synthesized from 2,5-dimethoxy-4-bromophenylethylamine hydrochloride (2C-B, 1.1) and 3-methoxybenzaldehyde by general procedure A in 82% yield as a white powder. Characterization was in accordance to reported values.[5, 83]

2-(4-bromo-2,5-dimethoxyphenethyl)isoindoline-1,3-dione (7.1)
2,5-Dimethoxy-4-bromophenylethylamine (2C-B, 1.1*) was obtained by following the same procedure used for 1.1. Instead of the final precipitation as hydrochloride salt by HCl addition, the oil was dissolved in a large amount of diethyl ether and cooled to 2°C. The ether phase was decanted and concentrated under reduced pressure to deliver 2,5-dimethoxyphenylethylamine (2C-B, 1.1*) as a colorless oil (93%). The

latter (0.8 g, 3.076 mmol) was added to a solution of phthalic anhydride (0.651 g, 4.397 mmol) in toluene (15 ml), and the vessel was equipped with a Dean-Stark apparatus and refluxed overnight. The residue was concentrated under reduced pressure and recrystallized with minimum amounts of ethanol to deliver 2-(4-bromo-2,5-dimethoxyphenethyl)isoindoline-1,3-dione (7.1) as yellow needle crystals (0.816 g, 2.092 mmol, 68%).

4-(2-(1,3-dioxoisoindolin-2-yl)ethyl)-2,5-dimethoxybenzonitrile (7.2)
Cuprous cyanide (0.360 g, 2 eq., 4.02 mmol) was added to a stirring solution of 2-(4-bromo-2,5-dimethoxyphenethyl)isoindoline-1,3-dione (0.816 g, 1 eq., 2.092 mmol, 7.1) in DMF (40 ml), and the mixture was refluxed at 160°C for two days. Once the reaction reached room temperature, a solution of iron(III) chloride hexahydrate (1.087 g, 2 eq., 4.02 mmol) in aqueous HCl 50% (10 ml) was added and the reaction was stirred for 30 minutes at 60°C. The mixture was poured in 100 ml of water and extracted with DCM (3 x 50 ml). The organic extracts were combined, washed with NH_3 3M in water (50 ml), water (20 ml), brine (20 ml), and dried over Na_2SO_4. After drying *in vacuo*, the brown residue was recrystallized with minimum amounts of ethanol to deliver 4-(2-(1,3-dioxoisoindolin-2-yl)ethyl)-2,5-dimethoxybenzonitrile (7.2), as a white powder (0.640 g, 1.904 mmol, 91%).

2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6)
Hydrazine (1.22 ml, 38.01 mmol) was added to a stirring solution of 4-(2-(1,3-dioxoisoindolin-2-yl)ethyl)-2,5-dimethoxybenzonitrile (0.640 g, 1.904 mmol, 7.2) in THF/EtOH (1:1, 50 ml) and the solution was kept stirring at room temperature for 3 days. After filtration, the filtrate was concentrated under vacuum, dissolved in water (10 ml), and extracted with ethyl acetate (3 x 10 ml). The combined organic extracts were washed with water (10 ml), brine (10 ml), dried over $MgSO_4$ and concentrated under reduced pressure to deliver 2,5-dimethoxy-4-cyanophenylethylamine (2C-CN) as a yellow oil (free base). The latter was dissolved in a large amount of diethyl ether and precipitated upon HCl 2N in diethyl ether addition, as hydrochloride salt. The product was collected by filtration and dried *in vacuo*, delivering 1.6 as a white powder (0.287 g, 1.181 mmol, 62%, 1.6). Characterization was in accordance to reported values.[5, 62]

4-cyano-2,5-dimethoxy-N-(2-hydroxybenzyl)phenethylamine hydrochloride (25CN-NBOH, 1.61)
Synthesized from 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) and 2-hydroxybenzaldehyde by general procedure A in 73% yield as a white powder. Characterization was in accordance to reported values.[5, 45c]

4-cyano-2,5-dimethoxy-N-(2-methoxybenzyl)phenethylamine hydrochloride (25CN-NBOMe, 1.62)
Synthesized from 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) and 2-methoxybenzaldehyde by general procedure A in 72% yield as a white powder. Characterization was in accordance to reported values.[5, 45c]

4-cyano-2,5-dimethoxy-N-(2-fluorobenzyl)phenethylamine hydrochloride (25CN-NBF, 1.63)

Synthesized from 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) and 2-fluorobenzaldehyde by general procedure A in 75% yield as a white powder. Characterization was in accordance to reported values.[5, 45c]

N-(benzo[d][1,3]dioxol-4-ylmethyl)-2-(2,5-dimethoxy-4-cyanophenyl)ethan-1-amine hydrochloride (25CN-NBMD, 1.64)

Synthesized from 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) and 2,3-(methylenedioxy)benzaldehyde by general procedure A in 71% yield as a white powder. Characterization was in accordance to reported values.[5, 45c]

4-cyano-2,5-dimethoxy-N-(3-hydroxybenzyl)phenethylamine hydrochloride (1.65)

Synthesized from 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) and 3-hydroxybenzaldehyde by general procedure A in 76% yield as a white powder. Characterization was in accordance to reported values.[5]

4-cyano-2,5-dimethoxy-N-(3-methoxybenzyl)phenethylamine hydrochloride (1.66)

Synthesized from 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) and 3-methoxybenzaldehyde by general procedure A in 83% yield as a white powder.

^{1}H NMR (600 MHz, DMSO) δ 8.88 (br. s, 2H), 7.38 (s, 1H), 7.36 (t, *J* = 7.9, 1H), 7.10 (s, 1H), 7.09 (t, *J* = 2.0 Hz, 1H), 7.05 (d, *J* = 7.6 Hz, 1H), 7.00 (m, *J* = 2.6 Hz, 1H), 4.16 (t, *J* = 5.8 Hz, 2H), 3.87 (s, 3H), 3.79 (s, 3H), 3.78 (3H), 3.15 (m, *J* = 5.5 Hz, 2H), 2.99 (t, *J* = 7.9 Hz, 2H). ^{13}C NMR (151 MHz, DMSO) δ 159.87, 155.71, 151.45, 133.73, 133.19, 130.43, 122.39, 116.83, 115.96, 115.51, 115.13, 114,93, 99.15, 57.00, 56.79, 55.69, 50.47, 46.06, 27.51.

4-cyano-2,5-dimethoxy-N-(3-methylbenzyl)phenethylamine hydrochloride (1.67)

Synthesized from 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) and 3-methylbenzaldehyde by general procedure A in 80% yield as a white powder.

^{1}H NMR (600 MHz, DMSO) δ 8.81 (br. s, 2H), 7.38 (s, 1H), 7.34 (d, *J* = 7.6 Hz, 1H), 7.27 (m, *J* = 7.9 Hz, 3H), 7.10 (s, 1H), 4.14 (t, *J* = 5.7 Hz, 2H), 3.87 (s, 3H), 3.79 (s, 3H), 3.15 (m, *J* = 5.5 Hz, 2H), 2.98 (t, *J* = 7.9 Hz, 2H), 2.34 (s, 3H).). ^{13}C NMR (151 MHz, DMSO) δ 155.71, 151.45, 138.47, 133.19, 132.23, 130.92, 130.14, 129.18, 127.44, 116.83, 115.51, 115.13, 99.15, 57.01, 56.80, 50.59, 46.09, 27.52, 21.40.

2-(4-cyano-2,5-dimethoxyphenyl)-N-(2-hydroxy-3-methylbenzyl)ethanamine hydrochloride (1.68)

Synthesized from 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) and 2-hydroxy-3-methylbenzaldehyde by general procedure A in 72% yield as a white powder. Characterization was in accordance to reported values.[5, 84]

2-(4-cyano-2,5-dimethoxyphenyl)-N-(2-hydroxy-3-ethylbenzyl)ethanamine hydrochloride (1.69)

Synthesized from 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) and 2-hydroxy-3-ethylbenzaldehyde by general procedure A in 80% yield as a white powder. Characterization was in accordance to reported values.[5, 84]

2-(4-cyano-2,5-dimethoxyphenyl)-N-(2-hydroxy-3-methoxybenzyl)ethanamine hydrochloride (1.71)

Synthesized from 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) and 2-hydroxy-3-methoxybenzaldehyde by general procedure A in 81% yield as a white powder. Characterization was in accordance to reported values.[5, 84]

1,4-dimethoxy-2-(trifluoromethyl)benzene (5.2)

A solution of 1-fluoro-4-methoxy-2-(trifluoromethyl)benzene (14.56 g, 1 eq, 75 mmol, 5.1) and sodium methoxide (40.52 g, 10 eq., 750 mmol) in DMSO (150 ml) was heated to 120°C and stirred overnight. The mixture was cooled to room temperature, poured in 700 ml of water, and extracted with diethyl ether (3 x 200 ml). The combined organic extracts were washed with water, dried over MgSO4, filtered and concentrated under reduced pressure to deliver 1,4-dimethoxy-2-(trifluoromethyl)benzene (5.2) as a colorless oil (15.173 g, 73.6 mmol, 98%).

4-trifluoromethyl-2,5-dimethoxybenzaldehyde (5.3)

In a flame-dried double-neck round bottom flask under Ar atmosphere, 1,4-dimethoxy-2-(trifluoromethyl)benzene (6,19 g, 1 eq., 30 mmol, 5.2) was dissolved in dry DCM (100 ml) and the vessel was cooled to -78°C. Titanium tetrachloride (8.24 ml, 2.5 eq., 75 mmol) was slowly added dropwise, followed by dichloromethyl methyl ether (8.13 ml, 3 eq., 90 mmol). The mixture was slowly allowed to reach room temperature over 5 hours, poured in cold water (400 ml, 0°C) and extracted with DCM (3 x 100 ml). The combined organic extracts were washed with water (100 ml), $NaHCO_3$ saturated solution (100 ml), brine (100 ml), dried thoroughly over $MgSO_4$, filtered, and concentrated under reduced pressure to deliver 4-trifluoromethyl-2,5-dimethoxybenzaldehyde (5.3) as a white powder (6.814 g, 29,1 mmol, 97%)

1,4-dimethoxy-2-(2-nitrovinyl)-5-(trifluoromethyl)benzene (5.4)

4-Trifluoromethyl-2,5-dimethoxybenzaldehyde (0.682 g, 1 eq., 2.91 mmol, 5.3) was added to 8 ml of 2-propanol and the vessel was heated to 75°C. Nitromethane (0.312 ml, 2 eq., 5.82 mmol) was added, followed by glacial acetic acid (86 µl, 0.5 eq, 1.5 mmol) and ethylenediamine (18 µl, 0.1 eq., 0.3 mmol). The mixture turned deep yellow as soon as the ethylenediamine was added and deep orange after 20 minutes of stirring. It was stirred under heating for 4 hours, when TLC staining with DNP confirmed full conversion of the starting material. Once lifted the vessel from the oil bath, nucleation occurred. The orange solid was washed with cold 2-propanol (3 x 5

ml, 2°C) and dried under reduced pressure to deliver 1,4-dimethoxy-2-(2-nitrovinyl)-5-(trifluoromethyl)benzene (5.4) as yellow needle crystals (0.4921 g, 1.775 mmol, 61%).

2,5-dimethoxy-4-trifluoromethylphenylethylamine hydrochloride (2C-TFM, 1.12)
This compound was synthesized by following the same procedure used for 1.3 (new method). However, after conversion of 1,4-dimethoxy-2-(2-nitrovinyl)-5-(trifluoromethyl)benzene (0.2772 g, 1 mmol, 5.4) to 2,5-dimethoxy-4-trifluoromethylphenylethylamine hydrochloride (1.12), the latter was isolated by use of a NaOH 25% aqueous solution and purified by acetone washing as a white powder (0.203 g, 0.71 mmol, 71%). (Manuscript III)
^{1}H NMR (600 MHz, DMSO) δ 7.64 (br. s, 3H), 7.15 (s, 1H), 7.13 (s, 1H), 3.85 (s, 3H), 3.82 (s, 3H), 3.03 (t, *J* = 7.6 Hz, 2H), 2.89 (t, *J* = 7.6 Hz, 2H).

4-trifluoromethyl-2,5-dimethoxy-N-(2-hydroxybenzyl)phenethylamine hydrochloride (25TFM-NBOH, 5.5)
Synthesized from 2,5-dimethoxy-4-trifluoromethylphenylethylamine hydrochloride (2C-TFM, 1.12) and 2-hydroxybenzaldehyde by general procedure A in 68% yield as a white powder. Characterization was in accordance to reported values.[65]

4-trifluoromethyl-2,5-dimethoxy-N-(2-methoxybenzyl)phenethylamine hydrochloride (25TFM-NBOMe, 5.6)
Synthesized from 2,5-dimethoxy-4-trifluoromethylphenylethylamine hydrochloride (2C-TFM, 1.12) and 2-methoxybenzaldehyde by general procedure A in 74% yield as a white powder. Characterization was in accordance to reported values.[65]

4-trifluoromethyl-2,5-dimethoxy-N-(2-fluorobenzyl)phenethylamine hydrochloride (25TFM-NBF, 5.7)
Synthesized from 2,5-dimethoxy-4-trifluoromethylphenylethylamine hydrochloride (2C-TFM, 1.12) and 2-fluorobenzaldehyde by general procedure A in 69% yield as a white powder. Characterization was in accordance to reported values.[65]

N-(benzo[d][1,3]dioxol-4-ylmethyl)-2-(2,5-dimethoxy-4-(trifluoromethyl)phenyl)ethan-1-amine hydrochloride (5.8)
Synthesized from 2,5-dimethoxy-4-trifluoromethylphenylethylamine hydrochloride (2C-TFM, 1.12) and 2,3-(methylenedioxy)benzaldehyde by general procedure A in 60% yield as a white powder. Characterization was in accordance to reported values.[65]

2-((2,5-dimethoxy-4-(methylthio)phenethylamino)methyl)phenol hydrochloride (8.1)
Synthesized from 2,5-dimethoxy-4-(methylthio)phenylethylamine (2C-T, 1.3) and 2-hydroxybenzaldehyde by general procedure A in 70% yield as a white powder. Characterization was in accordance to reported values.[65]

2-(2,5-dimethoxy-4-(methylthio)phenyl)-N-(2-methoxybenzyl)ethanamine hydrochloride (8.2)
2,5-dimethoxy-4-(methylthio)phenylethylamine (2C-T, 1.3) and 2-methoxybenzaldehyde by general procedure A in 75% yield as a white powder. Characterization was in accordance to reported values.[65]

2-((2,5-dimethoxy-4-(ethylthio)phenethylamino)methyl)phenol hydrochloride (8.3)
2,5-dimethoxy-4-(ethylthio)phenylethylamine (2C-T-2, 1.9) and 2-hydroxybenzaldehyde by general procedure A in 65% yield as a white powder. Characterization was in accordance to reported values.[65]

2-(2,5-dimethoxy-4-(ethylthio)phenyl)-N-(2-methoxybenzyl)ethanamine hydrochloride (8.4)
2,5-dimethoxy-4-(ethylthio)phenylethylamine (2C-T-2, 1.9) and 2-methoxybenzaldehyde by general procedure A in 59% yield as a white powder. Characterization was in accordance to reported values.[65]

2-((2,5-dimethoxy-4-(propylthio)phenethylamino)methyl)phenol hydrochloride (8.5)
2,5-dimethoxy-4-(n-propylthio)phenylethylamine (2C-T-7, 1.10) and 2-hydroxybenzaldehyde by general procedure A in 58% yield as a white powder. Characterization was in accordance to reported values.[65]

2-(2,5-dimethoxy-4-(propylthio)phenyl)-N-(2-methoxybenzyl)ethanamine hydrochloride, 8.6)
2,5-dimethoxy-4-(n-propylthio)phenylethylamine (2C-T-7, 1.10) and 2-methoxybenzaldehyde by general procedure A in 52% yield as a white powder. Characterization was in accordance to reported values.[65]

methyl 4-(2-aminoethyl)benzoate hydrochloride (9.2)
To a stirring solution of 4-(2-Aminoethyl)benzoic acid hydrochloride (0.5 g, 2.48 mmol, 9.1) in methanol (20 ml), HCl 4N in dioxane (1 ml) was added and the mixture was refluxed at 70°C overnight. The latter was cooled to room temperature, concentrated under reduced pressure, and washed with diethyl ether (20 ml) to deliver methyl 4-(2-aminoethyl)benzoate hydrochloride (9.2) as bright white crystals (0.523 g, 2.43 mmol, 98%).
^{1}H NMR (400 MHz, MeOD) δ 8.01 (d, *J* = 7.9 Hz, 2H), 7.41 (d, *J* = 8.0 Hz, 2H), 3.90 (s, 3H), 3.22 (t, *J* = 7.7 Hz, 2H), 3.04 (t, *J* = 7.6 Hz, 2H).

methyl 4-(2-((*tert*-butoxycarbonyl)amino)ethyl)benzoate (9.3)
To a stirring mixture containing methyl 4-(2-aminoethyl)benzoate hydrochloride (0.5 g, 1 eq., 2.318 mmol, 9.2) in dry THF/MeOH (2:1, 9 ml) under N_2 atmosphere, DIPEA was added (0.418 ml, 1 eq., 2.4 mmol), followed by di-*tert*-butyl dicarbonate (4.64 ml, 2 eq., 4.8 mmol). The mixture was stirred at room temperature for 5 hours, concentrated under reduced pressure, and dissolved in ethyl acetate (50 ml). The

organic layer was washed with water (30 ml) and brine (30 ml), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was triturated in a large amount of diethyl ether and dried in vacuo to deliver methyl 4-(2-((*tert*-butoxycarbonyl)amino)ethyl)benzoate (9.3) as a white powder (0.605 g, 2.165 mmol, 93%).

tert-butyl (4-carbamoylphenethyl)carbamate (9.4)
Methyl 4-(2-((*tert*-butoxycarbonyl)amino)ethyl)benzoate (55.9 mg, 0.2 mmol, 9.3), NH_3 25% aqueous solution (1 ml) and dioxane (0.5 ml) were poured in a tube, sealed thereafter, and the mixture was stirred at 160°C for 40 minutes under micro-wave irradiation. Upon cooling to room temperature, the precipitate was collected by filtration, washed with water (1 ml), and dried *in vacuo* as *tert*-butyl (4-carbamoylphenethyl)carbamate (18 mg, 68 µmol, 34%).

4-(2-aminoethyl)benzamide hydrochloride (9.5)
A solution of HCl 4N in dioxane (0.4 ml) was added to *tert*-butyl (4-carbamoylphenethyl)carbamate (79.3 mg, 0.3 mmol, 9.4) in dioxane (0.5 ml) and the reaction was stirred for 2 hours. The residue was concentrated under reduced pressure, dissolved in a minimum amount of 2-propanol, and poured in diethyl ether (15 ml). The precipitate was collected by filtration and dried *in vacuo* delivering 4-(2-aminoethyl)benzamide hydrochloride (9.5) as a white powder (47.1 mg, 0.287 mmol, 96%).

methyl (3-(cyanomethyl)phenyl)carbamate (9.7)
A stirring solution of 2-(3-aminophenyl)acetonitrile (0.236 ml, 1 eq., 2 mmol, 9.6) and DIPEA (0.5 ml, 1.45 eq., 2.9 mmol) in DCM (3 ml) was cooled to 0°C. A freshly prepared solution of methyl chloroformate (0.2 ml, 1.25 eq., 2.5 mmol) in DCM (0.4 ml) was slowly added dropwise and the vessel was allowed to reach room temperature and stirred for 16 hours. The mixture was poured in water (6 ml), and the organic layer was separated and washed with saturated citric acid aqueous solution (3 ml), saturated $NaHCO_3$ aqueous solution (3 ml), and brine (3 ml). After stirring for 15 minutes with a generous amount of activated carbon, the organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to deliver methyl (3-(cyanomethyl)phenyl)carbamate (9.7) as light pink crystals (0.379 g, 1.993 mmol, 99%).
^{1}H NMR (600 MHz, $CDCl_3$) δ 7.37 (s, 1H), 7.24 (m, J = 6.6 Hz, 2H), 6.98 (d, J = 7.0 Hz, 1H), 6.57 (br. s, 1H), 3.72 (s, 3H), 3.67 (s, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 152.8, 137.6, 130.0, 128.9, 121.8, 117.1, 117.0, 116.6, 51.5, 22.6.

methyl (3-(2-((*tert*-butoxycarbonyl)amino)ethyl)phenyl)carbamate (9.8)
In a sealed flame-dried round bottom flask under N_2 atmosphere, a stirring solution of methyl (3-(cyanomethyl)phenyl)carbamate (0.220 g, 1.16 mmol, 9.7) in dry methanol (15 ml) was cooled to 0°C. Di-*tert*-butyl dicarbonate (0.436 g, 2 mmol) and nickel(II) chloride hexahydrate (24 mg, 0.2 mmol) were added, followed by portion-wise $NaBH_4$ (0.265 g, 7 mmol) addition over 30 minutes. The mixture was allowed to reach room temperature and kept stirring for 1 hour, when complete conversion of 9.7 was

confirmed by TLC staining with ninhydrin solution (EtOAc/Heptane, 9:1). The mixture was stirred for 30 minutes upon addition of DETA (108 µl, 1 mmol) and concentrated under reduced pressure. The purple residue was dissolved in ethyl acetate (50 ml), which was washed with $NaHCO_3$ saturated aqueous solution (2 x 50 ml) and dried thoroughly over $MgSO_4$. After drying *in vacuo*, methyl (3-(2-((*tert*-butoxycarbonyl)amino)ethyl)phenyl)carbamate (9.8) was isolated as colorless oil with large white crystals (0.246 g, 0.835 mmol, 72%).

Structurally constrained derivatives

NMR spectra were recorded on Bruker Avance III HD 600 MHz spectrometer. Residual solvent peaks (Methanol-d_4, D_2O, $CDCl_3$, and DMSO-d_6) were used as internal standard (4.78, 4.87, 7.26, and 2.50 ppm for ^1H, respectively, and 49.15, 77.06, and 39.51 ppm for ^{13}C). Thin-layer chromatography (TLC) was performed on aluminum sheets precoated with silica gel F254. Compounds were visualized by UV radiation (254 nm) and again after staining with ninhydrin solution (1.5 g ninhydrin, 100 ml ethanol, 3.0 ml AcOH). Semi-preparative and preparative HPLC separation was performed on Dionex Ultimate 3000 HPLC system (Thermo) with a Gemini NX C18 (250 × 21 mm) column, Flow rate: 20 ml/min, mobile phase A: H_2O/TFA (100:0.1) (v/v); B: ACN/H_2O/TFA (90:10:0.1) (v/v/v). All the samples were dissolved in H_2O/ACN (1:1). HPLC separation was achieved with a Chiralpak IA column (250 * 20 mm, 5 mkm); mobile phase: Hexane:IPA:MeOH, (98:1:1) Flow Rate: 12 mL/min; Column Temperature: 24°C; Wavelength: 205 nm, 225 nm. Data acquisition was controlled by MassLynx ver. 4.1, and data analysis was done using Waters OpenLynx browser ver. 4.1. Solvents were commercial HPLC grade and used without further purification. $NaCO_3$ aqueous solutions were prepared by mixing H_2O/$NaHCO_3$ saturated solution in 2:3 ratio.

General procedure (B) for the synthesis of conformationally constrained derivatives

Glacial acetic acid (3 eq., 0.34 ml) was added to a suspension of the targeted amine hydrochloride (1 eq., 2 mmol) in MeOH/THF (2:1, 15 ml). 4-chromanone (2.5 eq., 740.8 mg) or 3-coumarone (3 eq., 804.8 mg) was added, and the reaction mixture was stirred at room temperature until formation of the corresponding imine seemed complete by TLC (DCM/MeOH/TEA, 98:2:0.2). $NaBH_3CN$ 1.0 M solution in THF (3 eq., 6 ml) was added and the reaction mixture was stirred for 30 minutes - 3 hours until the formation of the product seemed to be completed according to TLC. The mixture was quenched by addition of $NaHCO_3$ aqueous solution (5 ml), and the residue was extracted with ethyl acetate (3 x 15 ml). The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated under reduced pressure.

(±)-N-(4-bromo-2,5-dimethoxyphenethyl)-2,3-dihydrobenzofuran-3-amine trifluoroacetate (11.1)

Synthesized as TFA salt of its racemic mixture from 2,5-dimethoxy-4-bromophenylethylamine hydrochloride (2C-B, 1.1) and 3-coumaranone by use of general procedure B and purified by HPLC (multistep with 15-100%B gradient) in

52% yield as a bright white solid. Characterization was in accordance to reported values.[5]

(±)-N-(4-bromo-2,5-dimethoxyphenethyl)chroman-4-amine trifluoroacetate (11.2)

Synthesized as TFA salt of its racemic mixture from 2,5-dimethoxy-4-bromophenylethylamine hydrochloride (2C-B, 1.1) and 4-chromanone by use of general procedure B and purified by HPLC (multistep with 15-100%B gradient) in 58% yield as a bright white solid. Characterization was in accordance to reported values.[5]

(±)-4-(2-((2,3-dihydrobenzofuran-3-yl)amino)ethyl)-2,5-dimethoxybenzonitrile trifluoroacetate (11.3)

Synthesized as TFA salt of its racemic mixture from 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) and 3-coumaranone by use of general procedure B and purified by HPLC (multistep with 15-100%B gradient) in 55% yield as a bright white solid. Characterization was in accordance to reported values.[5]

(±)-4-(2-(chroman-4-ylamino)ethyl)-2,5-dimethoxybenzonitrile trifluoroacetate (11.4)

Synthesized as TFA salt of its racemic mixture from 2,5-dimethoxy-4-cyanophenylethylamine hydrochloride (2C-CN, 1.6) and 4-chromanone by use of general procedure B and purified by HPLC (multistep with 15-100%B gradient) in 69% yield as a bright white solid.

^{1}H NMR (600 MHz, D_2O) δ 7.50 (t, *J* = 7.8 Hz, 1H), 7.43 (d, *J* = 7.7 Hz, 1H), 7.40 (s, 1H), 7.21 (s, 1H), 7.15 (t, *J* = 7.5 Hz, 1H), 7.08 (d, *J* = 8.4 Hz, 1H), 4.72 (m, *J* = 2.0 Hz, 1H), 4.47 (m, *J* = 5.3 Hz, 1H), 4.40 (m, *J* = 5.1 Hz, 1H), 4.02 (s, 3H), 3.94 (s, 3H), 3.52 (m, *J* = 4.2 Hz, 2H), 3.22 (t, *J* = 7.5 Hz, 2H), 2.52 (m, *J* = 4.9 Hz, 1H), 2.44 (m, *J* = 4.5 Hz, 1H). ^{13}C NMR (151 MHz, D_2O) δ 155.82, 154.44, 151.24, 129.70, 121.27, 117.52, 117.22, 115.37, 114.90, 98.99, 61.68, 56.67, 56.12, 51.35, 44.18, 27.73, 24.11.

(±)-N-(2,5-dimethoxyphenethyl)chroman-4-amine trifluoroacetate (11.5)

Synthesized as TFA salt of its racemic mixture from 2,5-dimethoxyphenyletylamine hydrochloride (2C-H, 1.13) and 4-chromanone by use of general procedure B and purified by HPLC (multistep with 15-100%B gradient) in 79% yield as a bright white solid.

^{1}H NMR (600 MHz, MeOD) δ 7.31 (dd, *J* = 1.3, 7.8 Hz, 1H), 7.27 (m, *J* = 2.9 Hz, 1H), 6.94 (dt, *J* = 1.0, 7.5 Hz, 1H), 6.87 (m, *J* = 3.0 Hz, 2H), 6.77 (m, *J* = 4.3 Hz, 2H), 4.51 (t, *J* = 4.8 Hz, 1H), 4.28 (m, *J* = 4.2 Hz, 1H), 4.22 (m, *J* = 3.6 Hz, 1H), 3.75 (s, 3H), 3.69 (s, 3H), 3.28 (m, *J* = 3.0 Hz, 2H), 2.96 (m, *J* = 4.3 Hz, 2H), 2.29 (m, *J* = 3.3 Hz, 2H). ^{13}C NMR (151 MHz, MeOD) δ 162.81, 162.58, 156.89, 155.46,

153.19, 132.58, 131.13, 126.67, 122.12, 119.19, 118.16, 117.29, 114.02, 112.91, 62.61, 56.44, 56.26, 52.99, 46.29, 28.91, 25.78.

(±)-N-(2,5-dimethoxyphenethyl)-2,3-dihydrobenzofuran-3-amine trifluoroacetate (11.6)

Synthesized as TFA salt of its racemic mixture from 2,5-dimethoxyphenyletylamine hydrochloride (2C-H, 1.13) and 3-coumaranone by use of general procedure B and purified by HPLC (multistep with 15-100%B gradient) in 70% yield as a bright white solid.

(±)-N-(2,5-dimethoxy-4-(trifluoromethyl)phenethyl)-2,3-dihydrobenzofuran-3-amine trifluoroacetate (11.7)

Synthesized as TFA salt of its racemic mixture from 2,5-dimethoxy-4-trifluoromethylphenylethylamine (2C-TFM, 1.12) and 3-coumaranone by use of general procedure B and purified by HPLC (multistep with 15-100%B gradient) in 42% yield as a bright white solid.

(±)-N-(2,5-dimethoxy-4-(trifluoromethyl)phenethyl)chroman-4-amine trifluoroacetate (11.8)

Synthesized as TFA salt of its racemic mixture from 2,5-dimethoxy-4-trifluoromethylphenylethylamine (2C-TFM, 1.12) and 4-chromanone by use of general procedure B and purified by HPLC (multistep with 15-100%B gradient) in 46% yield as a bright white solid.

^{1}H NMR (600 MHz, MeOD) δ 7.33 (dd, *J* = 1.4, 7.8 Hz, 1H), 7.28 (m, *J* = 2.9 Hz, 1H), 7.11 (s, 1H), 7.04 (s, 1H), 6.96 (dt, *J* = 1.1, 7.5 Hz, 1H), 6.87 (dd, *J* = 1.0, 8.3 Hz, 1H), 4.54 (t, *J* = 4.8 Hz, 1H), 4.29 (m, *J* = 4.2 Hz, 1H), 4.23 (m, *J* = 3.7 Hz, 1H), 3.82 (s, 3H), 3.81 (s, 3H), 3.33 (m, *J* = 3.0 Hz, 2H), 3.08 (m, *J* = 4.1 Hz, 1H), 3.02 (m, *J* = 4.2 Hz, 1H), 2.31 (m, *J* = 3.6 Hz, 2H). ^{13}C NMR (151 MHz, MeOD) δ 156.90, 153.18, 152.46, 132.65, 131.34, 131.14, 125.98, 124.17, 122.15, 119.23, 117.24, 116.83, 110.60, 62.59, 57.23, 53.17, 45.86, 28.85, 25.77.

(±)-*tert*-butyl chroman-4-yl(4-cyano-2,5-dimethoxyphenethyl)carbamate (12.3)

(±)-4-(2-(Chroman-4-ylamino)ethyl)-2,5-dimethoxybenzonitrile trifluoroacetate (102.5 mg, 1 eq., 226.5 µmol, 11.4) was added to a stirring mixture of di-*tert*-butyl dicarbonate (0.169 g, 3.1 eq., 0.78 mmol) and $NaHCO_3$ (57.2 mg, 3 eq., 0.68 mmol) in methanol (20 ml). The vessel was stirred at 45 °C for 3 days, while monitoring the reaction by TLC (DCM/MeOH/TEA, 95:5:0.2, ninhydrin staining) and UPLC-MS, to deliver the boc-protected product, as colorless crystals in quantitative yield.

^{1}H-NMR (600 MHz, MeOD) δ 7.03 (m, *J* = 3.0 Hz, 1H), 6.99 (s, 1H), 6.90 (m, *J* = 1.7 Hz, 1H), 6.87 (d, *J* = 7.7 Hz, 1H), 6.76 m, *J* = 3.1 Hz, 1H), 6.68 (q, *J* = 3.2 Hz, 1H), 4.22 (m, *J* = 3.9 Hz, 1H), 4.04 (q, *J* = 11.0 Hz, 1H), 3.76 (d, *J* = 24.5 Hz, 3H), 3.63 (br. s, 3H), 3.23 (t, *J* = 1.6 Hz, 2H), 2.86 (m, *J* = 5.3 Hz, 2H), 2.09 (m, *J* = 7.6 Hz, 1H), 1.92 (q, *J* = 6.8 Hz, 1H), 1.47 (br. s, 6H), 1.28 (br. s, 3H).

(±)-*Tert*-butyl chroman-4-yl(4-cyano-2,5-dimethoxyphenethyl)carbamate (12.3) was adsorbed on celite, purified by column chromatography (DCM/MeOH/TEA – 99:1:0.2), and isolated as white powder (45.7 mg, 104.2 µmol, 46%).

(+)-*tert*-butyl chroman-4-yl(4-cyano-2,5-dimethoxyphenethyl)carbamate, (-)-*tert*-butyl chroman-4-yl(4-cyano-2,5-dimethoxyphenethyl)carbamate (12.6, 12.7)
HPLC chiral separation was performed to obtain the separated boc-protected stereoisomers (12.6 and 12.7). Retention times: 10.5 and 14 minutes.

N-(chroman-4-yl)-2-nitrobenzenesulfonamide (14.2)
In a round-bottom flask under N_2 atmosphere, TEA (7.9 ml, 37.8 mmol) was added to a stirring solution of chroman-4-amine (5.64 g, 37.8 mmol, 14.1) in DCM (90 ml) and the vessel was cooled to 0°C. After dropwise addition of 2-nitrobenzenesulfonyl chloride (8.377 g, 37.71 mmol) in dry DCM (30 ml) over 15 minutes, the mixture was allowed to reach room temperature and monitored by TLC (EtOAc/Heptane, 1:1). Once the conversion seemed complete after overnight stirring, the mixture was poured in water (90 ml), and the organic layer was separated, washed with brine (60 ml), dried over $MgSO_4$, and concentrated under vacuum. N-(Chroman-4-yl)-2-nitrobenzenesulfonamide (14.2) was purified by recrystallization in EtOAc/Heptane (80 ml, 1:1) and in ethyl acetate (400 ml) as white powder (11.73 g, 31.64 mmol, 84%).
^{1}H-NMR (600 MHz, $CDCl_3$) δ 8.20 (m, *J* = 2.3 Hz, 1H), 7.87 (m, *J* = 2.3 Hz, 1H), 7.74 (m, *J* = 2.3 Hz, 2H), 7.09 (m, *J* = 4.3 Hz, 1H), 6.72 (m, *J* = 6.5 Hz, 3H), 4.53 (q, *J* = 5.1 Hz, 1H), 4.16 (m, *J* = 4.7 Hz, 2H), 2.14 (m, *J* = 2.7 Hz, 1H), 2.06 (m, *J* = 4.6 Hz, 1H).

N-(chroman-4-yl)-2-nitro-N-phenethylbenzenesulfonamide (14.3)
To a stirring solution of N-(chroman-4-yl)-2-nitrobenzenesulfonamide (167.17 mg, 1 eq., 0.5 mmol, 14.2) and K_2CO_3 (138.2 mg, 2 eq., 1 mmol) in anhydrous DMF (12 ml), (2-bromoethyl)benzene (75 µl, 1.1 eq., 0.55 mmol) was added dropwise after being diluted in dry DMF (1 ml) and the vessel was heated to 60°C. After 2 days the starting material did not seem to be fully alkylated by UPLC-MS and the reaction was stopped to quantify the extent of conversion. The mixture was diluted with more DMF (100 ml), extracted with diethyl ether (5 x 40 ml) and the combined organic extracts were washed with water (100 ml) and brine (100 ml). The residue was concentrated under reduced pressure and analyzed by NMR spectroscopy, which showed that the resulting light-yellow powder was composed of minor amounts of N-(chroman-4-yl)-2-nitro-N-phenethylbenzenesulfonamide (14.3) and, mostly, of the starting N-(chroman-4-yl)-2-nitrobenzenesulfonamide (14.2).

2,5-dimethoxyphenylethylamine hydrochloride (2C-H, 1.3)
A stirring solution of thiophenol (0.391 ml, 38.25 mmol) in dry DMF (10 ml) was cooled to 0°C when KOH (2.15 g, 38.25 mmol) was added portion-wise over 15 minutes. N-(chroman-4-yl)-N-(2,5-dimethoxyphenethyl)-2-nitrobenzenesulfonamide

(7.63 g, 15.3 mmol, 14.4) in DMF (10 ml) was added dropwise over 20 minutes and the vessel was allowed to reach room temperature meanwhile. The mixture was stirred at 50°C per 1 hour, cooled to room temperature, poured in water (40 ml), and extracted with DCM (3 x 50 ml). The combined organic extracts were washed with NaOH 25% aqueous solution (2 x 50 ml), brine (20 ml), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The light-yellow clear oil was dissolved in diethyl ether (50 ml) and precipitated by dropwise addition of HCl 2N in diethyl ether (8 ml) under stirring. After 30 minutes, 2,5-dimethoxyphenylethylamine hydrochloride (2C-H, 1.3) was filtered off and dried in vacuo as a white crystals (4.597 g, 13.14 mmol, 86%).

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APPENDIX

I. *Discovery of β-Arrestin-Biased 25CN-NBOH-Derived 5-HT2A Receptor Agonists - (published, 2022)*

II. *Structure-activity assessment and in-depth analysis of biased agonism in a set of phenethylamine 5-HT2AR agonists - (submitted and under revision) – not included in the printable version*

III. *Facile One-pot Reduction of Nitrostyrenes to Phenethylamines using Sodium Borohydride and Copper(II) - (submitted)*

NMR spectra

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Article

Discovery of β-Arrestin-Biased 25CN-NBOH-Derived 5-HT$_{2A}$ Receptor Agonists

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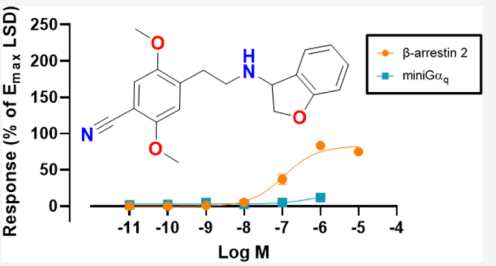
ABSTRACT: The serotonin 2A receptor (5-HT$_{2A}$R) is the mediator of the psychedelic effects of serotonergic psychedelics, which have shown promising results in clinical studies for several neuropsychiatric indications. The 5-HT$_{2A}$R is able to signal through the Gα_q and β-arrestin effector proteins, but it is currently not known how the different signaling pathways contribute to the therapeutic effects mediated by serotonergic psychedelics. In the present work, we have evaluated the subtype-selective 5-HT$_{2A}$R agonist 25CN-NBOH and a series of close analogues for biased signaling at this receptor. These ligands were designed to evaluate the role of interactions with Ser159$^{3\times36}$. The lack of interaction between this hydroxyl moiety and Ser159$^{3\times36}$ resulted in detrimental effects on potency and efficacy in both βarr2 and miniGα_q recruitment assays. Remarkably, Gα_q-mediated signaling was considerably more affected. This led to the development of the first efficacious βarr2-biased 5-HT$_{2A}$R agonists **4a−b** and **6e−f**, βarr2 preferring, relative to lysergic acid diethylamide (LSD).



INTRODUCTION

G protein-coupled receptors (GPCRs) form the largest protein family in the human genome, mediating signaling from the extracellular to the intracellular side of the cell membrane via a diverse range of neurotransmitters, hormones, and peptides.[1−3] These transmembrane proteins are also the most prominent drug targets, with nearly one-third of all FDA-approved drugs acting on GPCRs. These receptors typically transduce physiological signals through intracellular G protein(s). Upon binding of an agonist, the heterotrimeric G protein interacts with the receptor, and the G$_\alpha$ subunit dissociates and initiates the downstream G-protein-mediated signaling cascade. Despite being the most prevalent targets in drug discovery campaigns, there have been limitations in understanding the *in vivo* pharmacological response of GPCR ligands through *in vitro* assays.[4,5] Particularly the discovery of other effector proteins, such as β-arrestin 2 (βarr2), has shown the multifaceted nature of GPCR signaling.[6] To fully untangle this complexity, pathway-selective (or biased) agonists need to be developed for each signaling pathway, i.e., ligands that lead to a preferential (ideally specific) activation of one of the alternative G protein and/or beta-arrestin signaling pathways.

The serotonin 2A receptor (5-HT$_{2A}$R) is the most abundant excitatory serotonin receptor in the brain and the primary mediator of the psychedelic effects of serotonergic psychedelics. These psychedelics can be subdivided into three distinct chemotypes: ergolines, such as lysergic acid diethylamide (LSD),[7] tryptamines such as psilocin[8] (which was first isolated from *Psilocybe Mexicana*),[9] and phenylalkylamines, such as 2,5-dimethoxy-4-iodoamphetamine (DOI)[10] and mescaline[11,12] (which was first isolated from *Lophophora williamsii*).[13] In recent years, there has been increased scientific interest in these serotonergic psychedelics primarily based on the work with psilocybin, which has displayed promising effects in clinical studies focused on various neuropsychiatric indications, including depression and anxiety,[14−17] substance abuse,[18−20] and obsessive-compulsive disorder (OCD).[21] Besides psilocybin, there has also been a renewed interest in the medical use of LSD.[22,23] In addition to its 5-HT$_{2A}$R agonism, psilocybin exhibits high agonist potency at most of the serotonergic receptors[24,25] and LSD possesses high activity at an even broader range of monoaminergic receptors.[26] Despite these nonselective receptor profiles, the activation of 5-HT$_{2A}$R is considered essential for the psychedelic effects as well as the apparent therapeutic potential of these compounds. In general, the phenylalkylamines, and in particular *N*-benzylphenethylamines (NBOMe's), have shown selectivity toward 5-HT$_{2A}$R. The reader is referred to recent review articles for a more in-depth discussion on the historical overview of the NBOMe class.[27,28] Despite efforts from many, the success rate of developing truly selective 5-HT$_{2A}$R agonists has been

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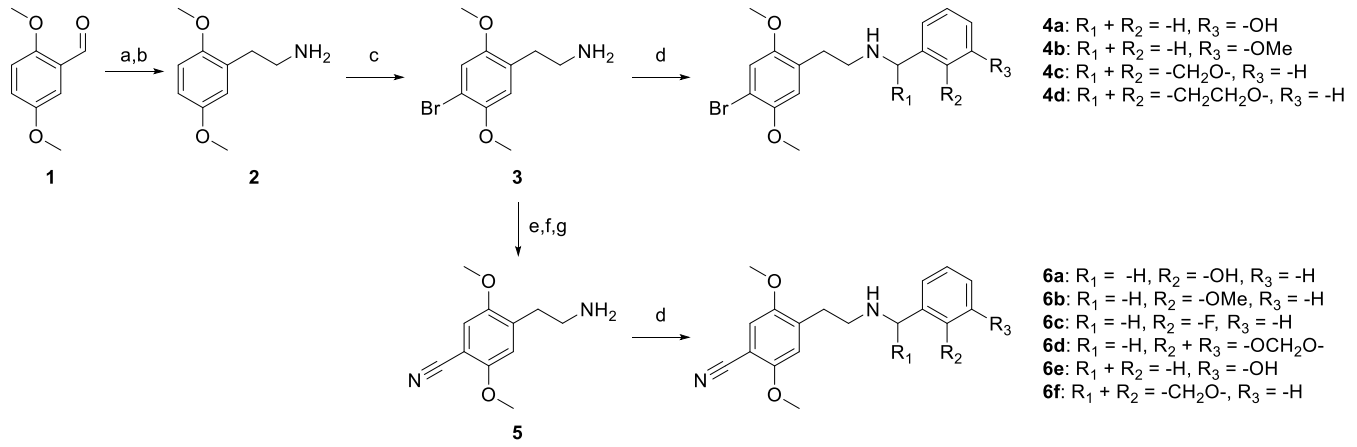
Scheme 1. Synthesis of *N*-Benzylphenethylamines[a]



[a]Reaction conditions: (a) nitromethane, NH_4OAc, 100 °C; (b) LAH, tetrahydrofuran (THF) reflux; (c) Br_2, AcOH, rt; (d) aldehyde, EtOH, rt or ketone, AcOH, MeOH/THF, rt; (ii) $NaBH_4$, EtOH, rt or $NaBH_3CN$, THF, rt; (e) phthalic anhydride, toluene, reflux; (f) Cu(I)CN, *N*,*N*-dimethylformamide (DMF), reflux; (g) hydrazine (aq.), THF, rt.

Table 1. Functional Properties of the Compounds (4a−d and 6a−f) at 5-HT_{2A}R in the βarr2 or miniGα_q Recruitment Assays[a]

	βarr2		miniGα_q		
5-HT_{2A}	EC_{50} (nM) [CI]	E_{max} (%) [CI]	EC_{50} (nM) [CI]	E_{max} (%) [CI]	β-factor
5-HT	12.1 [8.52−17.4]	110 [105−115]	130 [63.3−270]	222 [197−249]	0.576
LSD	12.9 [8.45−19.7]	99.7 [93.6−106]	13.2 [6.81−25.6]	100 [91.0−110]	0
4a	11.1 [7.65−16.2]	112 [105−118]	48.8 [13.0−157]	28.0 [22.1−34.7]	1.240
4b	11.1 [7.59−16.3]	113 [106−120]	44.4 [19.1−94.6]	38.8 [34.0−44.3]	1.100
(±)-**4c**	28.6 [18.9−43.3]	96.6 [90.4−103]	23.0 [12.0−44.6]	48.7 [44.0−53.7]	0.279
(±)-**4d**	132 [108−161]	121 [115−126]	174 [80.9−423]	47.7 [40.4−57.5]	0.558
6a (25CN-NBOH)	2.75 [1.73−4.40]	150 [141−160]	8.59 [3.87−18.1]	123 [110−136]	0.619
6b (25CN-NBOMe)	1.93 [1.17−3.28]	161 [151−171]	6.71 [3.82−11.4]	159 [148−170]	0.526
6c (25CN-NBF)	53.2 [36.8−75.7]	114 [107−121]	168 [77.7−363]	72.5 [62.8−82.9]	0.669
6d (25CN-NBMD)	17.0 [10.4−28.2]	114 [106−123]	45.1 [14.5−128]	83.0 [67.1−100]	0.638
6e	84.5 [64.0−111]	106 [101−112]	301 [46.1−1764]	22.5 [16.4−30.3]	1.250
(±)-**6f**	108 [68.6−169]	82.9 [75.9−90.2]	631 [n.d.]	18.0 [n.d.]	n.d.

[a]Data obtained in the βarr2 or miniGα_q recruitment assays, using the 2 h time−luminescence profile to calculate the AUC. The EC_{50} value is a measure of agonist potency, and the E_{max} value is a measure of agonist efficacy. The E_{max} values for the compounds are normalized to the E_{max} of LSD as the reference agonist (data for the compounds where the E_{max} are normalized to serotonin E_{max} values can be found in the Supporting Information). Data are combined from at least three independent experiments, each performed in duplicate. The reported β-factor is the average value of the three β-factors obtained in three independent experiments; β-factors derived from the "combined" EC_{50} and the E_{max} values can be found in Table S2. n.d. is not determined; see text for further details. CI: 95% confidence interval.

nominal.[29−31] The most notable exceptions are 25CN-NBOH[32−35] and (*S*,*S*)-DMBMPP,[36] which are the most selective 5-HT_{2A}R agonists reported to date, with a 52- to 100-fold and 124-fold selectivity over 5-HT_{2C}R, respectively.[33,36]

The 5-HT_{2A}R is able to signal through members of both Gα_q and G$\alpha_{i/o}$ protein families and also through beta-arrestin mediated pathways.[37,38] Several psychedelics and 5-HT_{2A}R agonists have been evaluated for their respective bias profiles toward the Gα_q and βarr2 transducers, by means of highly analogous functional complementation assays.[39−42] Recently, the first partial agonist (E_{max} = 13%) with bias toward the βarr2 over G$\alpha_{q\text{-}\gamma 9}$ pathway, compared to the reference 5-HT, has been disclosed (IHCH-7086).[43] However, no strongly biased agonist for the G-protein-mediated signaling pathway has been identified. Herein, we have profiled the subtype-selective agonist 25CN-NBOH and a series of close analogues for functional selectivity at 5-HT_{2A}R in Gα_q- and βarr2-based functional assays and have evaluated the role of the simultaneous interaction of $Ser159^{3\times36}$ with the ammonium and the benzylic hydroxyl of the ligands,[44] which led to the discovery of the first efficacious βarr2-biased agonists for this receptor, relative to LSD.

RESULTS AND DISCUSSION

Chemistry. The corresponding phenethylamine analogues were all prepared according to previously described methods.[10,32,45,46] In short, aldehyde **1** was condensed with nitromethane and subsequently reduced with lithium aluminum hydride (LAH), to yield phenethylamine **2** (2C-H) (Scheme 1). Compound **3** (2C-B) was prepared via subsequent bromination of the 4-position.[10] The corresponding *N*-benzyl derivatives (**4a−b**) were prepared via reduction amination of **3**, in the presence of the appropriate benzylaldehyde.[45] Analogues **4c−d** were prepared from condensation of **3** with 3-coumaranone or 4-chromanone, respectively. The resulting imines were reduced with $NaBH_3CN$, which yielded the racemic secondary amines in

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Table 2. Functional Properties of the Tested Compounds (4a−b and 6a, c, e−f) at the 5-HT$_{2A}$R S159A-Mutated Receptor in the βarr2 or miniGα_q Recruitment Assays[a]

	β-arr2		miniGα_q		
5-HT$_{2A}$-S159A	EC$_{50}$ (nM) [CI]	E_{max} (%) [CI]	EC$_{50}$ (nM) [CI]	E_{max} (%) [CI]	β-factor
5-HT	661 [415−1025]	77.4 [71.7−83.4]	1672 [728−4550]	49.3 [41.3−60.4]	0.550
LSD	5.19 [3.20−8.25]	99.9 [93.7−106]	5.38 [2.85−9.81]	99.5 [91.6−108]	0
4a	172 [74.8−443]	89.8 [75.8−108]	154 [n.d.]	21.0 [n.d.]	n.d.
4b	81.9 [52.6−126]	106 [97.5−114]	112 [40.1−327]	44.7 [36.9−53.9]	0.565
6a (25CN-NBOH)	37.7 [23.3−59.9]	114 [105−123]	137 [76.2−247]	70.3 [62.2−78.9]	0.733
6c (25CN-NBF)	661 [504−853]	86.6 [81.5−92.0]	1126 [253−3869]	20.4 [14.1−30.3]	0.731
6e	939 [614−1388]	68.2 [62.6−74.3]	2064 [n.d.]	12.9 [n.d.]	n.d.
(±)-**6f**	1025 [673−1517]	90.8 [81.9−101]	1653 [n.d.]	13.8 [n.d.]	n.d.

[a]Data obtained in the βarr2 or miniGα_q recruitment assays, using the 2 h time−luminescence profile to calculate the AUC. The EC$_{50}$ value is a measure of agonist potency, and the E_{max} value is a measure of agonist efficacy. The E_{max} values for the compounds are normalized to E_{max} of LSD as the reference agonist (data for the compounds where E_{max} are normalized to serotonin E_{max} values can be found in the Supporting Information). Data are combined from at least three independent experiments, each performed in duplicate. The reported β-factor is the average value of the three β-factors obtained in three independent experiments; β-factors derived from the "combined" EC$_{50}$ and E_{max} values can be found in Table S2. n.d. is not determined; see text for further details. CI: 95% confidence interval.

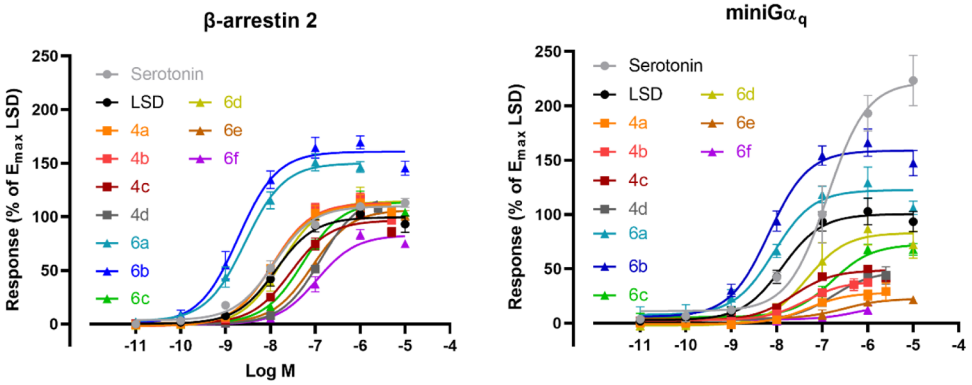

Figure 1. Concentration−response curves of the tested compounds (**4a−d**, and **6a−f**) at the 5-HT$_{2A}$R in the βarr2 or miniGα_q recruitment assays. Overlay of the concentration−response curves for each of the tested substances in the two assay formats. The E_{max} values for the compounds are normalized to E_{max} of LSD as the reference agonist (data for the compounds where E_{max} are normalized to serotonin E_{max} values can be found in the Supporting Information). Each point represents the mean of three independent experiments, each performed in duplicate ± standard error of the mean (SEM). Curves represent three parametric, nonlinear fits.

52−58% isolated yield (Scheme 1). To obtain phenethylamine **5** (2C-CN), compound **3** was converted to the corresponding phthalimide. Subsequent copper-catalyzed cyanation on the 4-bromo moiety and the phthalimide deprotection with NH_2NH_2[46] led to **5**, from which the corresponding *N*-benzyl derivatives (**6a−e**) were prepared via reduction amination in the presence of the appropriate benzylaldehyde.[32,45] **6f** was prepared from condensation of **5** with 3-coumaranone. The resulting imine was reduced with $NaBH_3CN$, which yielded the racemic secondary amine in 55% (Scheme 1).

Pharmacological Characterization. The functional characteristics of **4a−d** and **6a−f** at the 5-HT$_{2A}$R were determined by bioassays using the Nanoluciferase Binary Technology (NanoBiT).[40,41] Briefly, the two nonfunctional parts of the nanoluciferase are each fused to one of the two interacting proteins, in this case the 5-HT$_{2A}$R and the cytosolic proteins, βarr2 or miniGα_q, i.e., the GTPase domain of the Gα_q subunit.[47−49] Upon receptor activation, the cytosolic proteins are recruited to the intracellular parts of the receptor, leading to the functional complementation of the split-nanoluciferase and generation of a luminescent signal, in the presence of the enzyme's substrate.[50] Both the potency and efficacy of the evaluated compounds were determined with this setup. To allow the comparison of the obtained results with previous results, LSD was chosen as the reference agonist for E_{max} and β-factor calculations, and serotonin (5-HT) was included as a positive control.[41] The functional data normalized to 5-HT as a reference agonist is included in the Supporting Material (Table S1). To obtain the data given in Table 1, the area under the curve (AUC) of the full (standard) 2 h activation (time−luminescence) profiles was used to generate concentration−response curves. For a more detailed comparison of biased agonism of (psychedelic) phenethylamines with various incubation times, the reader is referred to Pottie and Poulie et al.[51]

The EC$_{50}$ and E_{max} values (normalized to E_{max} of LSD), as a measure of potency and efficacy, respectively, for the compounds are summarized in Table 1. Additionally, the EC$_{50}$ and E_{max} values of compounds **4a−b** and **6a,c,e−f** were also determined at the 5-HT$_{2A}$R S159A-mutated receptor, and these data are summarized in Table 2. The S159A residue was mutated because of its double interaction with 25CN-NBOH (**6a**) in the deposited cryo-EM structure: Ser159 interacts simultaneously with both its ammonium and its *ortho*-OH moiety on the benzyl ring.[52] Additionally, Kim et al.[52] previously reported that **6a** and serotonin show 161- and 157-fold decreases in potency in a Gα_q-dissociation BRET assay at 5-HT$_{2A}$R, respectively, with the introduction of the

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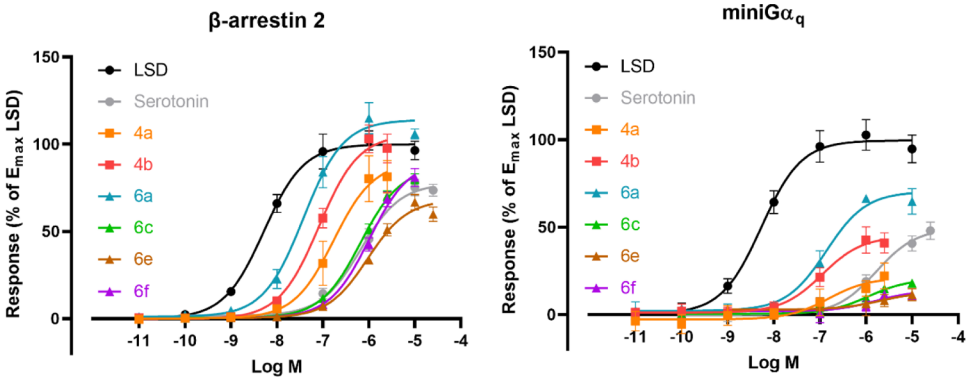

Figure 2. Concentration−response curves of the compounds (**4a−b** and **6a,c,e−f**) at the 5-HT_{2A}R- S159A mutated receptor in the βarr2 or miniGα_q recruitment assays. Overlay of the concentration−response curves for each of the tested substances in the two assay formats. The E_{max} values for the compounds are normalized to E_{max} of LSD as the reference agonist (data for the compounds where E_{max} are normalized to serotonin E_{max} values can be found in the Supporting Information). Each point represents the mean of three independent experiments, each performed in duplicate ± standard error of the mean (SEM). Curves represent three parametric, nonlinear fits.

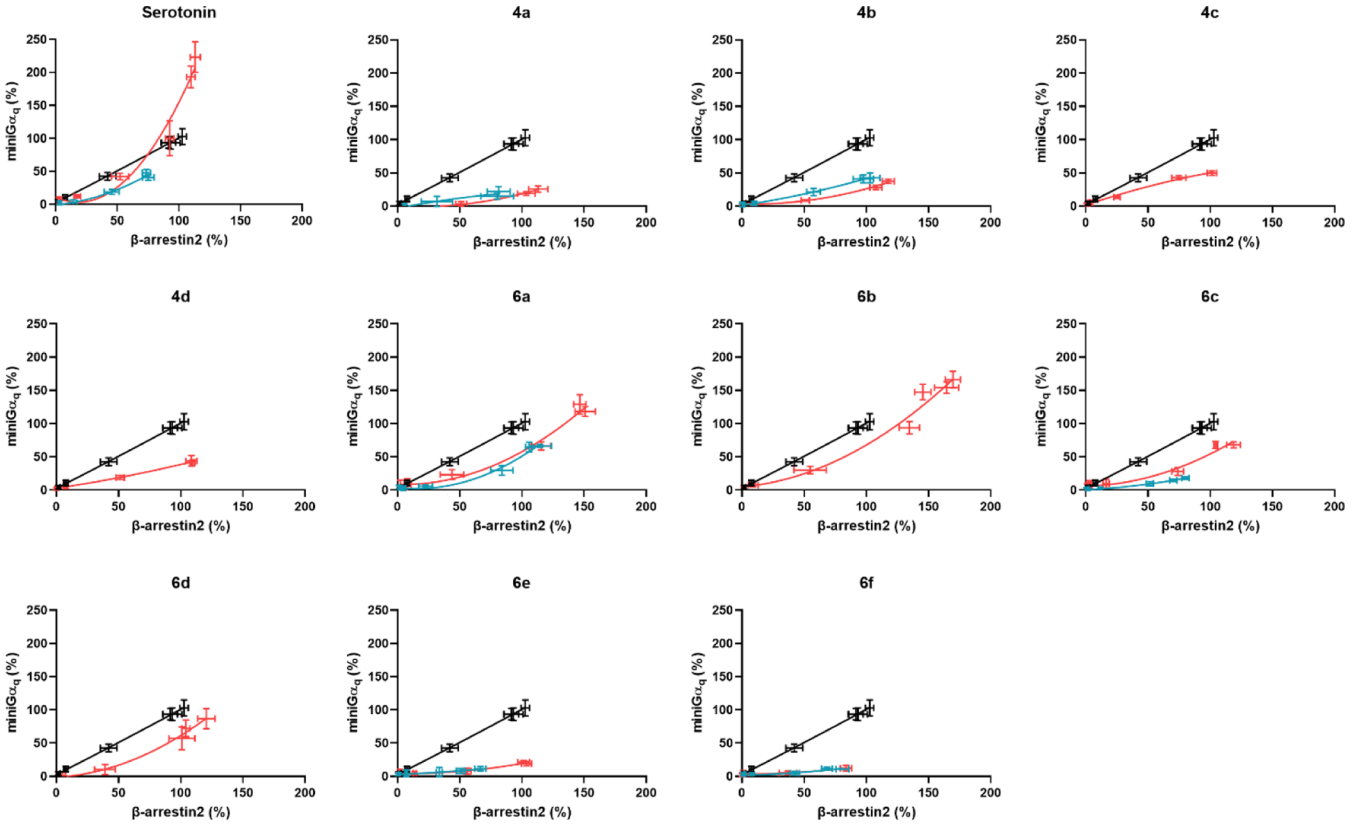

Figure 3. Qualitative bias plots, where each panel shows the centered second-order polynomial fit of the activation values at equimolar concentrations of the substance in the respective assays in red, and that of the reference agonist (LSD) in black (WT and S159A mutated receptor overlap for LSD). Red is data for WT receptor, and blue is data for S159A mutated receptor. Error bars represent the SEM of the individual data points per concentration.

S159A mutation, while the efficacy of the two agonists remained roughly unchanged for serotonin and decreased by a quarter, for **6a**. Most of the evaluated ligands lack the possibility to interact with $Ser159^{3\times36}$, making it compelling to investigate the influence of this residue on the biased agonism of these ligands. The agonist concentration−response curves of all compounds are presented in Figure 1A (βarr2) and Figure 1B (miniGα_q) for the wild-type receptor, and Figure 2A,B, respectively, for the S159A mutated receptor. Figure 3 illustrates the bias plots of the respective ligands evaluated, and Figure 4 shows the overview of the Kruskal−Wallis analysis of the bias factors. These data with serotonin as reference can be found in Figures S2−6 and Table S1, in the Supporting Information.

The 4-bromo analogues (**4a−c**) displayed nanomolar agonist potency at 5-HT_{2A}R in both the βarr2 (EC_{50}: 11−29 nM) and the miniGα_q (EC_{50}: 23−49 nM) recruitment assays, which is in line with previously reported values for 4-halogen-substituted analogues, such as 25I-NBOMe and 25I-NBOH.[41] Interestingly, the E_{max} values exhibited by these analogues were

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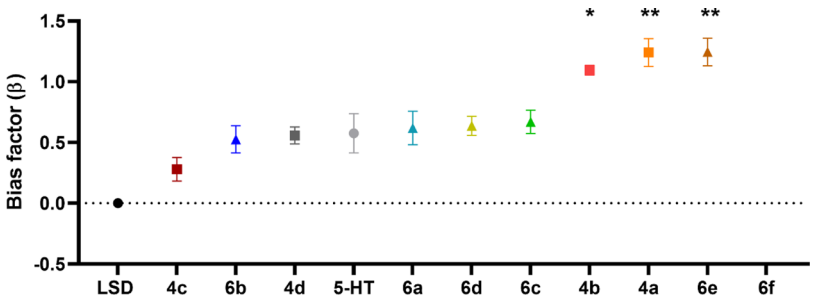

Figure 4. Visual representation of the bias factors (β), where * stands for $p < 0.05$ and ** stands for $p < 0.01$ in the nonparametric Kruskal–Wallis analysis of significance. Compound **6f** is omitted, as no bias factor could be calculated. LSD is used as the reference agonist (data for the compounds where serotonin is used as the reference agonist can be found in the Supporting Information).

reduced compared to 25I-NBOMe and 25I-NBOH, albeit not as pronounced in the βarr2 assay (E_{max}: 97–113% vs 135–141%, respectively) as in the miniGα_q assay (E_{max}: 28–49% vs 111–160%, respectively) (Table 1). Interestingly, extension of the dihydrobenzofuran ring of **4c** with one carbon markedly reduced the potency of **4d** in both the βarr2 and miniGα_q recruitment assays (EC_{50}: 132 and 174 nM, respectively) compared to that of **4c**, whereas this modification had little influence on the agonist efficacies in either assay (121 and 48%, respectively) compared to **4a–c**. Despite minor variations in the potencies and efficacies of the four bromo analogues (**4a–d**), there was a marked difference in their calculated β-factor. For example, **4a–b** were statistically significant βarr2-preferring agonists, with β-factors of 1.24 and 1.10, respectively (Table 1), relative to LSD in contrast to **4c** with a β-factor of 0.279, which is in line with most other NBOMes.[41]

The 4-cyano analogues (**6a–f**) displayed more mixed potency profiles compared to the 4-bromo analogues (**4a–d**). Compounds **6a–b** displayed agonist potencies in the low nanomolar range at 5-HT_{2A}R in both the βarr2- (EC_{50}: 2.8 and 1.9 nM, respectively) and the miniGα_q-recruitment assays (EC_{50}: 8.6 and 6.7 nM), with E_{max} values of 150 and 161% in the βarr2-assay and 123 and 159% in the miniGα_q-assay, respectively. This is in line with the reported values for 25H-NBOH and 25H-NBOMe.[41,42] Compound **6c** followed the same trend as **6a–b**, albeit with significantly lower potencies and efficacies at the receptor for the recruitment of both cytosolic mediators. Interestingly, **6d** displayed reduced potencies and efficacies in the βarr2 and miniGα_q assays compared to those of **6a–b**, but both were increased or the same compared to **6c**, respectively. This tendency is similar to what is observed with the 4-bromo analogues (**4a–b**), which also lack a hydrogen-bond acceptor in the ortho-position on the benzylic ring.

Compounds **6a–d** displayed slightly lower E_{max} values in the miniGα_q than in the βarr2 recruitment assay, and interestingly, the efficacies displayed by the other compounds in the miniGα_q recruitment assay were only half or even lower than the corresponding efficacies in the βarr2 assay compared to other NBOMes.[41] This resulted in a particularly strong preference toward βarr2 recruitment for **4a–b** and **6e** with calculated β-factors ranging 1.10–1.25, relative to LSD. While no β-factor for **6f** could be calculated because of its low activity in the miniG$_{\alpha q}$ recruitment assay, judging from the bias plot (Figure 3), it is apparent that this ligand was highly biased for βarr2 recruitment, relative to LSD Table S2. This observation is numerically reflected when using a slightly different method of data analysis, as shown in Supplementary Table S2.

Of note, even though the obtained absolute bias factors are different when serotonin is taken as the reference agonist (Table S1 and Figure S6), these three compounds still show a preference toward βarr2 recruitment relative to serotonin. From the bias plot (Figure S5) of compound **6f**, also a strong preference toward βarr2 recruitment relative to serotonin can be deduced. For a more detailed comparison of biased agonism of (psychedelic) phenethylamines relative to reference agonists LSD and serotonin, the reader is referred to Pottie and Poulie et al.[51]

Regarding the S159A mutated 5-HT_{2A}R, it should first be noted that serotonin displayed a significant loss of potency and efficacy at this mutated receptor compared to the WT receptor in both the βarr2 and miniGα_q recruitment assays. The fact that the agonist potency of LSD at 5-HT_{2A}R was not affected by this mutation prompted us to use LSD as the reference agonist to enable comparisons between the WT and mutated receptor (Table 2 and Figure S8). The potency of **4a** was reduced at 5-HT_{2A}R S159A compared to WT 5-HT_{2A}R by factors of approximately 15 and 3 in the βarr2 and miniGα_q recruitment assays, respectively. On the other hand, agonist potency of **4b** in the βarr2 assay was only negatively affected by a factor of 7, which is to be expected from the loss of a hydrogen-bond interaction. Remarkably, the efficacy of **4b** remained largely unaffected by the S159A mutation, as neither its potency nor its efficacy in the miniGα_q recruitment assay was significantly altered. The agonist potency displayed by **6a** at the S159A mutated 5-HT_{2A}R in the βarr2 recruitment assay was likewise reduced (approximately 14-fold) as it has been reported previously.[52] In this case, the efficacy was also considerably decreased (E_{max}: 150 and 114% at WT 5-HT_{2A}R and 5-HT_{2A}R S159A, respectively). The same was observed in the miniGα_q recruitment assay, with substantially reduced agonist potency and a significant decrease in efficacy (EC_{50}: 8.6 and 137 nM, E_{max}: 123 and 70% at WT 5-HT_{2A}R and 5-HT_{2A}R S159A, respectively). The agonist potencies displayed by **6c** at 5-HT_{2A}R S159A were reduced by factors of 12 and 7 at the mutated receptor compared to the WT receptor in the βarr2 and miniGα_q recruitment assays, respectively, and its agonist efficacies also decreased substantially by the introduction of the mutation (Table 2). The agonist potencies of **6e–f** also decreased by factors of ~10 at the S159A mutated receptor in the βarr2 recruitment assay, and the potency of **6e** at the mutated receptor in the miniGα_q recruitment assay was more affected compared to that of **6f** (6.9-fold compared to 2.6-fold, respectively). Of note is that, although all experiments with the

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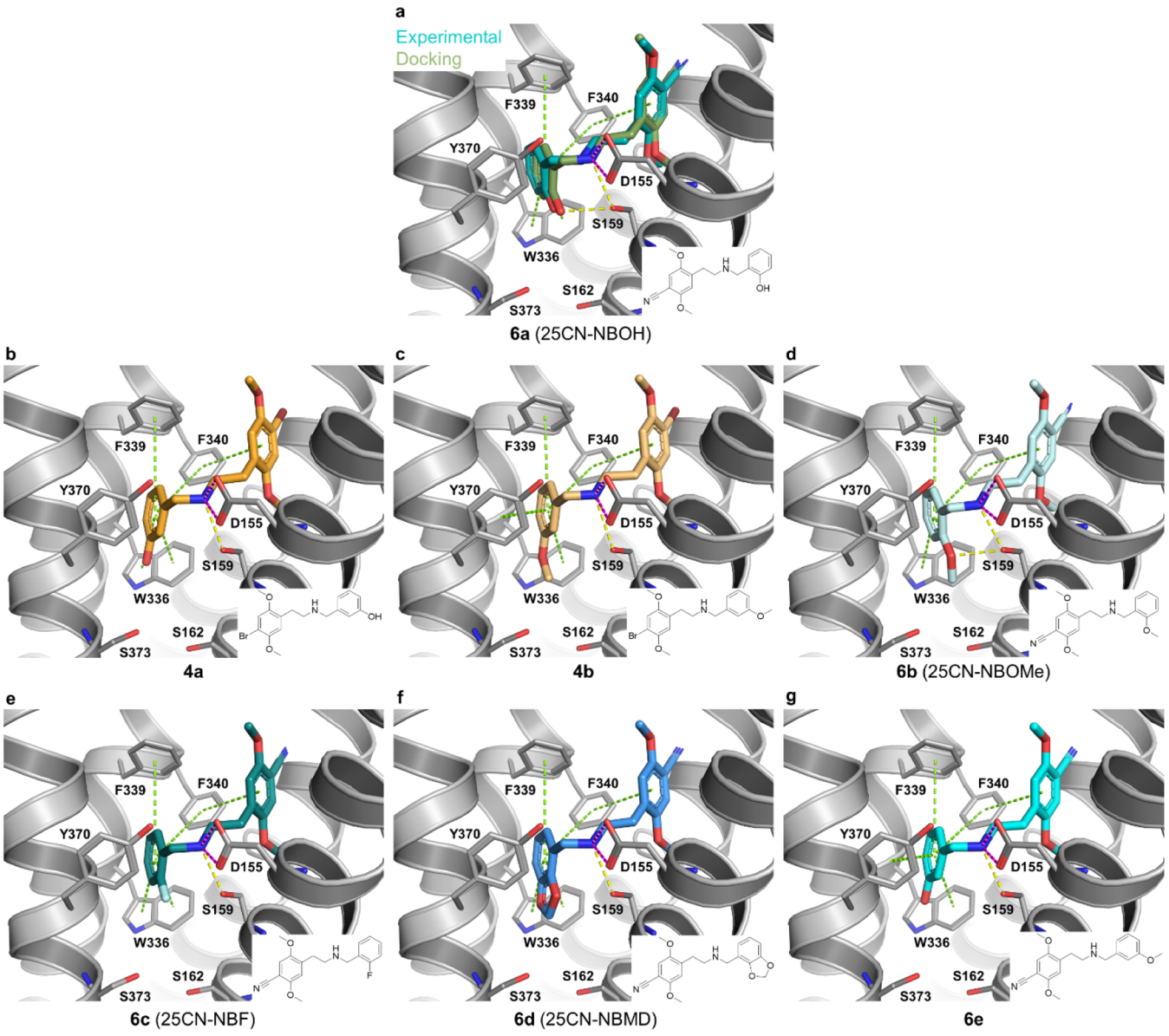

Figure 5. Experimental and predicted binding modes of **4a**−**b** and **6a**−**e** to the 5-HT_{2A}R. (A) Comparison between the experimental (cyan) and the redocking binding pose of **6a** in the cryo-EM structure of the G_q-coupled 5-HT_{2A}R (PDB ID 6WHA)[52] (RMSD of 0.57 Å for heavy atoms). (B−G) Predicted binding poses and ligand−receptor interactions for **4a**−**b** and **6b**−**e** to the 5-HT_{2A}R. The ligands are displayed as sticks, while the receptor is shown as gray lines and cartoon. Ligand−receptor interactions are displayed as dashed lines and colored in green (aromatic, $\pi-\pi$ staking), yellow (hydrogen bond), and pink (salt-bridge).

mutated receptor were conducted relative to reference agonists, we cannot fully exclude that different expression levels of the wild-type and mutated receptor constructs may have some impact.

Taken together, from these results it is apparent that regardless of the benzylic substituent, the 4-bromo analogues and the 4-cyano analogues do not exhibit the same structure−activity relationship (SAR). In particular, this is highlighted in the clear difference in the relative preference exhibited by these two analogue series when it comes to βarr2 recruitment to the 5-HT_{2A}R (Figure 1 and Table 1). An exception to this is the fact that **4a** and **6e** display the same trend (β-factors of 1.240 and 1.250, respectively). Furthermore, the change of the benzylic hydroxy in the ortho-position in **6a**, to the meta-position in **6e**, resulted in a significant loss of both agonist potency and efficacy in both the βarr2 and mini$G\alpha_q$ recruitment assays. However, the efficacy was more significantly reduced in the mini$G\alpha_q$ assay, which resulted in a β-factor signifying a stronger preference toward βarr2 recruitment for **6e**. This suggests that, at least for the 4-cyano analogues, this interaction with $Ser159^{3\times36}$ is desired for the recruitment of mini$G\alpha_q$.[32,52]

Binding Mode Analysis. As an attempt to investigate a hypothesis of a direct interaction from the *N*-benzyl moiety to $Ser159^{3\times36}$ as a determinant of bias and provide structural explanations for the experimental results, compounds **4a**−**4d** and **6a**−**6f** were docked (Figures 5 and 6) into the cryo-EM structure of the human 5-HT_{2A}R bound to **6a** (25CN-NBOH) and coupled to a miniG_q/G_{i2}-G_s protein chimera.[52] As validation of the docking protocol, the highest ranking binding pose of **6a** displayed a root-mean-square deviation (RMSD) of 0.57 Å for all heavy atoms, in comparison to the experimental

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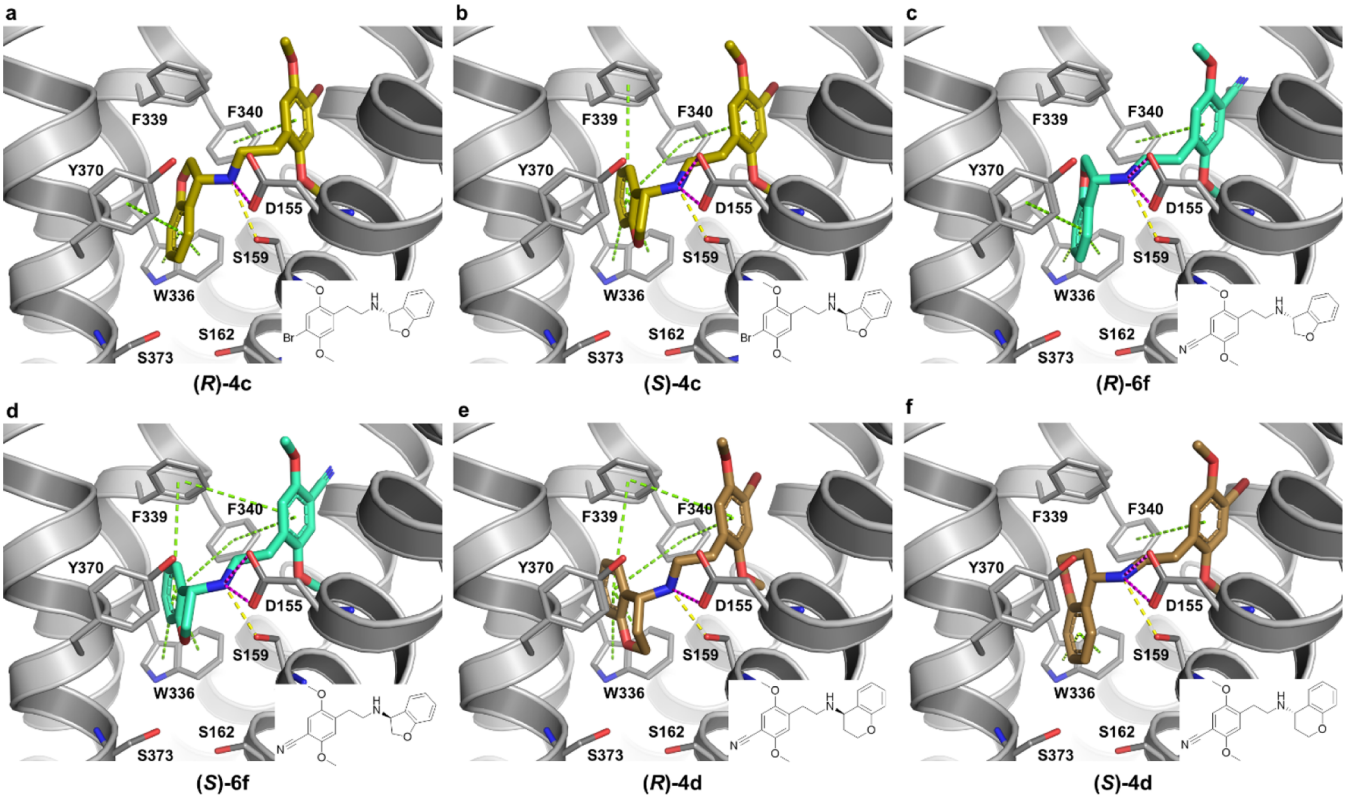

Figure 6. Predicted binding mode of both enantiomers of **4c**−**d** and **6f** to the 5-HT$_{2A}$R. A−F. Predicted binding poses and ligand−receptor interactions for the *S*- and *R*-isomers of dihydrobenzofuran (A−D) and chromane (E, F) substituted PEAs in the 5-HT$_{2A}$R. The receptor is shown as gray lines and cartoon, while ligands are displayed as sticks. Ligand−receptor interactions are displayed as dashed lines and colored in green (aromatic, $\pi-\pi$ staking), yellow (hydrogen bond), and pink (salt-bridge).

ligand coordinates and reproduced all major ligand−receptor interactions, i.e., the canonical salt-bridge to the Asp155$^{3\times32}$, two hydrogen bonds to Ser159$^{3\times36}$, and aromatic interactions to Trp336$^{6\times48}$, Phe339$^{6\times51}$, and Phe340$^{6\times52}$ (Figure 5A).[52] In general, the docking poses of the other compounds showed the same binding mode and interactions to the receptor as **6a**, with differences only in the pocket encompassing the *N*-benzyl moiety with differing substitution patterns (Figures 5A−G, 6A−F, and S7).

Focusing on Ser159$^{3\times36}$, only **6a** and **6b**, which both have oxygens in the ortho-position of the *N*-benzyl moiety (−OH or −OMe), establish the two hydrogen bonds to Ser159$^{3\times36}$ (Figure 5A,B). The fact that **6a** and **6b** display similar potencies in the two assays indicates that additional van der Waals interactions of the 2-methoxy of **6b** compensate for a weaker hydrogen bond relative to the 2-hydroxy substituent on the *N*-benzyl. While all other analogues, except **6c**, contain either a hydroxy group or an ether function, our docking poses of **4a**−**d** and **6d**−**f** do not show hydrogen bonds from the *N*-benzyl moiety to Ser159$^{3\times36}$ (Figures 5 and 6), corresponding to the lower potencies in both assays relative to **6a** and **6b** (Table 1). In fact, none of the three compounds that display significant bias (**4a**, **4b**, and **6e**—Figure 4) seem to form hydrogen bonds from the *N*-benzyl substituents to any of the surrounding receptor residues. The common trait between these three compounds is oxygen in the meta-position of the *N*-benzyl. While the docking poses showing placement of this functional group in a mainly hydrophobic receptor region (Figures 5b,c,g and S7) may explain the observed potency decreases (Table 1) compared to **6a** and **6b**, they do not provide a straightforward explanation for why the three compounds display bias. On the other hand, the meta-position points in the direction of Ser162$^{3\times39}$ and Ser373$^{7\times46}$, which could potentially change conformation when the ligands bind (induced-fit), something that the employed docking protocol does not account for. The substitution pattern of the *N*-benzyl may also impact the distribution of electron density of this aromatic ring and, thus, affect the interactions with the surrounding aromatic residues (Figures 5 and 6). Additionally, previous work has shown Leu362$^{7\times34}$ and Tyr370$^{7\times42}$ to play a role in bias.[43,53] However, Leu362$^{7\times34}$ is outside of contact distance for all docked compounds and while Tyr370$^{7\times42}$ does display aromatic interactions to the *N*-benzyl of some compounds, this is not consistent with whether they show bias or not, e.g., **4b** but not **4a** displays aromatic contact to Tyr370$^{7\times42}$ (Figure 5B,C).

While the importance of direct hydrogen bonds between **6a** and Ser159$^{3\times36}$ is supported by marked and similar drops in both β-arr2 and miniGα_q potencies (14- and 16-fold) and efficacies (36 and 53%) in the S159A-mutated 5-HT$_{2A}$R, the effect on the β-factor is remarkably subtle (0.619 vs 0.733, Tables 1 and 2). Since Ser159$^{3\times36}$ interacts with both the ammonium and the *N*-benzyl *ortho*-OH of **6a**, we cannot distinguish the influence of these two hydrogen bonds on the observed agonist potency and efficacy decreases. Regardless, this residue has little influence on β-arr2 vs miniGα_q bias for **6a**. Serotonin signaling is also markedly decreased by the S159A mutation in both assays (55-fold and 33% in β-arr2 plus 13-fold and 173% in miniGα_q), indicating that the removal of the interaction between Ser159$^{3\times36}$ and the protonated amine

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(Figure S8) is the root cause for this decrease. The differential effects by the S159A-mutated 5-HT_{2A}R on the two pathways observed for serotonin and the other compounds (excluding **6a**) may then be due to the lack of the additional hydrogen bond to $Ser159^{3\times36}$ seen in **6a**, which displays similar decreases. However, the effect of the S159A mutation on the β-factor for serotonin is again very small (0.576 vs 0.550—Tables 1 and 2). The only compound for which we have data showing a marked change in bias by the S159A mutation is **4b**, where the β-factor changes from 1.100 to 0.565. This does indicate that $Ser159^{3\times36}$ in combination with the *N*-benzyl substitution pattern in fact influences bias between β-arr2 and miniGα_q, but apparently not via a direct hydrogen bond to the *N*-benzyl substituent. A water-bridged interaction could potentially play a role, but such an analysis cannot be performed with the docking protocol used here.

Regardless of the docking failing to provide a detailed explanation for the differing β-factors and the fact that most compounds do not display bias between β-arr2 and miniGα_q, we can clearly see that the different *N*-benzyl substituents result in differential functional profiles in the β-arr2 and miniGα_q assays. Regarding **6b** as a reference (as it has similar potency and efficacy in the two assays), both potency and efficacy are in general higher in the β-arr2 vs the miniGα_q assay (Figure S9). Keeping in mind that the highest difference in potency between the two assays (for **6f**) only corresponds to a 6-fold change, this still indicates that the changes we made in the *N*-benzyl substitution in general have larger effects in the miniGα_q vs the β-arr2 assay, reflected in either potency and/or efficacy decrease. This demonstrates that alterations in the *N*-benzyl substitution pattern may be used to affect the preference between the two signaling pathways.

CONCLUSIONS

In summary, 10 5-HT_{2A}R ligands, based on the 5-HT_{2A}R selective agonist **6a** (25CN-NBOH), were successfully designed and synthesized, with the aims of delineating their functional selectivity profiles in assays for Gq- and βarr2-mediated 5-HT_{2A}R signaling and to evaluate the role of the hydrogen interaction of **6a** with $Ser159^{3\times36}$ in the receptor. The ligands were functionally characterized at 5-HT_{2A}R in the βarr2- and miniGα_q-recruitment assays. Compounds **4a**−**d**, **6c**, and **6e**−**f** lacked the possibility for simultaneous interaction of the ammonium and the *ortho*-oxygen on the benzyl moiety with $Ser159^{3\times36}$. The lack of interaction between the hydroxy and $Ser159^{3\times36}$ resulted in detrimental effects for both potency and efficacy, as assessed by βarr2 and miniGα_q recruitment assays. Remarkably, G_q-mediated signaling was considerably more affected by the compounds' lack of the ortho-hydrogen bond acceptor. The exact reasons for this observation could not be identified computationally, as the precise effect of the interaction of the benzylic hydroxyl and the interaction of the ammonium with $Ser159^{3\times36}$ could not be distinguished.

Regardless of the docking not being able to provide a detailed explanation for the differing β-factors and the fact that most compounds do not display bias between βarr2 and miniGα_q, we can clearly see that the different *N*-benzyl substituents result in differential functional profiles in the βarr2 and miniGα_q assays. Keeping in mind that the highest difference in potency between the two assays (for **6f**) only corresponds to a 6-fold change, this still indicates that the changes we made in the *N*-benzyl substitution in general have larger effects in the miniGα_q vs the βarr2 assay, reflected in either potency and/or efficacy decrease. This demonstrates that alterations in the *N*-benzyl substitution pattern can be used to affect the preference between the two signaling pathways. Overall, these insights led to the development of **4a-b** and **6e-f**, the first efficacious 5-HT_{2A}R agonists to be βarr2-biased, relative to LSD. Of special highlight is compound **4a** with potency and efficacy of 11.1 nM and 112%, respectively, for βarr2 recruitment, while in the miniGα_q-recruitment assay, **4a** had potency and efficacy of 48.8 nM and 28.0%, respectively, as referenced by LSD. Compound (±)−**6f** showed potency and efficacy of 108 nM and 82.9%, respectively, for βarr2, while in the miniGα_q-recruitment assay, compound (±)−**6f** exhibited potency and efficacy of 631 nM and 18.0%, respectively, as referenced by LSD. Therefore, **4a** and **6f** are interesting tool compounds to use for further evaluation of the role of signaling bias at the 5-HT_{2A}R.

EXPERIMENTAL SECTION

Organic Chemistry. All reactions involving dry solvents or sensitive agents were performed under a nitrogen atmosphere and glassware was dried prior to use. Commercially available chemicals were used without further purification. Solvents were dried prior to use with an SG water solvent purification system or dried by standard procedures, and reactions were monitored by analytical thin-layer chromatography (TLC, Merck silica gel 60 F_{254} aluminum sheets). Flash chromatography was carried out using Merck silica gel 60A (35−70 μm). ^{1}H NMR spectra were recorded on a 400 MHz Bruker Avance III or 600 MHz Bruker Avance III HD, and ^{13}C NMR spectra on a 101 MHz Bruker Avance III or 151 MHz Bruker Avance III HD. Analytical high-performance liquid chromatography (HPLC) was performed using an UltiMate HPLC system consisting of an LPG-3400A pump (1 mL/min), a WPS-3000SL autosampler, and a 3000 Diode Array Detector installed with a Gemini-NX C18 (250 mm × 4.60 mm, 3 μm) column. Solvent A: H_2O + 0.1% trifluoroacetic acid (TFA); Solvent B: MeCN-H_2O 9:1 + 0.1% TFA. For HPLC control, data collection, and data handling, Chromeleon software v. 6.80 was used. Ultrahigh-pressure liquid chromatography-mass spectrometry (UPLC-MS) spectra were recorded using an Acquity UPLC H-Class Waters series solvent delivery system equipped with an autoinjector coupled to an Acquity QDa and TUV detectors installed with an Acquity UPLCBEH C18 (50 mm × 2.1 mm, 1.7 μm) column. Solvent A: 5% aq MeCN + 0.1% HCO_2H: Solvent B: MeCN + 0.1% HCO_2H. Usually, gradients from A:B 1:0 to 1:1 (5 min) or A:B 1:0 to 0−50 (5 min) were performed depending on the polarity of the compounds. For data collection and data handling, MassLynx software was used. Optical rotations were determined in a thermostated cuvette on an Anton Paar MCP300 Modular Circular Polarimeter. Compounds were dried under high vacuum or freeze-dried using a ScanVac Cool Safe Freeze Drier. The purity of compounds submitted for pharmacological characterization was determined to be >95%, by HPLC analysis.

General Procedure (A) for the Synthesis of Secondary Amines. The aldehyde (1.1 equiv) was added to a suspension of the phenethylamine hydrochloride (1 equiv) and Et_3N (1.0 equiv) in EtOH. The reaction mixture was stirred until the formation of the imine was complete (30 min—3 h). After the addition of $NaBH_4$ (2.0 equiv), the mixture was stirred for 45 min and concentrated under reduced pressure. The residue was partitioned in CH_2Cl_2/H_2O (1:1 v/v), and the aqueous phase was further extracted with CH_2Cl_2 (2×). The organic layers were combined, dried over $NaSO_4$, filtered, and evaporated under reduced pressure. The secondary amine product was purified by column chromatography (CH_2Cl_2/MeOH/Et_3N, 98.2:1.4 + 0.24%) and precipitated by the addition of 4 M HCl in dioxane (1.5 equiv) under continuous stirring. The solid was filtered, dried under reduced pressure, dissolved in a minimum amount of MeOH, and precipitated by the addition of Et_2O. The product was collected by filtration and dried under high vacuum.

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General Procedure (B) for the Synthesis of Conformational Constrained Derivatives. Glacial acetic acid (3.0 equiv) was added to a suspension of the targeted amine hydrochloride (1.0 equiv) in methanol/THF (2:1 v/v). 4-Chromanone (2.5 equiv) or 3-coumaranone (3 equiv) was added, and the reaction mixture was stirred at room temperature until the formation of the corresponding imine was complete based on TLC (CH_2Cl_2/MeOH/Et_3N, 98.2:1.4 + 0.24%). $NaBH_3CN$ (in THF) (1.0 M, 3.0 equiv) was added and the reaction mixture was monitored by TLC and stirred for 30 min to 3 h. The mixture was quenched by the addition of $NaHCO_{3(aq)}$, and the residue was extracted with EtOAc (3×). The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The secondary amine product was purified by column chromatography (CH_2Cl_2/MeOH/Et_3N, 98.2:1.4 + 0.4%) and precipitated by the addition of 4 M HCl in dioxane (1.5 equiv) under continuous stirring. The solid was filtered, dried under reduced pressure, dissolved in a minimum amount of MeOH, and precipitated by the addition of Et_2O. The product was collected by filtration and dried under high vacuum.

*2-(2,5-Dimethoxyphenyl)ethan-1-amine Hydrochloride (**2**).* The title compound was prepared according to reported conditions.[10] Characterization was in accordance with reported values.[54]

*2-(4-Bromo-2,5-dimethoxyphenyl)ethan-1-amine Hydrochloride (**3**).* The title compound was prepared according to reported conditions.[10] Characterization was in accordance with reported values.[54]

*3-(((4-Bromo-2,5-dimethoxyphenethyl)amino)methyl)phenol Hydrochloride (**4a**).* The title compound was prepared according to General procedure A and in line with reported conditions, and the characterization was in accordance with reported values.[45]

*2-(4-Bromo-2,5-dimethoxyphenyl)-N-(3-methoxybenzyl)ethan-1-amine Hydrochloride (**4b**).* The title compound was prepared according to General procedure A and in line with reported conditions, and the characterization was in accordance with reported values.[45]

*(±)-N-(4-Bromo-2,5-dimethoxyphenethyl)-2,3-dihydrobenzofuran-3-amine Hydrochloride (**4c**).* The title compound was prepared according to General procedure B, which yielded the desired compound as a white solid in 52%. LCMS (ESI) m/z = 378.1 $[M + H]^+$; ^{1}H NMR (600 MHz, DMSO) δ 9.28 (s, 2H), 7.64 (d, J = 7.5 Hz, 1H), 7.38 (td, J = 7.8, 1.4 Hz, 1H), 7.22 (s, 1H), 7.04−6.99 (m, 2H), 6.99−6.96 (m, 1H), 5.10 (s, 1H), 4.75 (dd, J = 11.5, 2.7 Hz, 1H), 4.65 (dd, J = 11.4, 7.9 Hz, 1H), 3.80 (s, 4H), 3.76 (s, 4H), 3.14 (s, 2H), 2.95−2.84 (m, 2H). ^{13}C NMR (151 MHz, DMSO) δ 160.7, 151.5, 149.4, 131.7, 127.2, 125.1, 121.2, 121.0, 115.9, 115.1, 110.4, 109.1, 58.0, 56.7, 56.3, 56.2, 43.5, 26.7.

*(±)-N-(4-Bromo-2,5-dimethoxyphenethyl)chroman-4-amine Hydrochloride (**4d**).* The title compound was prepared according to General procedure B, which yielded the desired compound as a white solid in 58%. LCMS (ESI) m/z = 392.1 $[M + H]^+$; ^{1}H NMR (600 MHz, DMSO) δ 9.06 (s, 2H), 7.51 (d, J = 7.5 Hz, 1H), 7.32 (t, J = 7.7 Hz, 1H), 7.23 (s, 1H), 7.02 (s, 1H), 6.98 (t, J = 7.4 Hz, 1H), 6.89 (d, J = 8.2 Hz, 1H), 4.55 (s, 1H), 4.39−4.21 (m, 2H), 3.81 (s, 3H), 3.78 (s, 3H), 3.28−3.13 (m, 2H), 2.97 (dtd, J = 44.2, 12.4, 11.9, 5.4 Hz, 2H), 2.36−2.29 (m, 1H), 2.28−2.18 (m, 1H). ^{13}C NMR (151 MHz, DMSO) δ 154.9, 151.5, 149.4, 130.7, 130.6, 125.3, 120.2, 117.2, 116.6, 115.9, 115.0, 109.1, 61.2, 56.7, 56.3, 50.2, 43.7, 26.5, 23.7.

*4-(2-Aminoethyl)-2,5-dimethoxybenzonitrile Hydrochloride (**5**).* The title compound was prepared according to reported conditions, and the characterization was in accordance with reported values.[46]

*4-(2-((2-Hydroxybenzyl)amino)ethyl)-2,5-dimethoxybenzonitrile Hydrochloride (**6a**).* The title compound was prepared according to reported conditions, and the characterization was in accordance with reported values.[32]

*2,5-Dimethoxy-4-(2-((2-methoxybenzyl)amino)ethyl)-benzonitrile Hydrochloride (**6b**).* The title compound was prepared according to reported conditions, and the characterization was in accordance with reported values.[32]

*4-(2-((2-Fluorobenzyl)amino)ethyl)-2,5-dimethoxybenzonitrile Hydrochloride (**6c**).* The title compound was prepared according to reported conditions, and the characterization was in accordance with reported values.[32]

*4-(2-((Benzo[d][1,3]dioxol-4-ylmethyl)amino)ethyl)-2,5-dimethoxybenzonitrile Hydrochloride (**6d**).* The title compound was prepared according to reported conditions, and the characterization was in accordance with reported values.[32]

*4-(2-((3-Hydroxybenzyl)amino)ethyl)-2,5-dimethoxybenzonitrile Hydrochloride (**6e**).* The title compound was prepared according to General procedure A and in line with reported conditions, and the characterization was in accordance with reported values.[45]

*(±)-4-(2-((2,3-Dihydrobenzofuran-3-yl)amino)ethyl)-2,5-dimethoxybenzonitrile Hydrochloride (**6f**).* The title compound was prepared according to General procedure B, which yielded the desired compound as a white solid in 55%. LCMS (ESI) m/z = 325.2 $[M + H]^+$; ^{1}H NMR (600 MHz, DMSO) δ 9.33 (s, 2H), 7.64 (d, J = 7.5 Hz, 1H), 7.38 (d, J = 6.2 Hz, 2H), 7.14 (s, 1H), 7.02 (t, J = 7.4 Hz, 1H), 6.98 (d, J = 8.1 Hz, 1H), 5.10 (s, 1H), 4.80−4.72 (m, 1H), 4.65 (dd, J = 11.4, 7.9 Hz, 1H), 3.88 (s, 3H), 3.79 (s, 3H), 3.18 (s, 2H), 3.04−2.91 (m, 2H). ^{13}C NMR (151 MHz, DMSO) δ 160.7, 155.2, 150.9, 132.6, 131.7, 127.2, 121.0, 116.3, 115.0, 114.7, 110.4, 98.6, 72.3, 58.1, 56.6, 56.3, 43.2, 27.2.

Pharmacology. *Cell Culture and Transfection.* The potency and efficacy of the synthesized substances are assessed by means of two distinct yet highly analogous bioassays, monitoring the recruitment of either β-arrestin 2 (βarr2) or miniGα_q to the activated target receptor (5-HT_{2A}R). Essentially, the experimental procedures are carried out as described before, employing transiently transfected cells.[40−42] Human embryonic kidney (HEK) 293T cells are maintained in Dulbecco's modified Eagle's medium (DMEM) (supplemented with GlutaMAX), containing 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL of penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. The cells are routinely cultured and incubated at 37 °C, in a humidified atmosphere containing 5% CO_2. To quantify the activity of the ligands at the 5-HT_{2A}R, the cells are transfected with the receptor construct (either the wild type or the S159A mutated 5-HT_{2A}R fused to the LgBiT component of the NanoBiT system) and either SmBiT-βarr2 or SmBiT-miniGα_q. To this end, the cells are seeded in six-well plates at a density of 500 000 cells per well. After 24 h, a transfection mixture is prepared consisting of a total of 3.3 μg of plasmid DNA and FuGENE HD transfection reagent, in a 3:1 FuGENE:DNA ratio, in OptiMEM I Reduced Serum Medium, incubated for 10 min, and added to the cells, according to the manufacturer's protocol.

Assay Protocol. After overnight incubation of the transfected cells, the cells are reseeded in poly-D-lysine coated 96-well plates at a density of 50 000 cells per well. Following an additional 24 h incubation (in total, 48 h after transfection), the assay is started by rinsing the cells twice with 150 μL of Hank's Balanced Salt Solution (HBSS) and adding 100 μL of HBSS to each well. To this, 25 μL of NanoGlo Live Cell Reagent is added (diluted 1/20 in LCS Dilution Buffer, according to the manufacturer's protocol) and the plate is transferred to the Tristar2LB 942 multimode microplate reader (Berthold Technologies GmbH & Co, Germany), where the luminescent signal is measured during an equilibration phase. Upon signal stabilization, 10 μL of the 13.5 × concentrated agonist solutions is added to the wells—obtaining in-well concentrations of 25 μM−10 μM−1 μM−100 nM−10 nM−1 nM−100 pM−10 pM−1 pM, and the luminescence is monitored for 2 h. For each condition, the appropriate solvent controls are included. Each substance is tested in duplicate in at least three independent experiments, and reference substances LSD and serotonin are included in every experiment. For optimal comparability, the two assays are performed immediately after one another, using the same dilutions.

Cloning of the S159A-Mutated Receptor via Site-Directed Mutagenesis (SDM). To assess the influence of residue S159 on the potency and efficacy of a selected subset of the substances, an S159A mutated 5-HT_{2A}R was generated using a Phusion Site-Directed Mutagenesis kit, according to the manufacturer's protocol. In brief, 200 pg of the template DNA (5-HT_{2A}R-LgBiT) was mixed with the provided Phusion High Fidelity Mastermix and 0.5 μM of the

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forward primer (GTGCTCTTCGCCACGGCCTCCATCATGC) and reverse primer (GTCCAGGTAAATCCAGACTGCACAAAGCTTGC). The three-step polymerase chain reaction (PCR) was performed in a Mastercycler Nexus Thermal Cycler (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation (98 °C, 30 s), denaturation (98 °C, 10 s), annealing (71 °C, 20 s), extension (72 °C, 150 s), and final extension (72 °C, 5 min), of which the middle three steps were repeated 25 times. Following gel electrophoresis and purification, the linear product was religated with the provided T4 DNA ligase in the rapid ligation buffer and transformed into chemically competent *Escherichia coli* bacteria. After plasmid purification using the E.Z.N.A. Plasmid DNA Mini Kit (VWR International), the correctness of the construct was verified via Sanger sequencing.

Data Analysis. The resulting data were analyzed as described before in more detail.[55] In brief, the obtained time−luminescence profiles are corrected for interwell variability and used for the calculation of the area under the curve (AUC), from which the AUC of the corresponding solvent control is subtracted. Data are then normalized using GraphPad Prism software (San Diego, CA), where the maximal response of the reference agonist is arbitrarily set at 100%. After pooling the data of the individual experiments, the potency and efficacy values are calculated in GraphPad Prism through three parametric nonlinear regression analysis. To quantify the tendency of the measured substances toward preferentially inducing one pathway or the other, bias factors are calculated via the "intrinsic relative activity approach.[56,57] In this approach, an RA_i value is calculated for each substance in each of the measured assays, relative to a reference agonist, using the following formula

$$RA_{i,\text{reference agonist}}^{\text{pathway}} = \frac{\frac{E_{\max,i}}{EC_{50,i}}}{\frac{E_{\max,\text{REF}}}{EC_{50,\text{REF}}}} = \frac{EC_{50,\text{REF}} \times E_{\max,i}}{E_{\max,\text{REF}} \times EC_{50,i}}$$

The obtained values for the respective pathways are then combined into a bias factor, β_i

$$\beta_i = \log\left(\frac{RA_{i,\text{REF}}^{\beta\text{arr2}}}{RA_{i,\text{REF}}^{\text{miniG}\alpha\text{q}}}\right)$$

This formula implies that the value of β_i for the reference agonist is 0. A positive bias factor indicates a preference toward the recruitment of βarr2 over miniGα_q, compared to the respective reference agonist. A negative bias factor then points to a relative preference toward the recruitment of miniGα_q over βarr2. To assess whether the obtained bias factors are statistically significant from 0, a Kruskal−Wallis analysis (which is the nonparametric counterpart of one-way analysis of variance (ANOVA), selected *a priori* to avoid presumptuous conclusions) with post hoc Dunn's multiple comparison was carried out in GraphPad Prism. To qualitatively visualize the possible preference of a certain substance towards recruiting either one cytosolic protein or the other, bias plots were generated via GraphPad Prism. To this end, the normalized AUC values obtained in the βarr2 assay are plotted on the *x*-axis, and those obtained in the miniGα_q assay are plotted on the *y*-axis. On each plot, both the respective reference agonist and one substance of interest are plotted, and a curve is fitted through the centered second-order (quadratic) polynomial fitting.[58]

Computational Methods. All molecular modeling calculations were performed in the Schrödinger Drug Discovery Suite (Release 2021−4, Schrödinger LLC, New York, NY, 2021). The ligands (**4a−d**, **6a−f**, and serotonin) were sketched in Maestro with the two-dimensional (2D) Sketcher tools, then the three-dimensional (3D) coordinates, charges, ionization states at pH 7.0 ± 2.0, and minimized conformations were generated with LigPrep using the default settings and the OPLS4 force field.[59] For the ligands with multiple protonation states at physiological pH, only the state with a positive charge in the amino group (and a total charge of +1.0) was kept, as the salt-bridge interaction between the positive amine Asp155$^{3\times32}$ is crucial for ligand binding.[44]

The cryo-EM structure of 5-HT$_{2A}$R bound to 25CN-NBOH and in complex to a mini-Gα_q protein chimera (accession code 6WHA)[52] and the crystallographic structure of the LSD-bound 5-HT$_{2A}$R (accession code 6WGT)[52] were imported from PDB. For the cryo-EM structure, the coordinates of the G-protein and other auxiliary proteins were deleted, while for the crystallographic structure, only one protein chain (chain A) was kept. The 5-HT$_{2A}$R structures were then prepared using Schrodinger's Protein Preparation Wizard[60] to add hydrogens, create disulfide bonds, generate protonation states for non-protein components using Epik v5.8[61] at pH 7.0 ± 2.0, and complete missing side chains using Prime.[62,63] For the bound ligands, 25CN-NBOH and LSD, the protonation state with the positive charge in the amine group was selected. The hydrogen-bond network of the protein was optimized with ProPKA[64,65] at pH 7.0 and using ProtAssign[60] to automatically optimize Asn, Gln, His, and hydroxyl side chains. This optimization was followed by two cycles of restrained minimization in the OPLS4 force field and with heavy atom convergence RMSD of 0.30 Å for each cycle, using Impact v9.3.[66]

The prepared 5-HT$_{2A}$R structures were used to generate the docking grids. The grids were centered around the experimental ligand (25CN-NBOH or LSD), with no van der Waals scaling factor applied to receptor atoms. The side chains of Ser159$^{3\times36}$ Thr160$^{3\times37}$, Ser239$^{5\times44}$, Ser242$^{5\times46}$, and Tyr370$^{7\times42}$ were allowed to rotate. No additional constraints were applied, and other settings were kept in default values. The ligands **4a−d** and **6a−f** were docked in the cryo-EM structure of 5-HT$_{2A}$R bound to 25CN-NBOH, while serotonin was docked in the crystallographic structure of the LSD-bound 5-HT$_{2A}$R. The dockings were performed in Glide v9.3[67,68] in extra precision mode and the OPLS4 force field.[69] The van der Waals radii of ligand atoms were not scaled, as the docking involved a congeneric series to the experimental ligand. The sampling of nitrogen inversions, ring conformations, and the use of enhanced planarity for conjugated π groups was allowed. Five docking poses were written per ligand, followed by a post-docking optimization with a rejection threshold of 0.50 kcal/mol with the application of strain correction. All other settings were kept in the default values, while docking poses were selected based on the lowest docking score and lowest RMSD to the experimentally bound ligand.

Ligand−receptor interaction and structural interaction fingerprints (SIFt) were calculated with the Pymol plugin Intermezzo (v1.2, Ochoa, et al., unpublished, available at http://mordred.bioc.cam.ac.uk/intermezzo), with a binding pocket definition comprising the residues within 5.0 Å of 25CN-NBOH (or LSD) in the docking template structure. PyMOL (The PyMOL Molecular Graphics System, Schrödinger LLC, New York, 2020) was also used to generate the figures. The GPCRdb numbering scheme was used to assign the generic residue numbers throughout the text and figures.[70]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.2c00702.

Concentration−response curves of each individual compound, for both βarr2 and miniGα_q; functional properties of the tested compounds, with serotonin as the reference agonist, the bias plots and Kruskal−Wallis analysis, with serotonin as the reference agonist; additional computational data and HPLC traces of a representative number of tested compounds (PDF)

CSV file of the docking models used in Figures 5 and 6 (CSV)

PDB file of the docking models used in Figure 5 (PDB)

PDB file of the docking models used in Figure 6 (PDB)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

5-HT_{2A}R, serotonin 2A receptor; βarr2, β-arrestin 2; 25CN-NBOH, 4-(2-((2-hydroxybenzyl)amino)ethyl)-2,5-dimethoxybenzonitrile; LSD, lysergic acid diethylamide; NBOMe, *N*-benzylphenethylamines

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Supporting Information

Discovery of β-Arrestin-Biased 25CN-NBOH-Derived 5-HT_{2A} Receptor Agonists

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Concentration-Response curves of individual compounds (referenced with LSD)

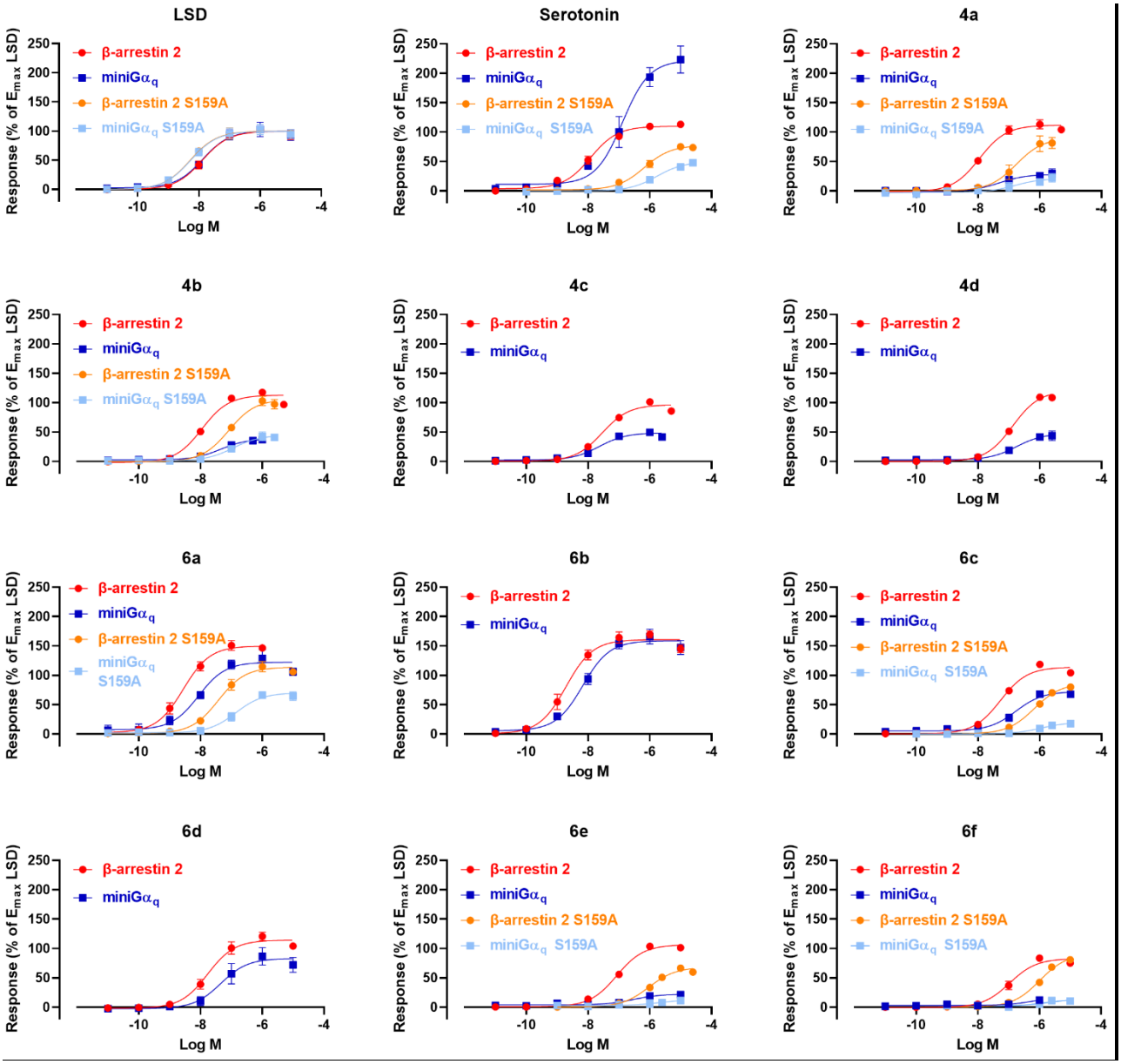


Figure S1. Concentration−response curves of the tested compounds (**4a–d**, and **6a–f**) in the 5-HT_{2A}R βarr2 and miniGα_q recruitment assays, with LSD as a reference agonist and concentration−response curves of the tested compounds (**4a–b**, and **6a, c, e–f**) in the S159A mutated 5-HT_{2A}R βarr2 and miniGα_q recruitment assays, with LSD as a reference agonist. Overlay of the concentration-response curves for each of the tested substances in the two assay formats. Each point represents the mean of three independent experiments, each performed in duplicate ± SEM (standard error of the mean). Curves represent three parametric, non-linear fits.

Concentration-Response curves of individual compounds (referenced with serotonin (5-HT))

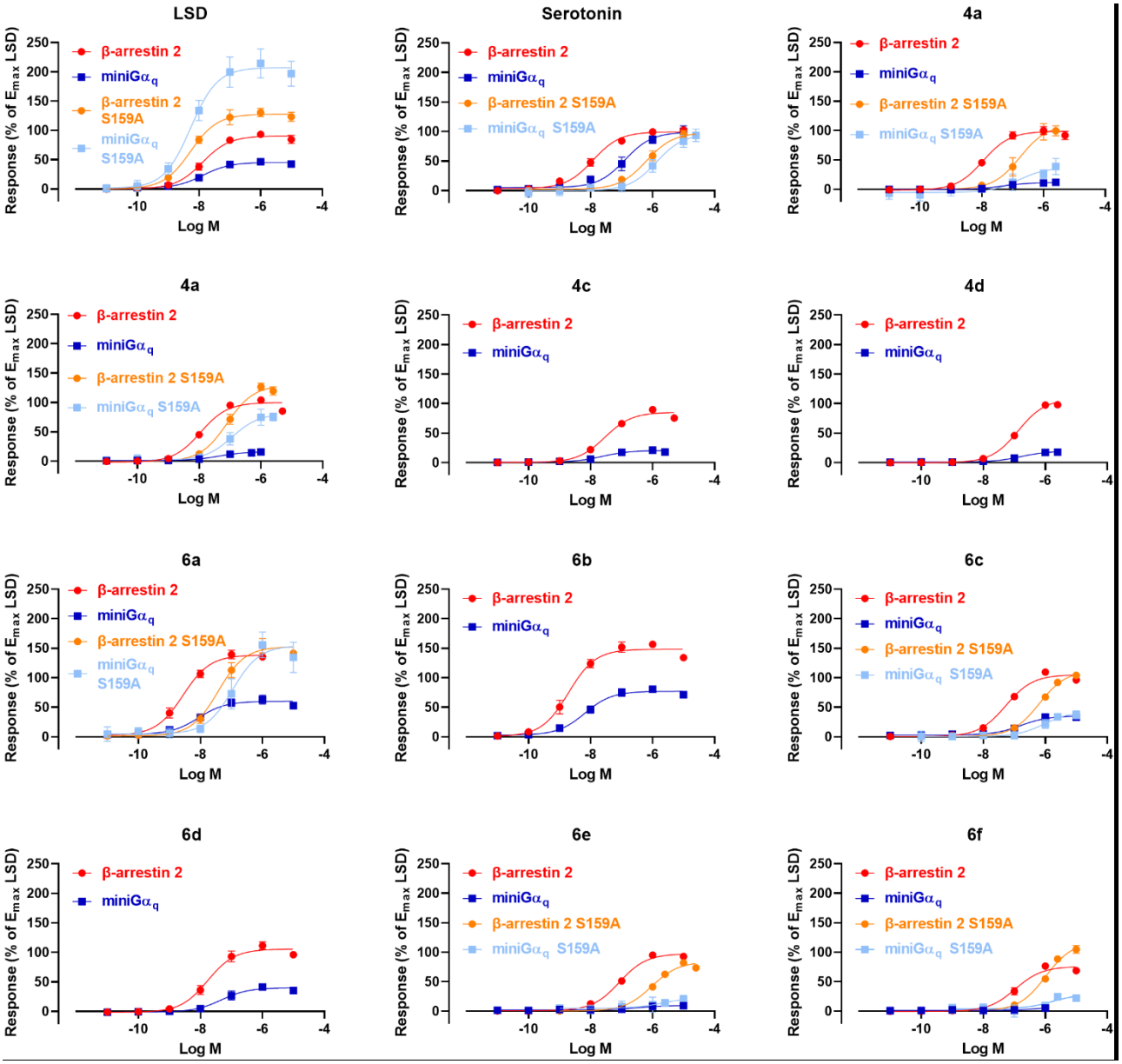


Figure S2. Concentration−response curves of the tested compounds (**4a–d**, and **6a–f**) in the 5-HT_{2A}R βarr2 and miniGα_q recruitment assays, with serotonin as a reference agonist and concentration−response curves of the tested compounds (**4a–b**, and **6a, c, e–f**) in the S159A mutated 5-HT_{2A}R βarr2 and miniGα_q recruitment assays, with serotonin as a reference agonist. Overlay of the concentration-response curves for each of the tested substances in the two assay formats. Each point represents the mean of three independent experiments, each performed in duplicate ± SEM (standard error of the mean). Curves represent three parametric, non-linear fits.

Functional properties of the tested compounds, with serotonin (5-HT) as the reference agonist

Table S1. Functional properties of the tested compounds (**4a–d**, and **6a–f**) on the 5-HT_{2A}R, and (**4a–b** and **6a, c, e–f**) on the S159A mutated 5-HT_{2A}R, in a βarr2 or mini$G\alpha_q$ recruitment assays, with serotonin as a reference agonist.[a]

5-HT_{2A}	**βarr2**		**mini$G\alpha_q$**		
	EC_{50} (nM) [CI]	**E_{max} (%) [CI]**	**EC_{50} (nM) [CI]**	**E_{max} (%) [CI]**	**β-factor**
5-HT	12.0 [8.41 - 17.1]	99.6 [95.1 – 104]	129 [64.9 – 258]	99.2 [88.4 – 111]	0
LSD	12.9 [8.45 - 19.9]	90.7 [85.0 - 96.4]	13.4 [6.82 - 26.5]	45.4 [41.1 - 49.9]	-0.718
4a	11.1 [7.45 - 16.4]	98.9 [92.8 – 105]	46.9 [11.9 – 158]	11.7 [9.10 - 14.6]	0.530
4b	11.1 [7.29 - 16.8]	100 [93.3 – 107]	43.3 [16.7 – 102]	16.2 [14.0 - 18.8]	0.386
(±)-4c	28.7 [19.5 - 42.4]	85.5 [80.2 - 90.9]	24.5 [12.1 - 50.1]	20.7 [18.6 - 22.9]	-0.431
(±)-4d	134 [111 – 161]	108 [104 – 113]	168 [78.7 – 402]	19.8 [16.8 - 23.7]	-0.152
6a (25CN-NBOH)	2.76 [1.78 - 4.31]	139 [130 – 147]	8.66 [3.26 - 21.5]	60.1 [52.7 - 67.8]	-0.126
6b (25CN-NBOMe)	1.94 [1.19 - 3.22]	148 [140 – 157]	6.53 [3.56 - 11.5]	77.1 [71.4 - 82.9]	-0.221
6c (25CN-NBF)	53.3 [37.3 - 75.0]	105 [99.2 – 111]	164 [61.0 – 437]	35.3 [29.5 - 41.7]	-0.077
6d (25CN-NBMD)	17.1 [10.5 - 28.0]	106 [97.9 – 114]	47.3 [17.7 – 117]	40.1 [33.4 - 47.3]	-0.107
6e	84.5 [64.0 – 111]	97.5 [92.9 – 102]	299 [43.9 - 1819]	9.75 [7.05 - 13.2]	0.548
(±)-6f	108 [68.6 – 169]	76.0 [69.5 - 82.6]	608 [n.d.]	7.72 [n.d.]	n.d.
5-HT_{2A}-S159A	**βarr2**		**mini$G\alpha_q$**		
	EC_{50} (nM) [CI]	**E_{max} (%) [CI]**	**EC_{50} (nM) [CI]**	**E_{max} (%) [CI]**	**β-factor**
5-HT	629 [385-995]	98.2 [90.6-106]	1317 [516-3745]	96.3 [79.5-118]	0
LSD	5.26 [3.21-8.44]	128 [120-136]	5.35 [2.32-11.7]	207 [186-229]	-0.533
4a	175 [82.3-411]	110 [94.4-130]	156 [n.d.]	36.8 [n.d.]	n.d.
4b	80.4 [55.1-116]	130 [121-139]	118 [44.3-332]	81.0 [67.5-97.1]	0.102
6a (25CN-NBOH)	37.3 [22.9-59.9]	153 [141-165]	109 [39.5-279]	155 [126-185]	0.187
6c (25CN-NBF)	645 [508-811]	113 [107-119]	899 [184-3332]	42.7 [29.3-63.7]	0.186
6e	938 [624-1364]	84.1 [77.4-91.2]	2060 [n.d.]	22.9 [n.d.]	n.d.
(±)-6f	1031 [695-1491]	118 [107-130]	1652 [n.d.]	28.9 [n.d.]	n.d.

[a] Data obtained in the βarr2 or mini$G\alpha_q$ recruitment assays, using the 2h time-luminescence profile to calculate the AUC. The EC_{50} value is a measure of agonist potency, and the E_{max} value is a measure of agonist efficacy. The E_{max} values for the compounds are normalized to E_{max} of serotonin as the reference agonist. Data are combined from at least three independent experiments, each performed in duplicate. The reported β-factor is the average value of the three β-factors obtained in three independent experiments. n.d. is not determined. CI: 95% confidence interval.

Table S2: Alternative method of calculation of the bias β-factor.[a]

5-HT$_{2A}$	Referenced with LSD		Referenced with 5-HT	
	β-factor	**Alternative β-factor**	**β-factor**	**Alternative β-factor**
4a	1.240	1.240	0.530	0.520
4b	1.100	1.060	0.386	0.348
4c	0.279	0.194	-0.431	-0.486
4d	0.558	0.516	-0.152	-0.198
6a (25CN-NBOH)	0.619	0.572	-0.126	-0.172
6b (25CN-NBOMe)	0.526	0.538	-0.221	-0.223
6c (25CN-NBF)	0.669	0.687	-0.077	-0.072
6d	0.638	0.553	-0.107	-0.169
6e	1.250	1.220	0.548	0.516
6f	n.d.	1.420	n.d.	0.711
5-HT$_{2A}$-S159A	**Referenced with LSD**		**Referenced with 5-HT**	
	β-factor	**Alternative β-factor**	**β-factor**	**Alternative β-factor**
4a	n.d.	0.566	n.d.	0.096
4b	0.565	0.494	0.102	0.043
6a (25CN-NBOH)	0.733	0.753	0.187	0.131
6c (25CN-NBF)	0.731	0.842	0.186	0.237
6e	n.d.	1.050	n.d.	0.577
6f	n.d.	1.010	n.d.	0.486

[a] Both β-factors are obtained via the formulas specified in the Materials and Methods section. The column 'β-factor' is the same as that reported in Table 1 and Table S1, and is the average of the β-factors calculated in each individual experiment. The column 'Alternative β-factor' is obtained by applying the same formulas on the 'combined' EC_{50} and E_{max} values, as reported in Table 1, 2 and Table S1. n.d. is not determined.

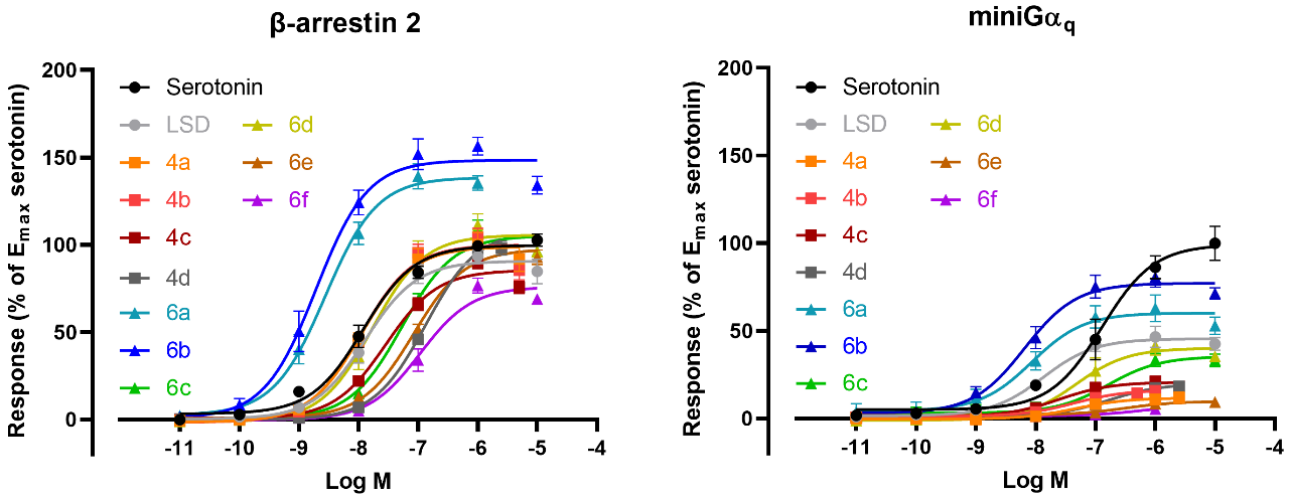


Figure S3. Concentration−response curves of the tested compounds (**4a–d**, and **6a–f**) in the 5-HT$_{2A}$R βarr2 or miniGα$_q$ recruitment assays, with serotonin as a reference agonist. Overlay of the concentration-response curves for each of the tested substances in the two assay formats. Each point represents the mean of three independent experiments, each performed in duplicate ± SEM (standard error of the mean). Curves represent three parametric, non-linear fits.

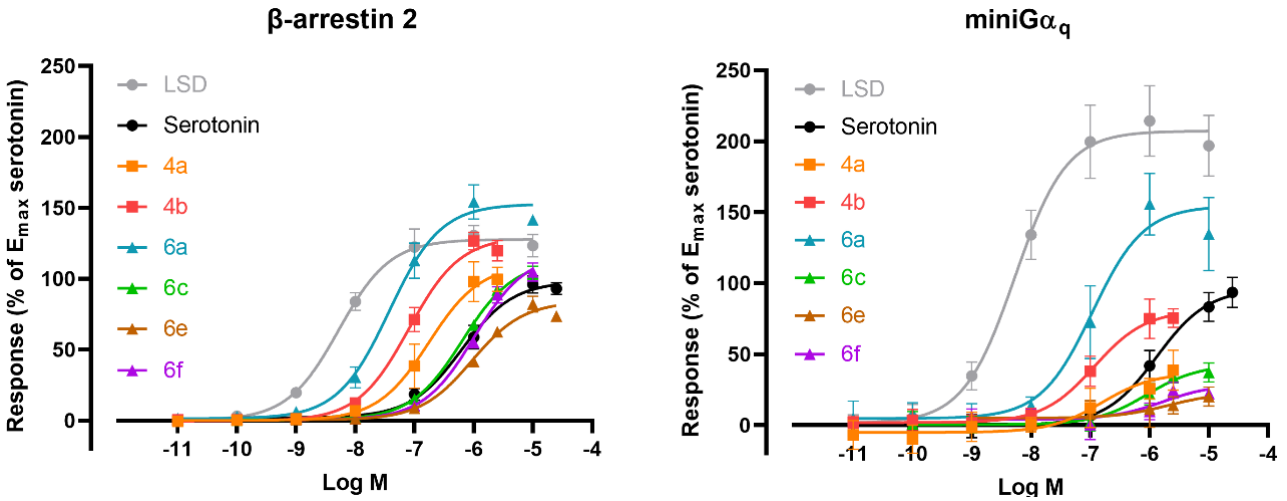


Figure S4. Concentration–response curves of the tested compounds (**4a–b**, and **6a, c, e–f**) in the S159A mutated 5-HT_{2A} R receptor βarr2 or mini$G\alpha_q$ recruitment assays, with serotonin as a reference agonist. Overlay of the concentration-response curves for each of the tested substances in the two assay formats. Each point represents the mean of three independent experiments, each performed in duplicate ± SEM (standard error of the mean). Curves represent three parametric, non-linear fits.

Bias plots and Kruskal-Wallis analysis, with serotonin (5-HT) as the reference agonist

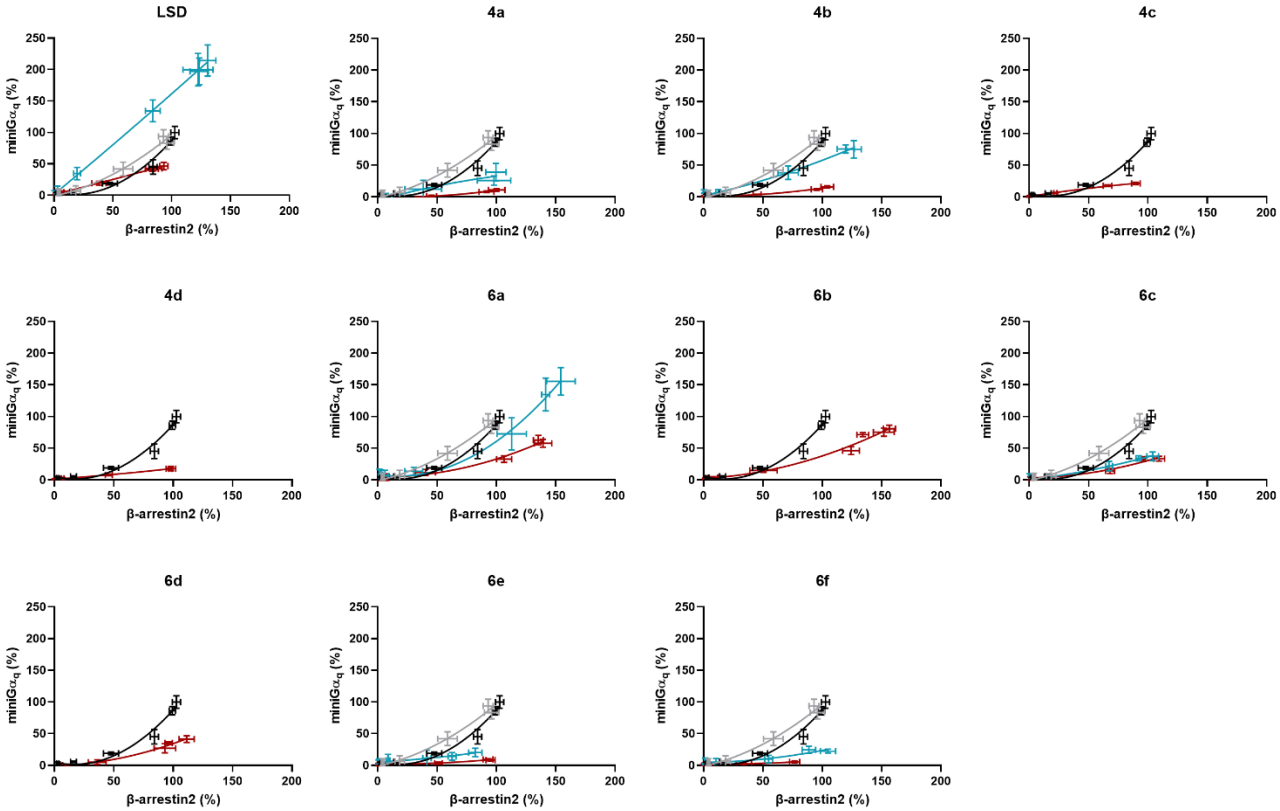


Figure S5. Qualitative bias plots, where each panel shows the centered second order polynomial fit of the activation values at equimolar concentrations of the substance in the respective assays in red, and that of the reference agonist (serotonin) in black for WT receptor and gray is reference (serotonin) data for the S159A mutated receptor. Red is data for WT receptor and blue is data for the S159A mutated receptor. Error bars represent the SEM of the individual data points per concentration.

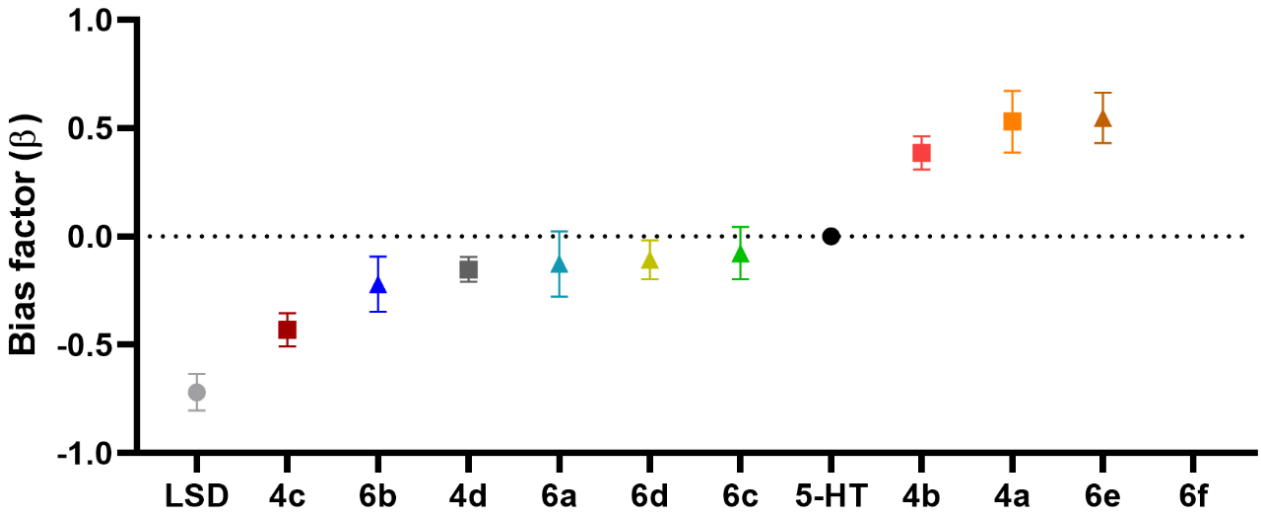
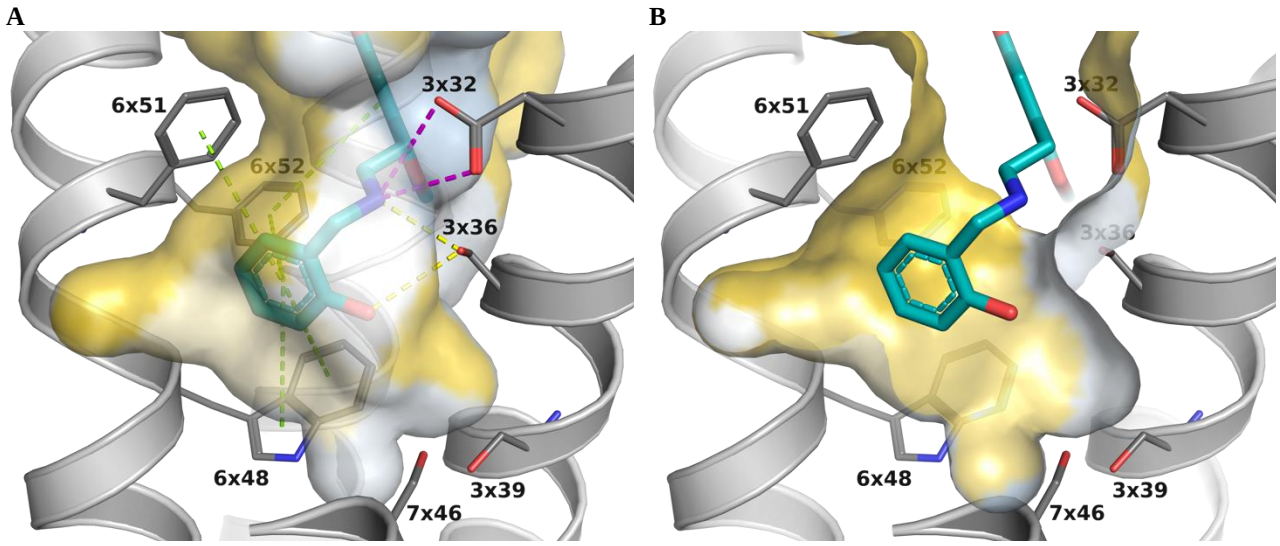


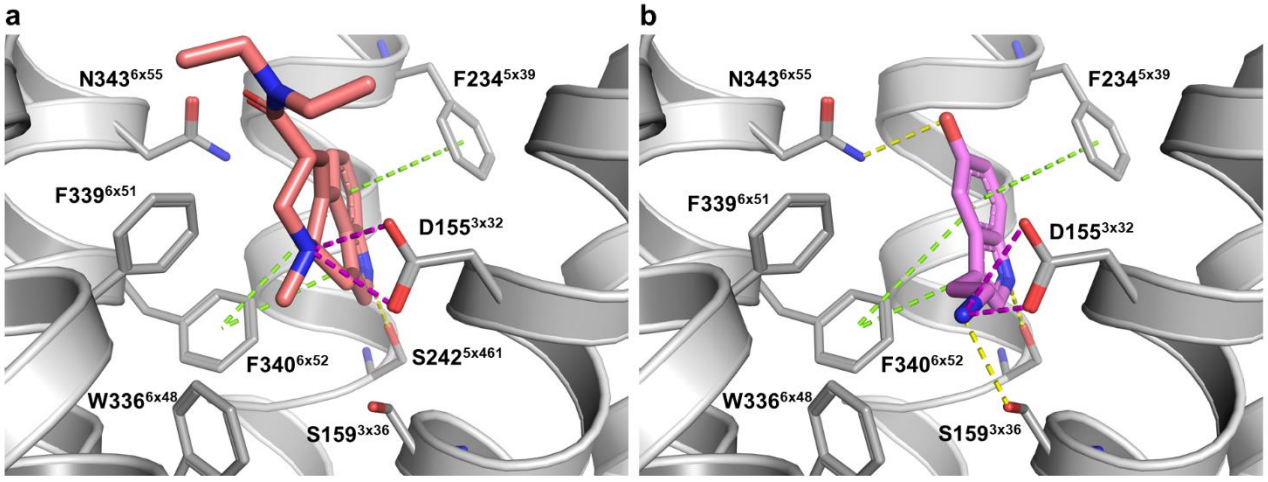
Figure S6. Visual representation of the bias factors (β) ± SEM, with serotonin as a reference agonist.

Additional Computational Data



6a (25CN-NBOH) **6a** (25CN-NBOH)

Figure S7. *N*-benzyl rings of NBOMes occupy a distinctive subpocket in the 5-HT$_{2A}$ which mediates Gα_q protein signaling via hydrophilic interactions. Interactions between receptor (gray lines and cartoon) and the *N*-benzyl moiety of **6a** (25CN-NBOH) (cyan sticks) in the cryo-EM structure of the Gα_q-coupled 5-HT$_{2A}$ (PDB ID 6WHA),[1] with the *N*-benzyl subpocket displayed as a closed (A) surface and colored according to the Eisenberg hydrophobicity scale,[2] from highly hydrophilic (blue) to highly hydrophobic (yellow).



Lysergic acid diethylamine (LSD) **Serotonin (5-hydroxytryptamine, 5-HT)**

Figure S8. Experimental binding mode of LSD and predicted binding mode of serotonin at the 5-HT$_{2A}$ receptor reveal differential engagements to Ser159^{3x36}, reflected in the miniGα_q recruitment response. A. Interactions between receptor (gray lines and cartoon) and LSD (salmon sticks) in the crystallographic structure of the 5-HT$_{2A}$ (PDB ID 6WGT)[1]; B. Predicted binding pose of serotonin (pink sticks) and ligand-receptor interactions in the same structure. The interactions are displayed as dashed lines and colored in green (aromatic, π-π staking), yellow (hydrogen bond), and pink (salt-bridge).

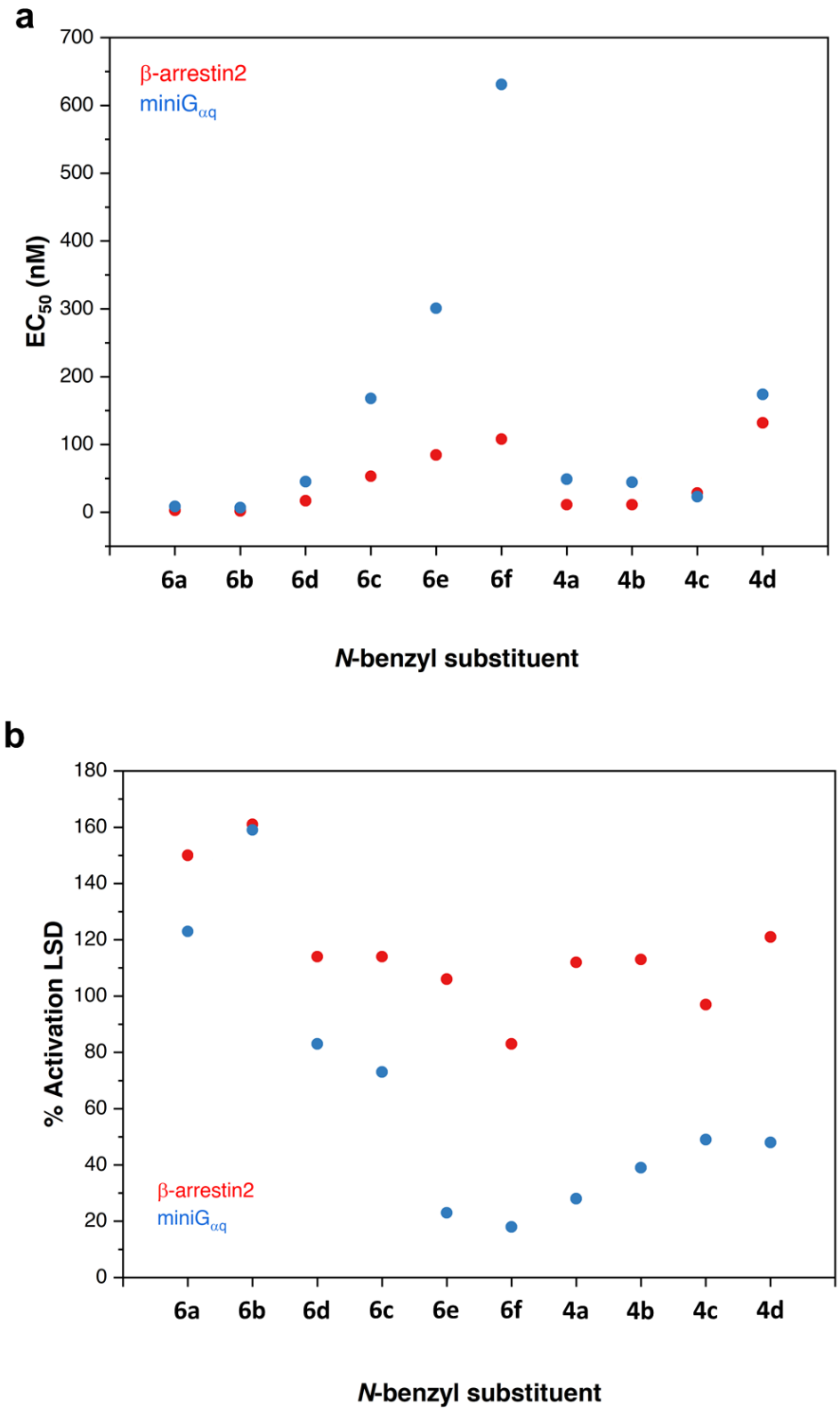
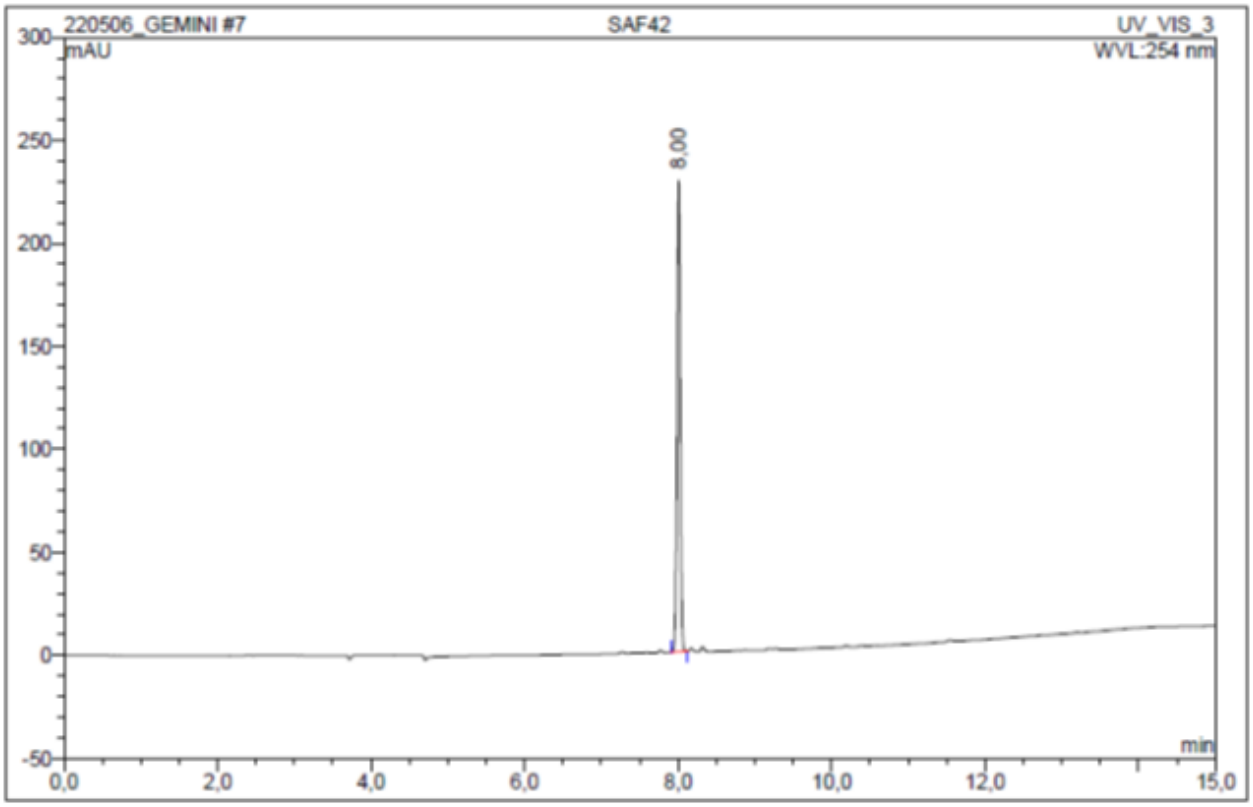


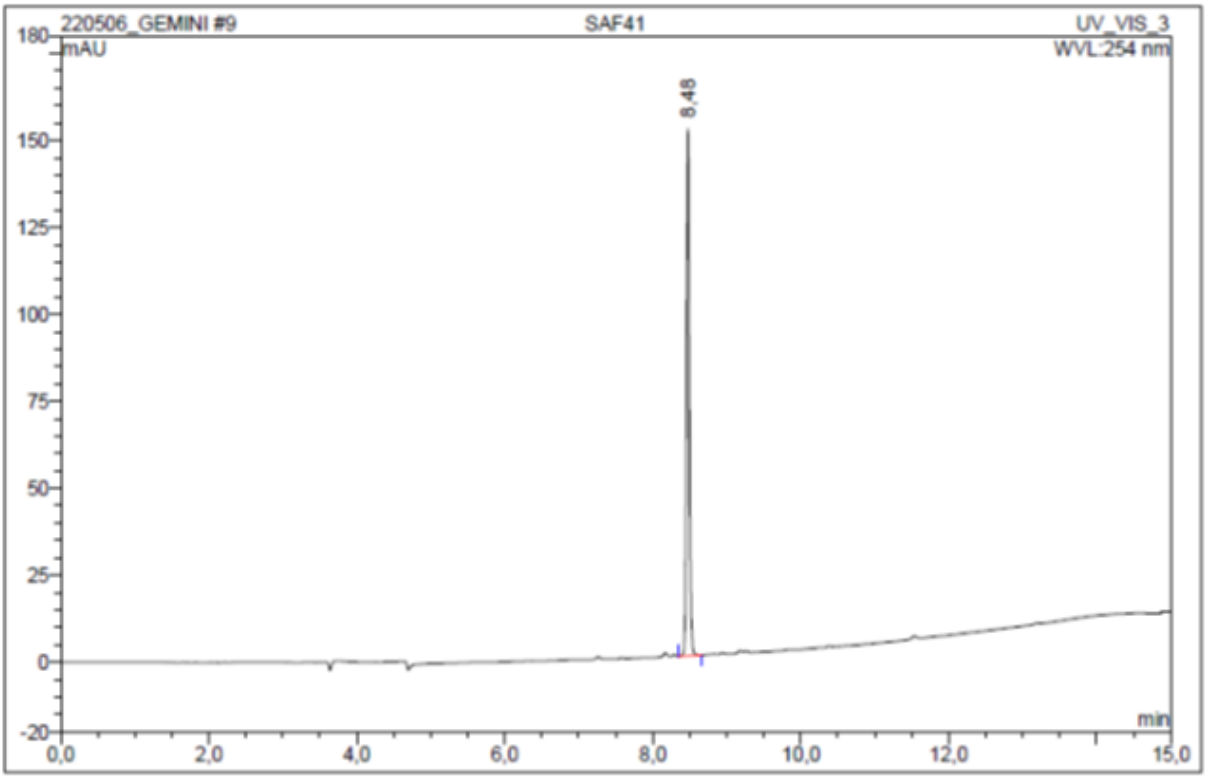
Figure S9. Interactions between the *N*-benzyl substituents and Ser159^{3x36} drive the preferential recruitment of Gα_q. (**A**) Potency (EC_{50}) and (**B**) efficacy (E_{max} relative to LSD) in the functional complementation assays for β-arrestin recruitment (red) and miniGα_q recruitment (blue) of the evaluated agonists.

HPLC traces



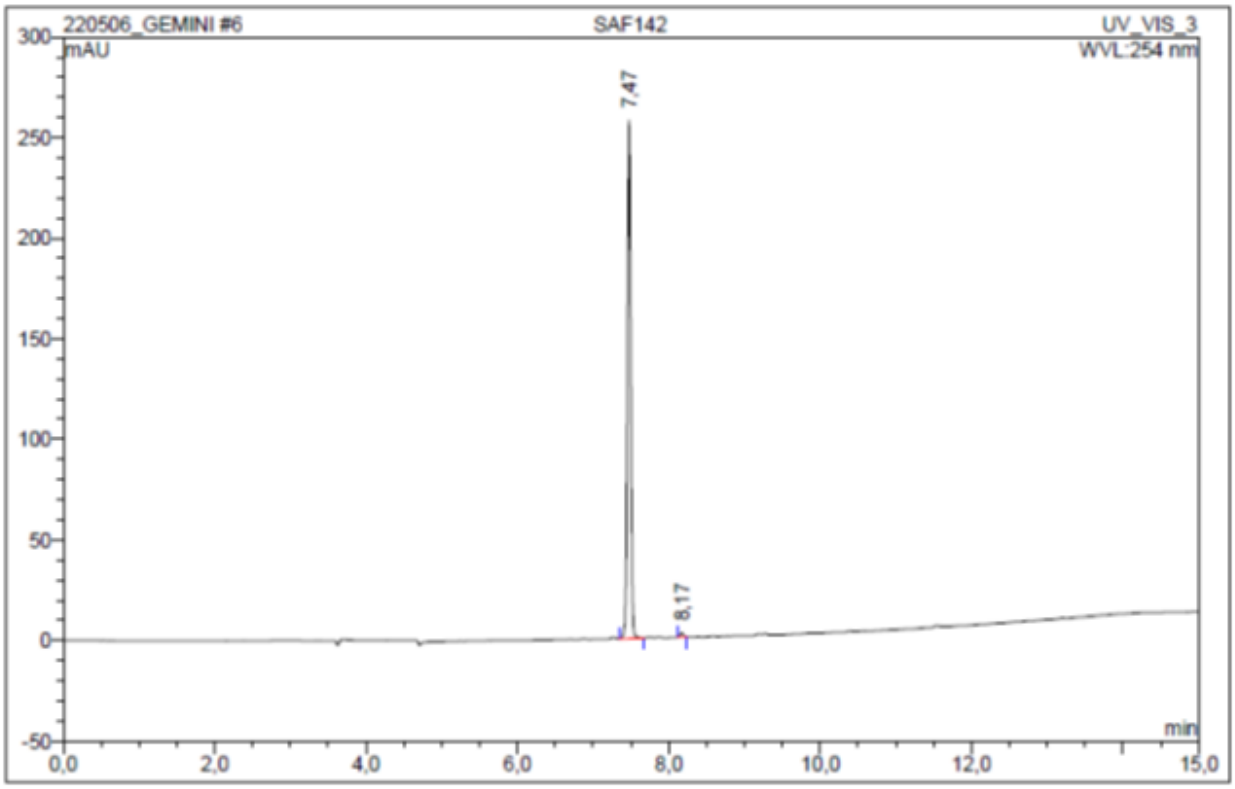
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Resolution(EP)
1	8,00	n.a.	228,896	10,694	100,00	n.a.	n.a.
Total:			228,896	10,694	100,00	0,000	

Figure S10. HPLC traces of compounds **4a**.



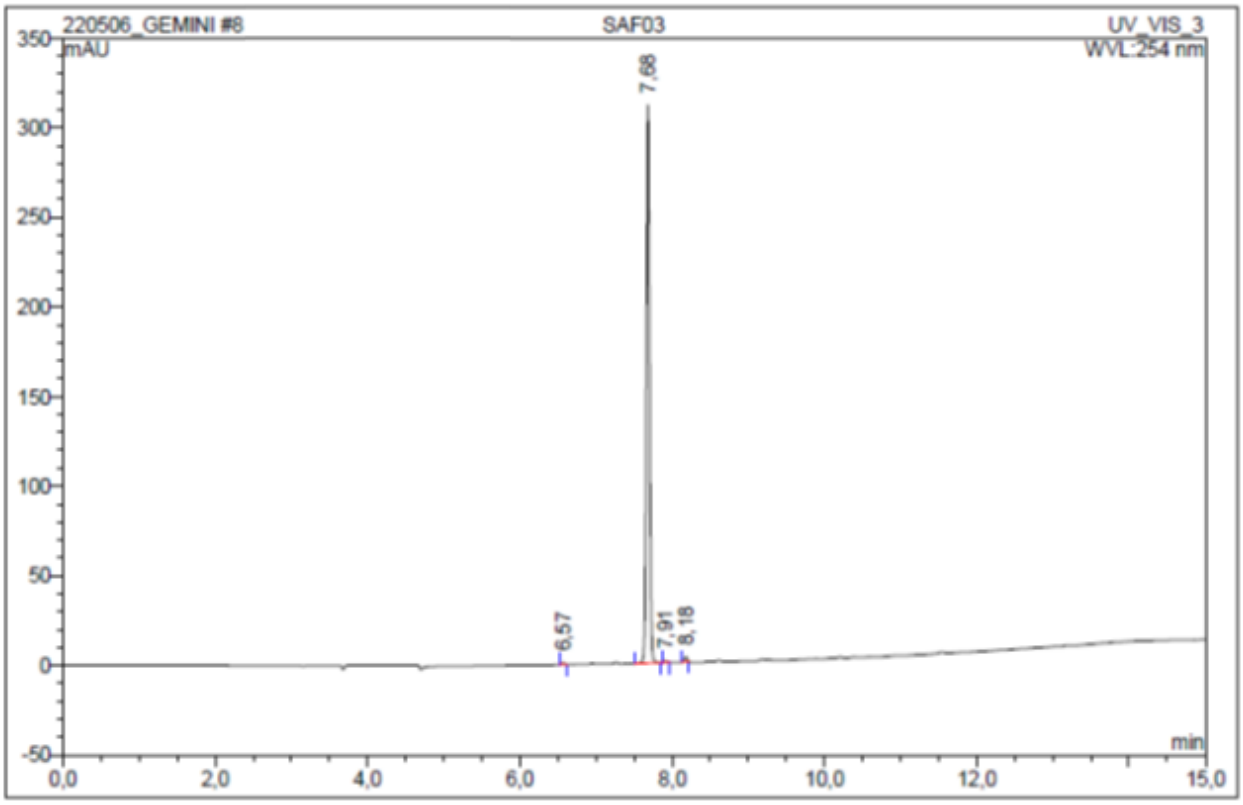
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Resolution(EP)
1	8,48	n.a.	151,474	7,020	100,00	n.a.	n.a.
Total:			151,474	7,020	100,00	0,000	

Figure S11. HPLC traces of compounds **4b**.



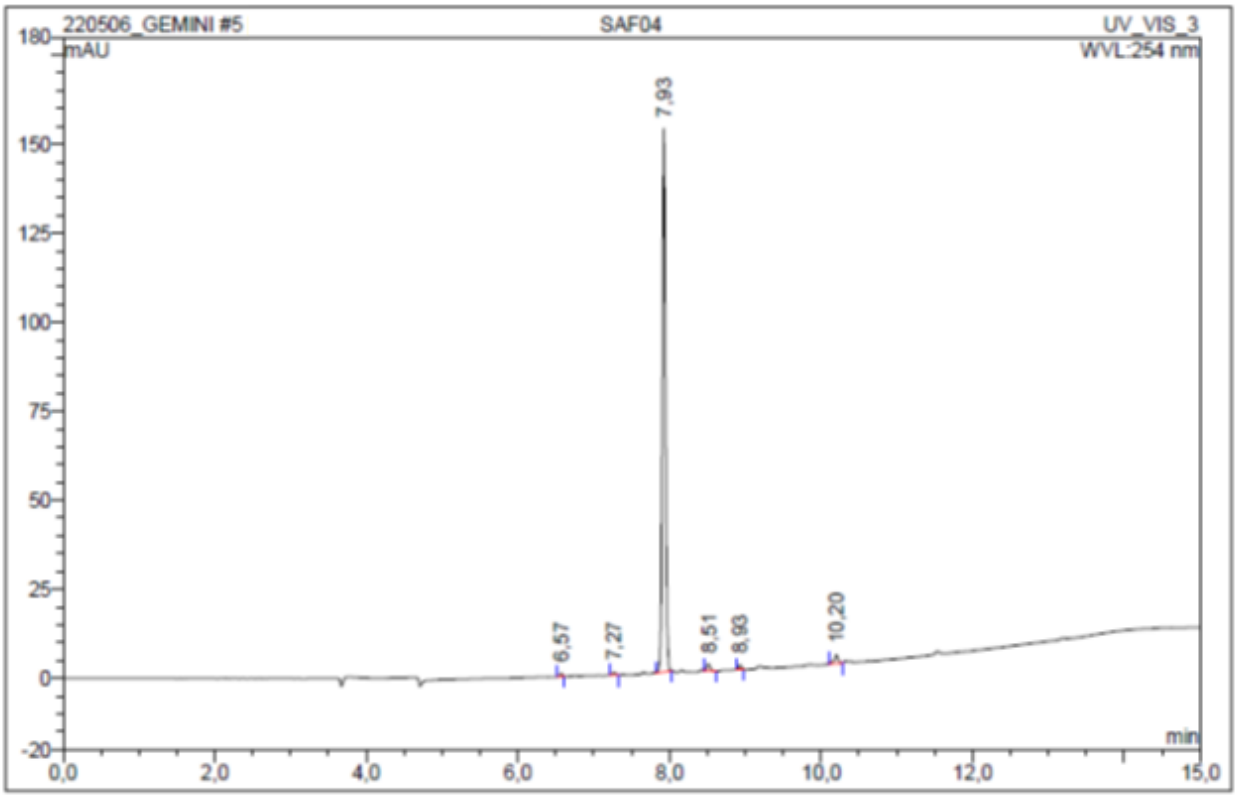
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Resolution(EP)
1	7,47	n.a.	257,553	13,240	99,28	n.a.	8,81
2	8,17	n.a.	1,980	0,097	0,72	n.a.	n.a.
Total:			259,533	13,336	100,00	0,000	

Figure S12. HPLC traces of compounds **4d**.



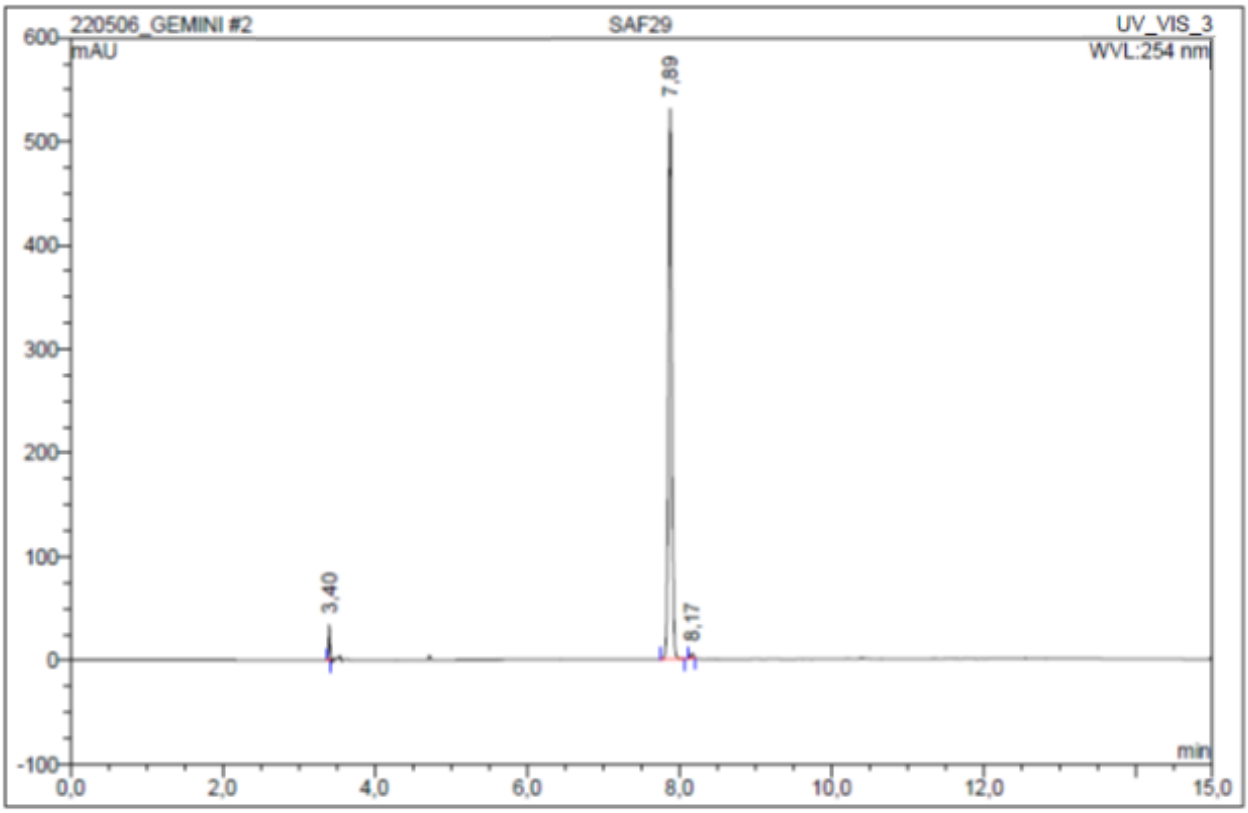
No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Resolution(EP)
1	6,57	n.a.	0,962	0,041	0,27	n.a.	15,32
2	7,68	n.a.	311,409	15,040	98,64	n.a.	2,97
3	7,91	n.a.	0,910	0,046	0,30	n.a.	3,58
4	8,18	n.a.	2,686	0,121	0,79	n.a.	n.a.
Total:			315,967	15,248	100,00	0,000	

Figure S13. HPLC traces of compounds **6a**.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Resolution(EP)
1	6,57	n.a.	0,831	0,037	0,48	n.a.	8,87
2	7,27	n.a.	0,672	0,034	0,44	n.a.	8,31
3	7,93	n.a.	152,573	7,343	95,40	n.a.	7,57
4	8,51	n.a.	1,892	0,096	1,25	n.a.	5,44
5	8,93	n.a.	1,241	0,054	0,71	n.a.	16,60
6	10,20	n.a.	2,301	0,133	1,73	n.a.	n.a.
Total:			159,511	7,697	100,00	0,000	

Figure S14. HPLC traces of compounds **6d**.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Resolution(EP)
1	3,40	n.a.	33,908	0,755	2,50	n.a.	72,47
2	7,89	n.a.	530,574	29,205	96,87	n.a.	3,52
3	8,17	n.a.	4,364	0,190	0,63	n.a.	n.a.
Total:			568,846	30,150	100,00	0,000	

Figure S15. HPLC traces of compounds **6d**.

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Facile One-pot Reduction of Nitrostyrenes to Phenethylamines using Sodium Borohydride and Copper(II) chloride

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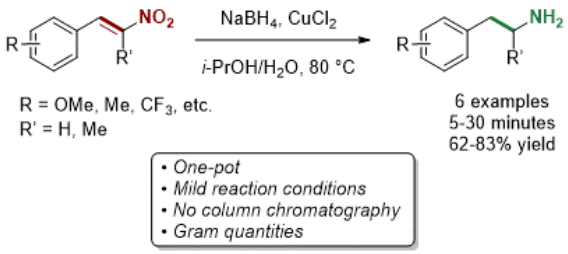

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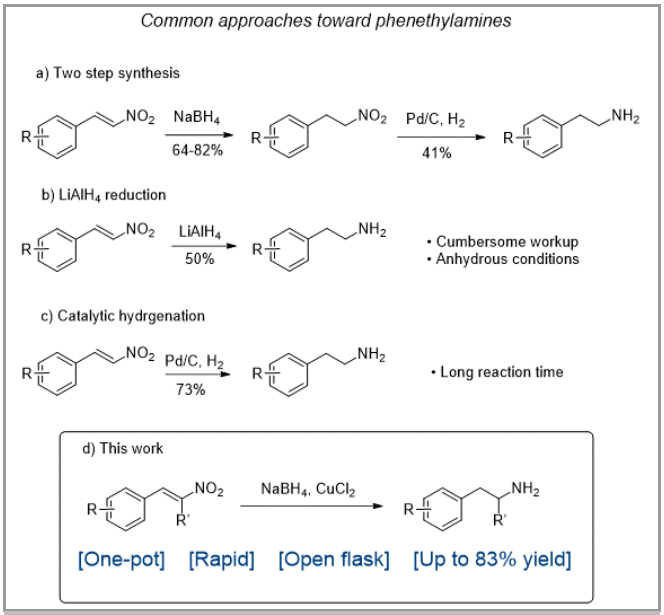
Abstract The preparation of phenethylamines and phenylisoproylamines of scientific relevance can be achieved with a $NaBH_4/CuCl_2$ system in 10 to 30 minutes via reduction of substituted β-nitrostyrenes. The method also reduces nitrobenzene and methyl benzoate in 92 to 97% yields, while has no effect on benzoic acid, benzaldehyde, and aromatic halides. This inexpensive and facile one-pot procedure allows the isolation of the products in good to excellent yields under mild conditions, without the need for special precautions and time-consuming purification techniques.

Key words phenethylamine, $NaBH_4$, β-nitrostyrene, 2C-X, $CuCl_2$, reductive amination, amphetamine

The phenethylamine scaffold represents a recurring motif among natural and synthetic drug molecules. The latter are mainly constituted by a varied class of substituted phenylethylamines exhibiting psychoactive properties and typically employed for medical and recreational use.[1] Representative examples include CNS stimulants (amphetamine), antidepressants and antiparkinson's agents (e.g., L-deprenyl)[2], hallucinogens and entactogens (e.g., 2,5-dimethoxy4-iodoamphetamine (DOI) and 3,4-methylenedioxy-N-methylamphetamine (MDMA)),[3] nasal decongestants (e.g., levomethamphetamine) and appetite suppressants (e.g. phentermine).[4]

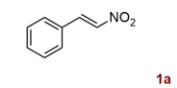
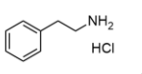
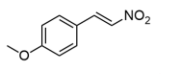
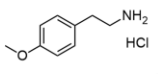
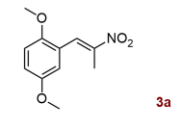
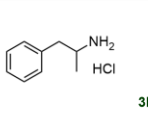
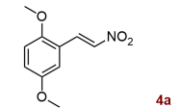
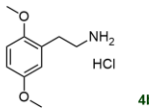
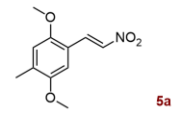
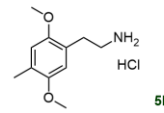
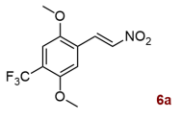
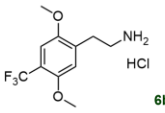
One of the most studied and inexpensive routes to synthesize substituted phenylethylamines involves the reduction of their α,β-unsaturated nitroalkene analogue (β-nitrostyrene), where both the double bond and the nitro group need to be reduced to deliver the corresponding primary amine. Their reduction can be accomplished via catalytic hydrogenation, involving stepwise reactions, use of additional reagents, and long reaction time.[5] Most commonly, metal hydrides are employed, typically lithium aluminium hydride[6], requiring inert atmosphere, special precautions, and final purification, due to the formation of side products, often with modest yields.[6a,7] (Scheme 1)



Scheme 1: Comparison between some examples of traditional synthetic routes for the preparation of substituted phenethylamines from β-nitrostyrene scaffolds and our work.

Sodium borohydride is a safe, inexpensive, and easy-to-handle reducing agent. Since the first attempts in 1967, $NaBH_4$ has been employed to reduce β-nitrostyrenes scaffolds to the corresponding nitroalkanes.[8] For this reason, several catalysts have been tested over the decades with $NaBH_4$ to facilitate full reduction to the phenethylamine, but to date no effective method for converting α,β-unsaturated nitroalkenes into aminoalkanes have been developed using NaBH4 as reducing agent.[8c, 9]

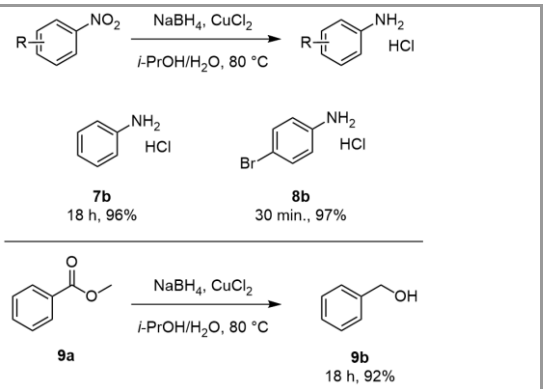
Herein, we demonstrate that $NaBH_4$ in combination with a catalytic amount of $CuCl_2$ is a simple and

Substrate	Product	Time (min.)	Yield (%)
 1a	 1b	30	83
 2a	 2b	10	82
 3a	 3b	30	62
 4a	 4b	10	82
 5a	 5b	30	65
 6a	 6b	30	71

Scheme 2: The β-nitrostyrene scaffolds with their corresponding products, reaction times and their yields.

high yielding method to synthesize phenethyl- and phenylisoproylamines from the corresponding nitroalkenes. Representative substituted β-nitrostyrene analogues were reduced via this method, including β-methyl-β-nitrostyrene **3a**, precursor of amphetamines, and 2,5-dimethoxy-β-nitrostyrene **4a**, precursor of most of the hallucinogenic 2C-X family and its derivatives.[6a,10] (Scheme 2)

The method was also tested on other types of scaffolds to investigate its potential general applications and effects on other substituents. As sodium borohydride per se does not reduce ester nor nitro functionalities,[11] the presence of the copper salt results in overcoming this issue, leading to excellent yields of their reduced derivatives (**7**-**9**). (Scheme 3)



Scheme 3: Nitrobenzene and methyl benzoate are reduced in excellent yields without dehalogenation.

Therefore, the $NaBH_4/CuCl_2$ system been proved to work on aromatic ester, nitro, and α,β-unsaturated nitroalkenes functionalities.

Our studies demonstrate that, up to 24 hours, the method shows some degree of functional group tolerance as the amido and carboxylic acid functionalities of benzamide and benzoic acid, respectively were left untouched, and the starting materials were finally fully recovered.

1-Bromo-4-nitrobenzene **8a** and 3-chlorophenol were used to test the effects of this method on halogenated aromatic structures and no dehalogenation was detected up to 24 hours stirring. The retainment of halogens atoms on aryl halides distinguishes this method from traditional techniques, such as those involving $LiAlH_4$, often causing dehalogenation.[12]

The role of the $CuCl_2$ salt is pivotal to the success of the method. Studies on the reduction of $CuCl_2$ by $NaBH_4$ suggests that copper(II) is promptly reduced to free Cu(0), composing up to 96% of the products. The remaining 4% consists of Cu_2O and negligible amounts of other copper species.[13] Consistently, once the chloride is added, the reduction to free Cu(0) is visually indicated by the immediate disappearance of the blue color of the copper(II) solution, and the formation of a fine suspended black powder. The latter, as metallic copper particles, acts as the actual catalyst.

Time seems to be a key factor for the phenethylamine structures, as the yields decrease over time after reaching their maximum. Once 2-propanol is evaporated, the products can also be isolated as free amines by dissolving the residue in ether, decanting it into another flask, and concentrating *in vacuo*.

In summary, the presented procedure represents a rapid, facile, high-yielding, scalable, and inexpensive alternative to the conventional reductive methods used to date for the synthesis of substituted phenethylamines from their α,β-unsaturated nitroalkene analogues. Furthermore, the $NaBH_4/CuCl_2$ system is effective at reducing nitro and ester functionalities on aromatic structures, while leaving intact benzoic acid, amido- and halogenated aromatic compounds.

Acknowledgment

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Supporting Information

YES (this text will be updated with links prior to publication)

Primary Data

NO.

Conflict of Interest

The authors declare no conflict of interest.

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Experimental section

NMR spectra were recorded on Bruker Avance 400 MHz or Bruker Avance III HD 600 MHz spectrometers. Residual solvent peaks (D_2O, Chloroform-d, Methanol-d_4, DMSO-d_6) were used as internal standard (4.79, 7.26, 3.31, and 2.50 ppm for ^{1}H, and 77.16, 49.15, and 39.51 ppm for ^{13}C, respectively). UPLC-MS analyses were performed on a Waters Acquity H-class UPLC with a Sample Manager FTN and a TUV dual wavelength detector coupled to a QDa single quadrupole analyser using electrospray ionization (ESI). UPLC separation was achieved with a C18 reversed-phase column (Acquity UPLC BEH C18, 2.1 mm × 50 mm, 1.7 µm) operated at 40 °C, using a linear gradient of the binary solvent system of buffer A (milliQ H_2O:MeCN:formic acid, 95:5:0.1 v/v%) to buffer B (MeCN:formic acid, 100:0.1 v/v%) from 0 to 100% B in 3.5 min, then 1 min at 100%B, Flow rate: 0.8 ml/min. Data acquisition was controlled by MassLynx ver. 4.1 and data analysis was done using Waters OpenLynx browser ver. 4.1. Solvents were commercial HPLC grade and used without further purification. The substrates 2a, 7a, 8a, and 10a-12a were commercially available and used without further purification. The substituted β-nitrostyrenes 1a and 3a-6a were prepared by use of literature.[14] 9a was prepared by modification of literature.[15]

General procedure

The desired substrate (**1a**-**12a**) (2 mmol, 1 eq.) was added in small portions to a stirring suspension of $NaBH_4$ (15 mmol, 7.5 eq.) in *i*-PrOH/H_2O (8:4ml). 0.1 ml of a freshly prepared $CuCl_2$ 2M solution were added dropwise but rapidly to the vessel. The reaction was monitored by TLC and refluxed at 80 °C for the time indicated in Table 1.

*General workup procedure of the amino products (**1**, **5**-**12**):* Once cooled to room temperature, 35% solution of NaOH (10 ml) was added under stirring. The mixture was extracted with i-PrOH (3 x 10 ml), and the organic extracts were combined, thoroughly dried over $MgSO_4$, and filtered.

(I) The residue was concentrated under reduced pressure and dissolved in a large amount of diethyl ether. The amino products were precipitated under stirring with an excess of HCl 2N in diethyl ether solution and the vessel was cooled to 5 °C. The solid was filtered, washed with cold diethyl ether, and dried under reduced pressure as the amine hydrochloride salt derivative.

(II) An excess of HCl 4N in dioxane solution was added and the filtrate was stirred for 30 minutes. The residue was concentrated under reduced pressure, suspended in dry cold acetone, and stirred vigorously for 1 hour. The suspension was filtered, washed with minimum amount of cold acetone to deliver the product as hydrochloride salt.

2-phenylethan-1-amine hydrochloride (**1b**) - The product was isolated by use of (II) as a as a colorless amorphous solid (83%).
^{1}H-NMR (600 MHz, Methanol-d4) δ 7.35 (t, *J* = 7.6 Hz, 2H), 7.28 (m, *J* = 5.0, 3H), 3.18 (q, *J* = 5.2, 2H), 2.97 (q, *J* = 5.2 Hz, 2H); ^{13}C-NMR (151 MHz, Methanol-d4) δ 136.53, 128.60, 128.39, 126.87, 40.59, 33.16.
MS (ESI): 121,1; Found $[M+1]^+$: 131,0.

2-(4-methoxyphenyl)ethan-1-amine hydrochloride (**2b**) - The product was isolated by use of (I) as a as a white solid (82%).
^{1}H-NMR (600 MHz, Methanol-d4) δ 7.19 (q, *J* = 2.9 Hz, 2H), 6.91 (q, *J* = 2.9 Hz, 2H), 3.78 (s, 3H), 3.13 (t, *J* = 7.7 Hz, 2H), 2.89 (t, *J* = 7.7 Hz, 2H); ^{13}C-NMR (151 MHz, Methanol-d4) δ 160.61, 130.93, 129.74, 115.57, 55.86, 42.29, 33.90.
MS (ESI): 151,1; Found $[M+1]^+$: 152,1.

1-(2,5-dimethoxyphenyl)propan-2-amine hydrochloride (**3b**) - The product was isolated by use of (II) as a as a colorless solid (62%).
^{1}H-NMR (600 MHz, Methanol-d4) δ 6.93 (d, *J* = 8.9 Hz, 1H), 6.84 (9, *J* = 3.8 Hz, 1H), 6.79 (d, *J* = 2.9 Hz, 1H), 3.81 (s, 3H), 3.75 (s, 3H), 3.55 (dt, *J* = 6.5, 20.0 Hz, 1H), 2.95 (q, *J* = 6.6 Hz, 1H), 2.82 (q, *J* = 6.9 Hz, 1H), 1.26 (d, *J* = 6.6 Hz, 3H); ^{13}C-NMR (151 MHz, Methanol-d4) δ 153.74, 151.77, 124.84, 117.23, 112.66, 111.41, 54.84, 54.72, 35.46, 17.17.
MS (ESI): 135,1; Found $[M+1]^+$: 136,2.

2-(2,5-dimethoxyphenyl)ethan-1-amine hydrochloride (**4b**) - The product was isolated by use of (I) as a as a white solid (82%).
^{1}H-NMR (600 MHz, DMSO-d6) δ 6.92 (d, *J* = 8.9 Hz, 1H), 6.81 (q, *J* = 4.0 Hz, 1H), 6.78 (d, *J* = 3.1 Hz, 1H), 3.75 (s, 3H), 3.70 (s, 3H), 2.97 (t, *J* = 7.8 Hz, 2H), 2.81 (t, *J* = 7.8 Hz, 2H). ^{13}C-NMR (151 MHz, DMSO-d6) δ 153.04, 151.24, 126.02, 116.44, 112.17, 111.77, 55.78, 55.03, 38.64, 28.13.
MS (ESI): 181.1; Found $[M+1]^+$: 182.1.

2-(2,5-dimethoxy-4-methylphenyl)ethan-1-amine hydrochloride (**5b**) - The product was isolated by use of (II) as a as a colorless solid (65%).
^{1}H-NMR (600 MHz, Methanol-d4) δ 6.83 (s, 1H), 6.78 (s, 1H), 3.82 (s, 3H), 3.80 (s, 3H), 3.14 (t, *J* = 7.4 Hz, 2H), 2.94 (t, *J* = 7.4 Hz, 2H), 2.20 (s, 3H); ^{13}C-NMR (151 MHz, Methanol-d4) δ 151.81, 151.25, 126.32, 121.97, 113.55, 112.83, 55.08, 54.93, 39.66, 28.40, 14.87.
MS (ESI): 195.1; Found $[M+1]^+$: 196.2.

2-(2,5-dimethoxy-4-(trifluoromethyl)phenyl)ethan-1-amine hydrochloride (**6b**) - The product was isolated by use of (II) as a as a colorless solid (71%).
^{1}H-NMR (400 MHz, Methanol-d4) δ 7.16 (s, 1H), 7.10 (s, 1H), 3.88 (s, 3H), 3.86 (s, 3H), 3.18 (t, *J* = 7.4 Hz, 2H), 3.03 (t, *J* = 7.4 Hz, 2H); ^{13}C-NMR (151 MHz, Methanol-d4) δ 152.98, 152.41, 131.62, 125.88, 124.08, 116.83, 110.40, 57.10, 56.57, 40.45, 29.85.
MS (ESI): 249.1; Found $[M+1]^+$: 250.1.

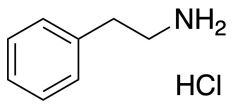
Aniline hydrochloride (**7b**) - The product formation was monitored by TLC using Hex:EtOAc:TEA (3:7:0.1). The product was isolated by use of (I) as a white solid (96%).^{1}H-NMR (600 MHz, D_2O) δ 7.54 (m, *J* = 3.2 Hz, 2H), 7.47 (m, *J* = 4.0 Hz, 1H), 7.37 (q, *J* = 2.8 Hz, 2H); ^{13}C-NMR (151 MHz, D_2O) δ 130,03, 122,03, 109,59.
MS (ESI): 93.1; Found $[M+1]^+$: 94,2.

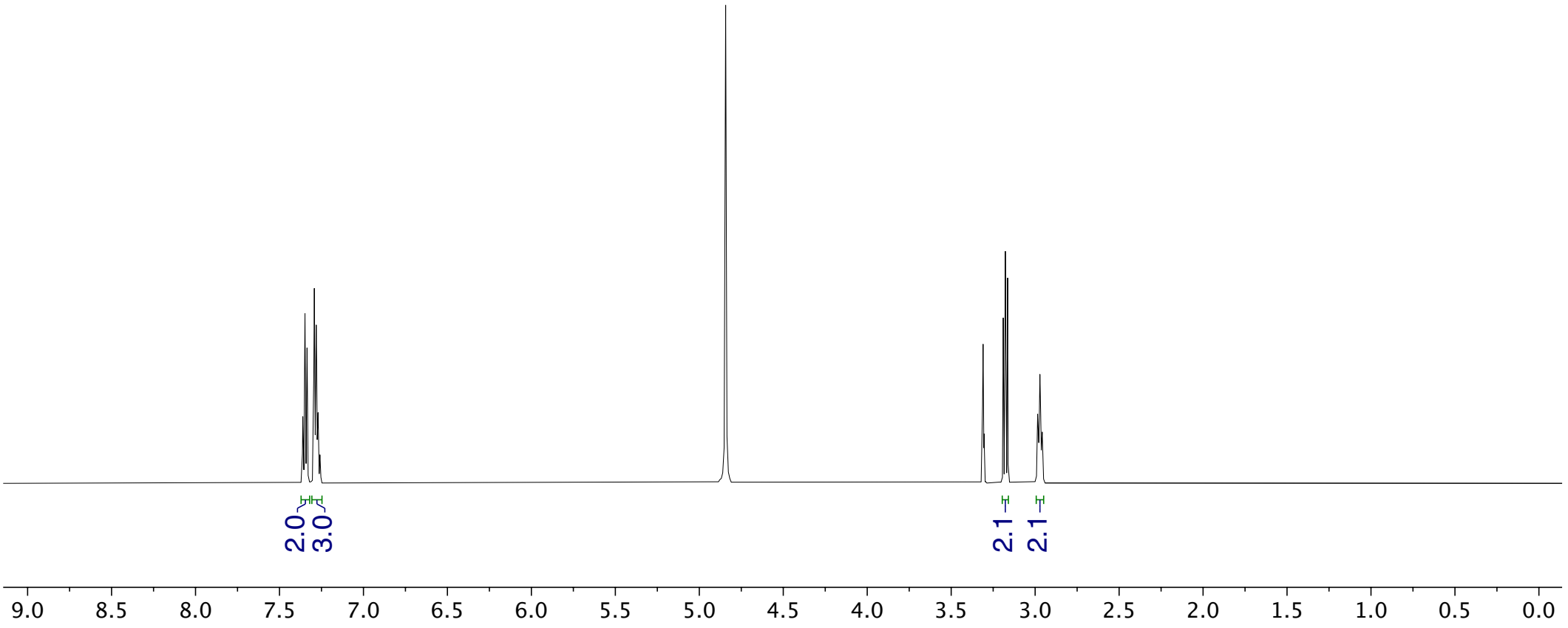
***p*-bromo-aniline hydrochloride** (**8b**) - The product formation was monitored by TLC using pure pentane. The product was isolated by use of (I) as a bright white powder (97%).
^{1}H-NMR (600 MHz, D_2O) δ 7.42 (d, *J* = 8.8 Hz, 2H), 6.87 (d, *J* = 8.8 Hz, 2H); ^{13}C-NMR (151 MHz, D_2O) δ 132.11, 119.07, 109.58.
MS (ESI): 171,0; Found $[M+1]^+$: 171.1.

Benzyl alcohol (**9b**) - The product formation was monitored by TLC using Hex:EtOAc (6:1). Once cooled to room temperature, the mixture was acidified with HCl 20% solution and extracted with DCM (3 x 15 ml). The organic extracts were combined, dried over $MgSO_4$, and concentrated under reduced pressure to deliver **9b** as colorless liquid (92%).

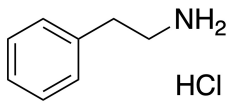
^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.37 (d, *J* = 4.6 Hz, 4H), 7,31 (m, *J* = 2,7 Hz, 1 H), δ 4.69 (s, 2H), δ 1.87 (s, 1H); ^{13}C-NMR (151 MHz, $CDCl_3$) δ 140,87, 128,59, 127,69, 127,02, 65,38.
MS (ESI): 108.1; Found $[M+1]^+$: 109.1.

^{1}H NMR (CD_3OD, 600 MHz)

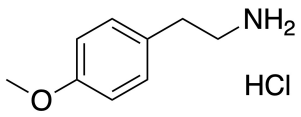




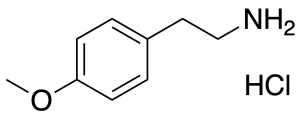
^{13}C NMR (CD_3OD, 151 MHz)



^1H NMR (CD_3OD, 600 MHz)



^{13}C NMR (CD_3OD, 151 MHz)



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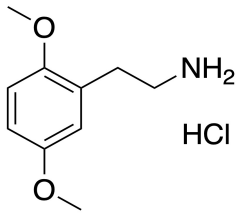
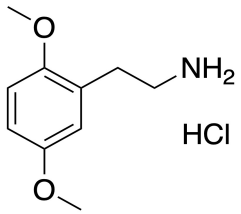
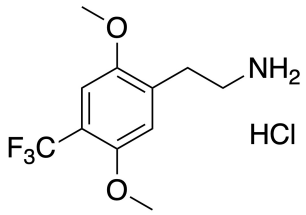


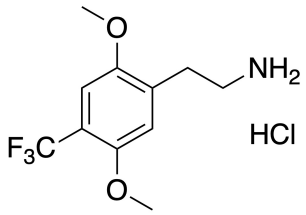
^{13}C NMR (CD_3OD, 151 MHz)



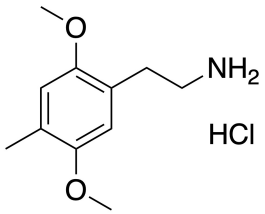
^1H NMR (CD_3OD, 400 MHz)



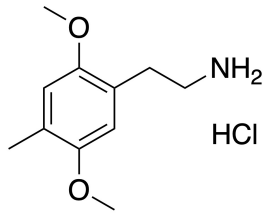
^{13}C NMR (CD_3OD, 151 MHz)



^1H NMR (CD_3OD, 600 MHz)



^{13}C NMR (CD_3OD, 151 MHz)



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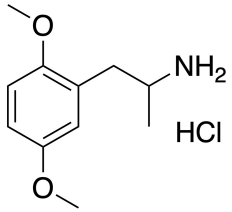
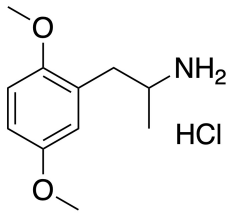
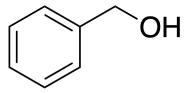
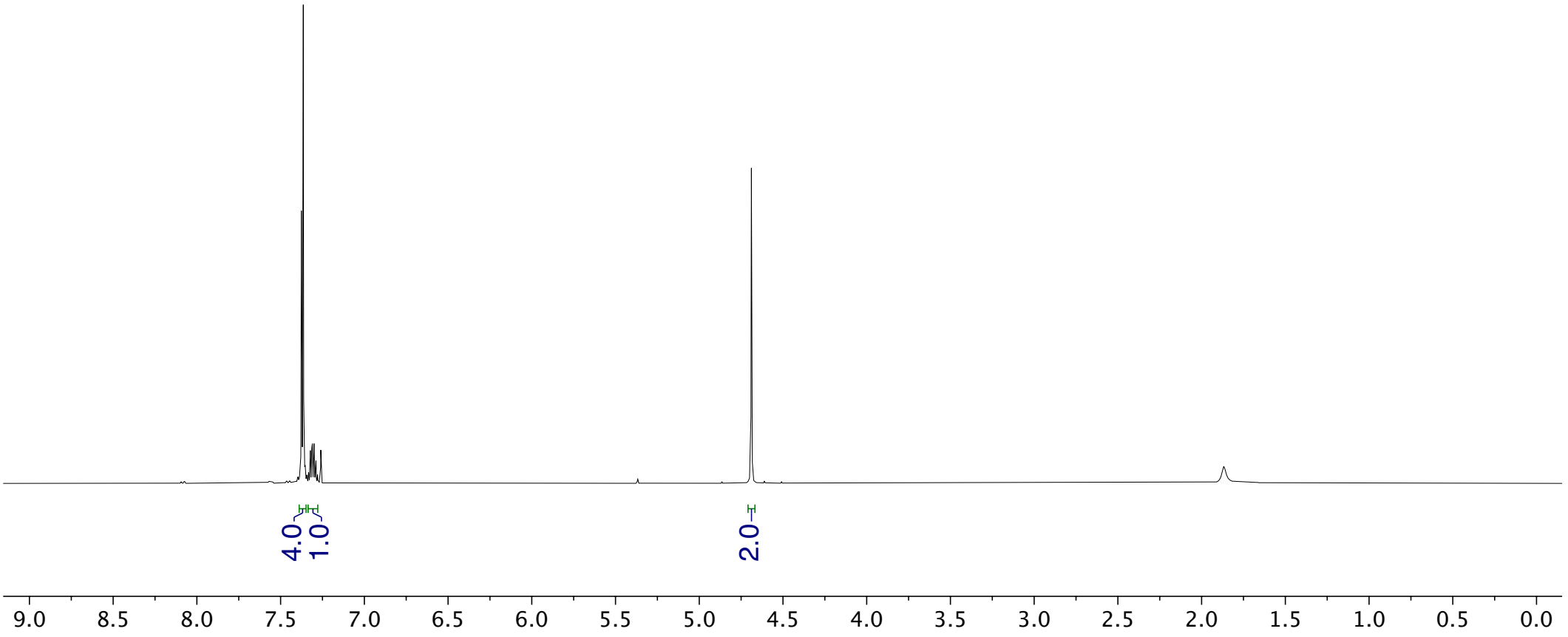


^{13}C NMR (CD_3OD, 151 MHz)

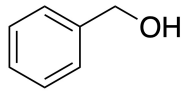


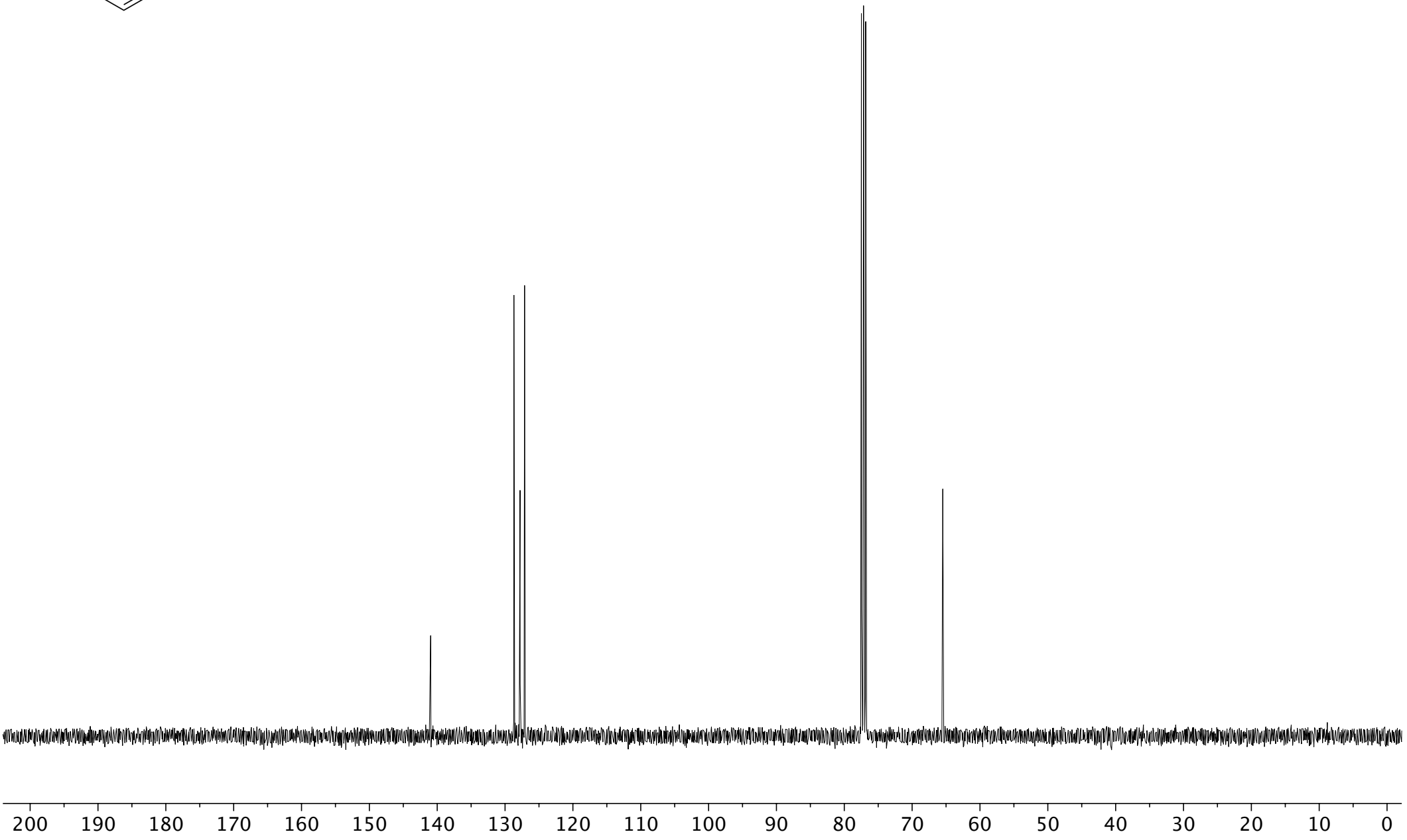
^{1}H NMR ($CDCl_3$, 400 MHz)



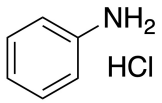


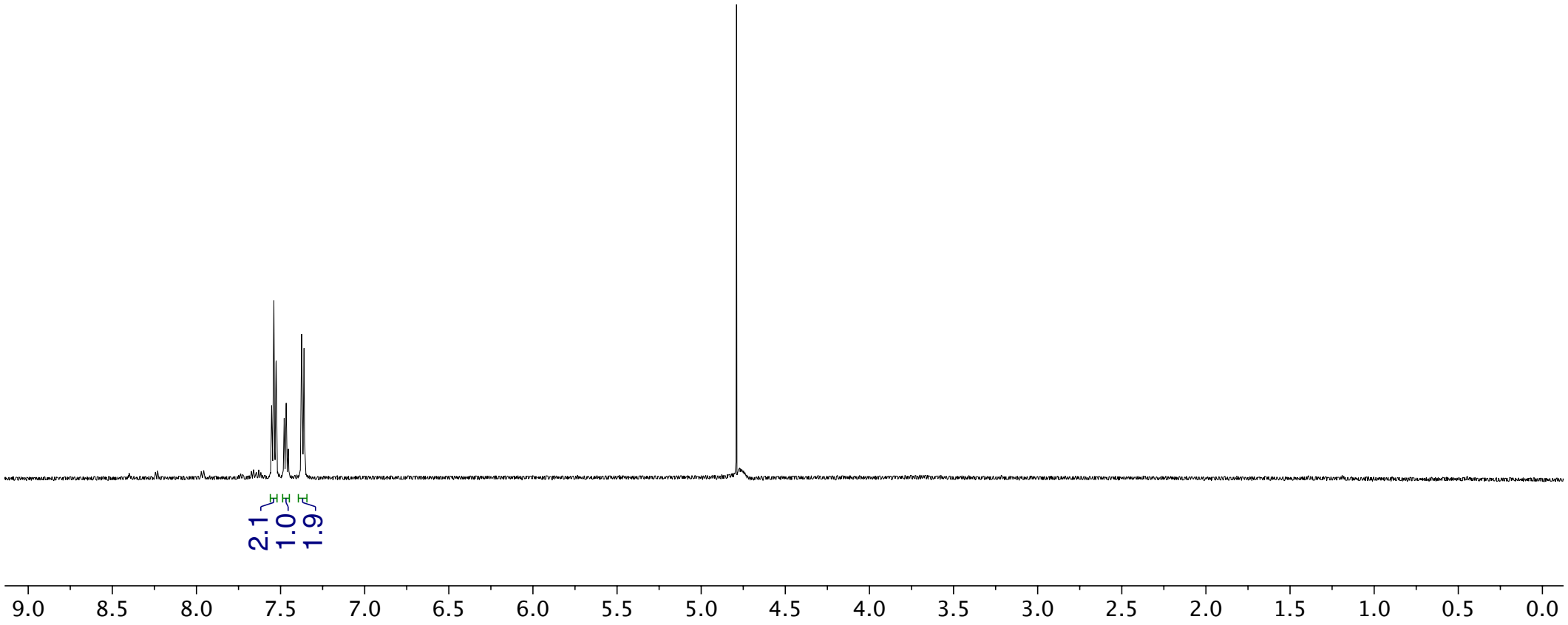
^{13}C NMR ($CDCl_3$, 151 MHz)



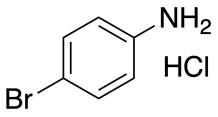


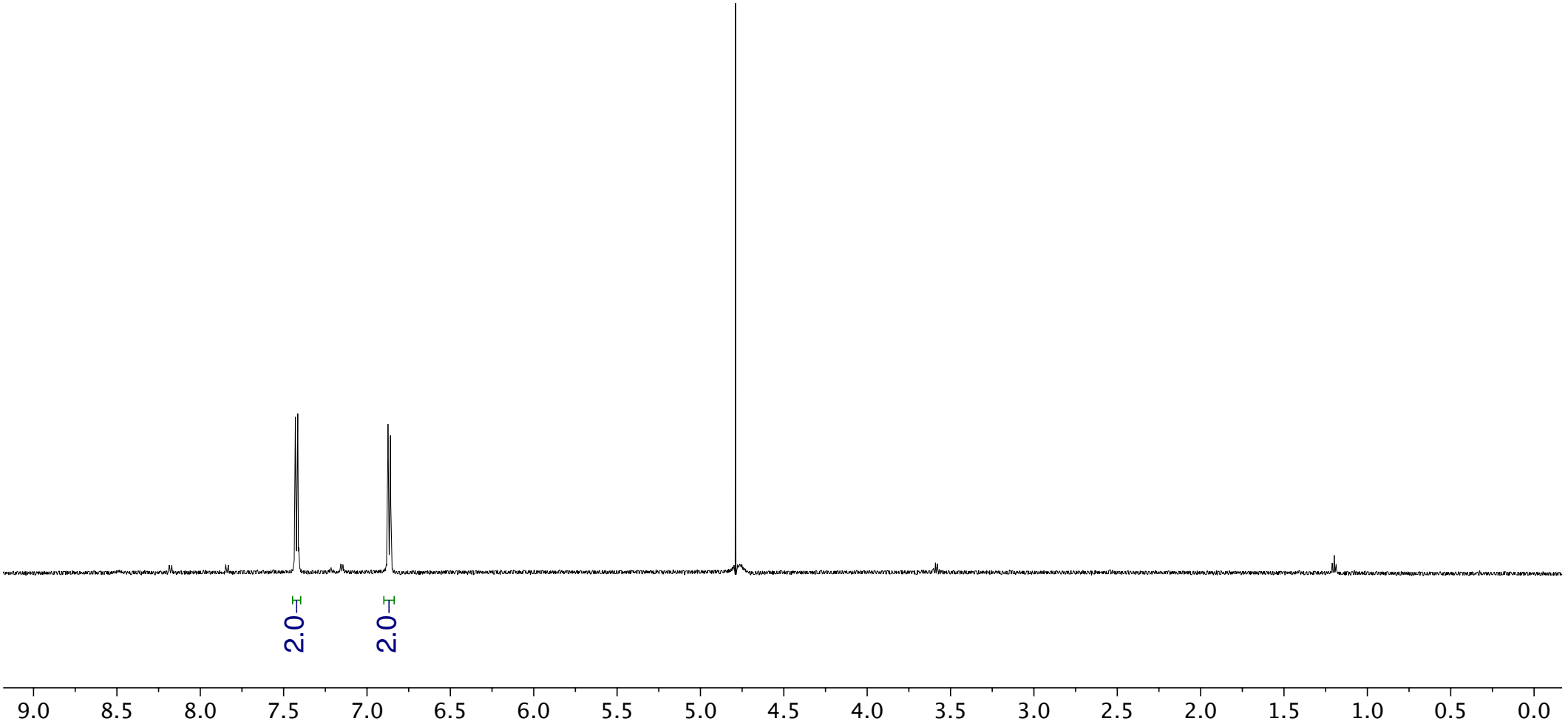
^1H NMR (D_2O, 600 MHz)





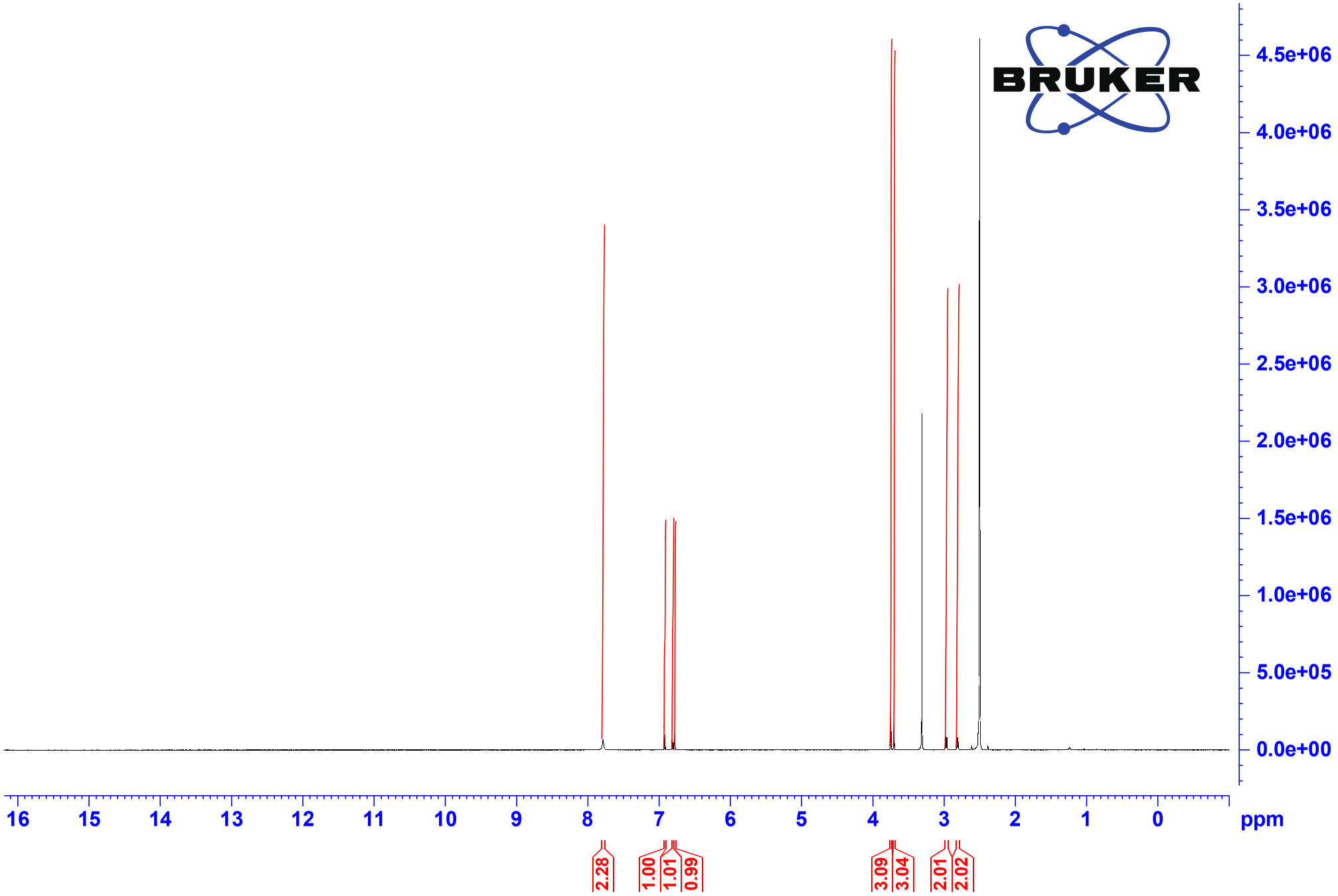
^{1}H NMR (D_2O, 600 MHz)



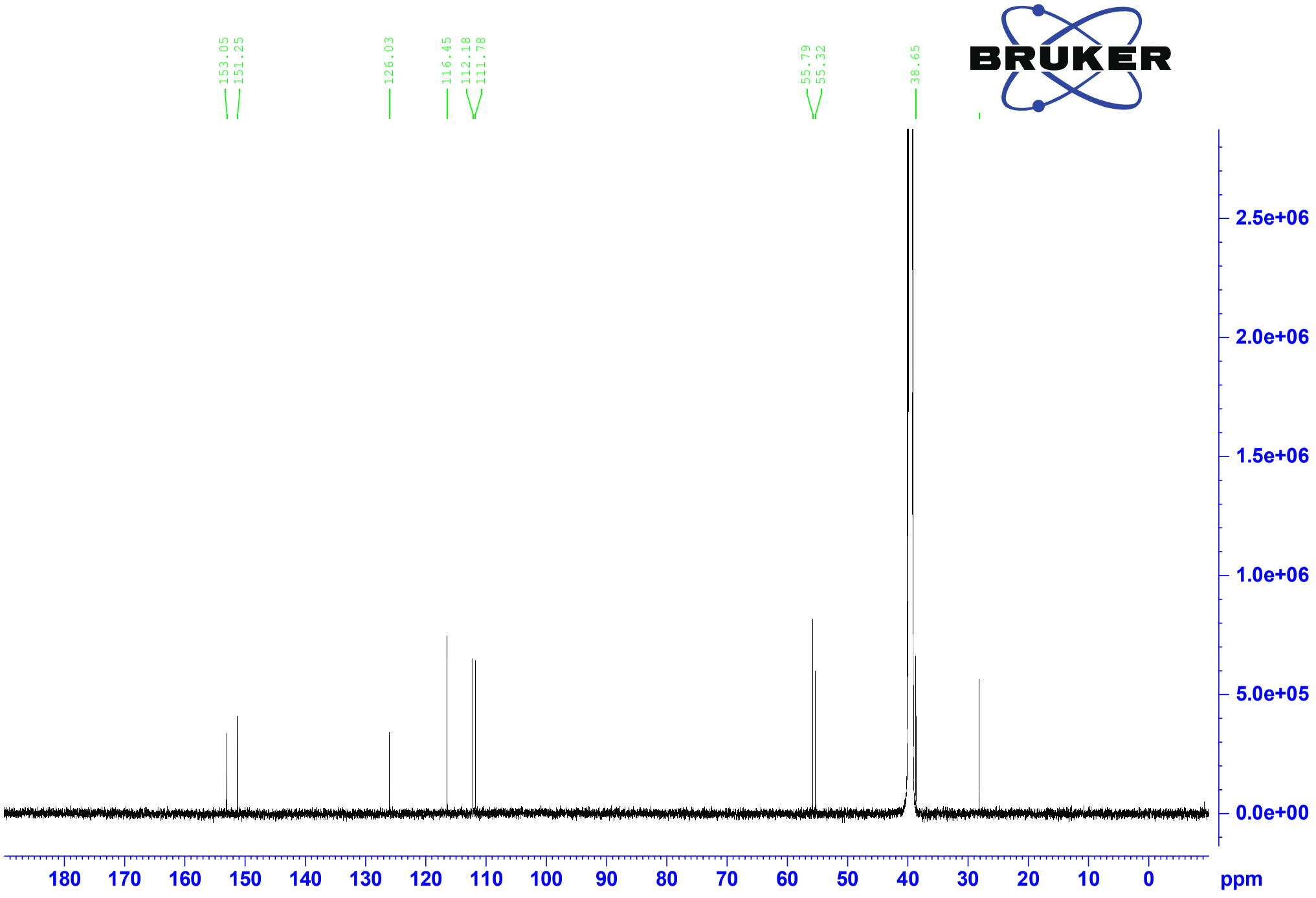


NMR spectra

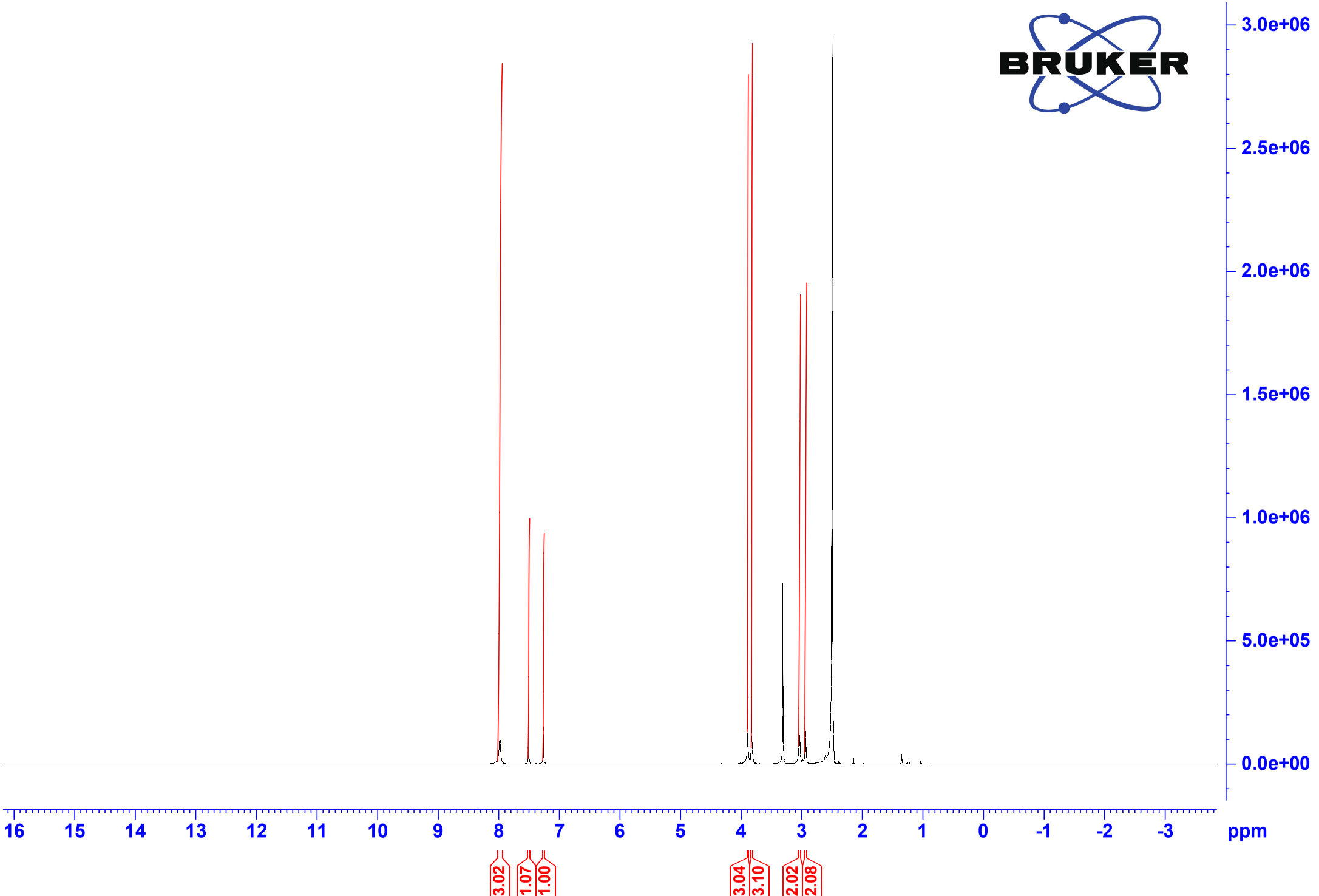
2,5-dimethoxyphenylethylamine hydrochloride (2C-H, 1.3)



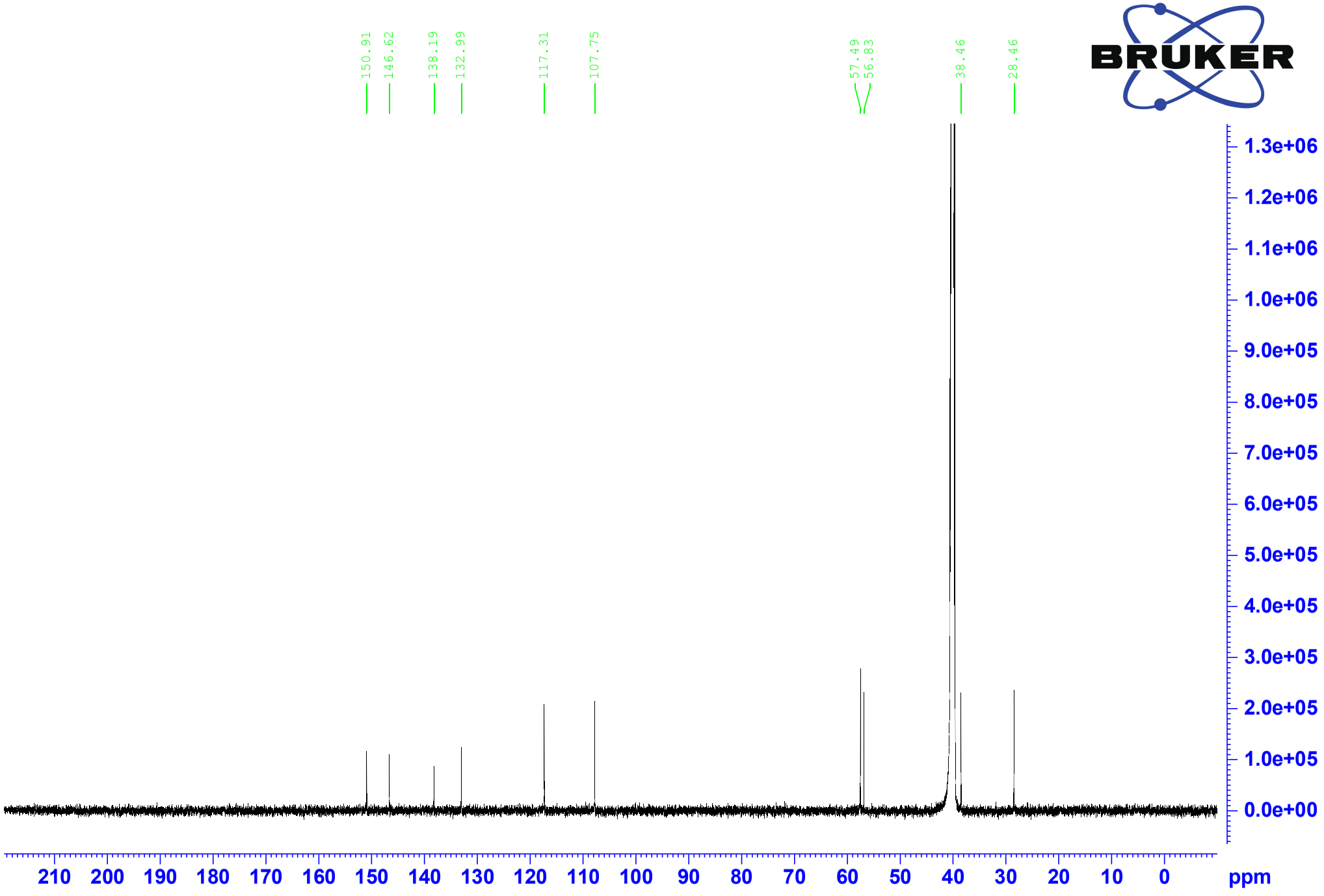
2,5-dimethoxyphenylethylamine hydrochloride (2C-H, 1.3)



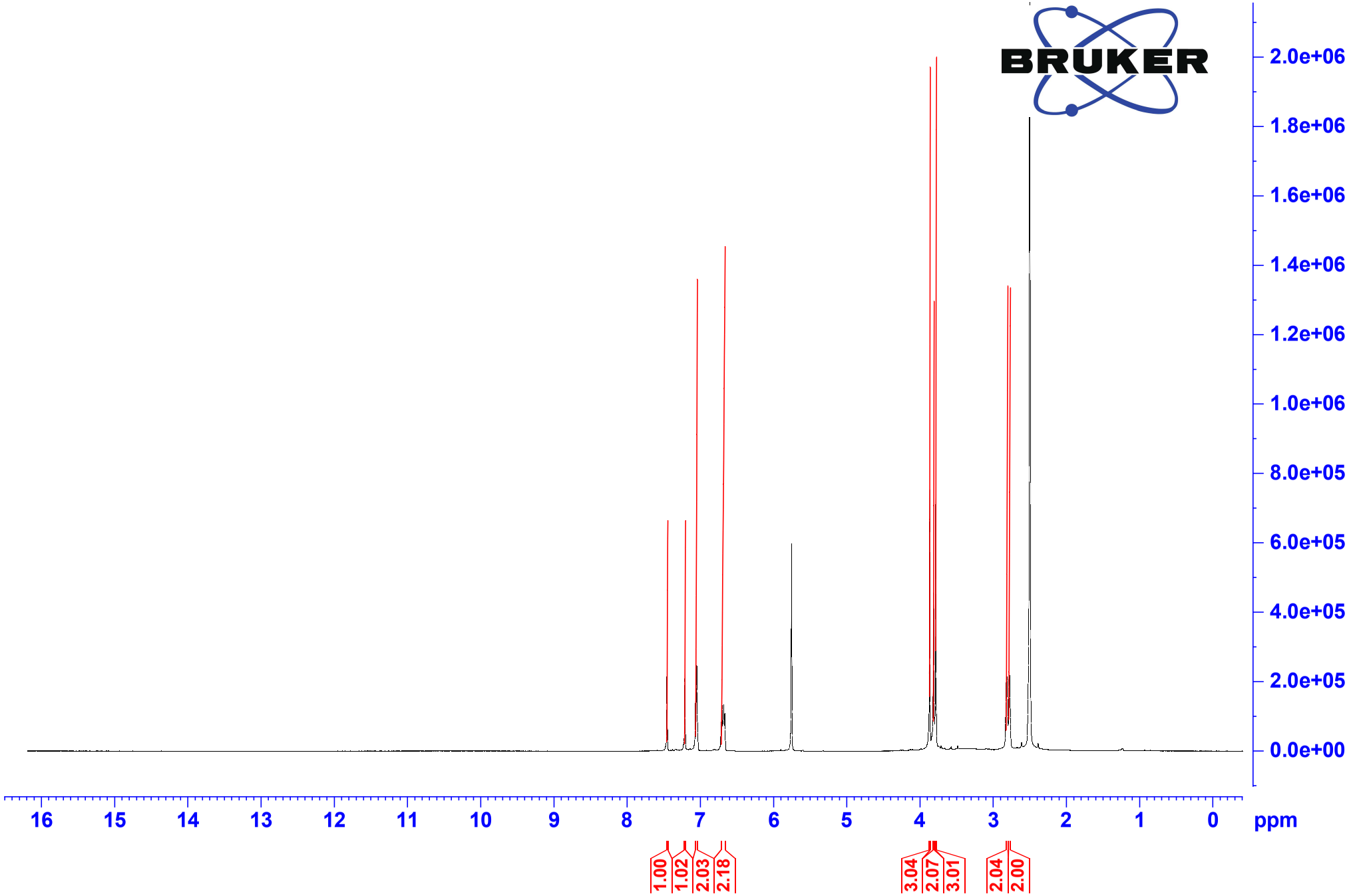
2,5-dimethoxy-4-nitrophenylethylamine hydrochloride (2C-N, 1.8)



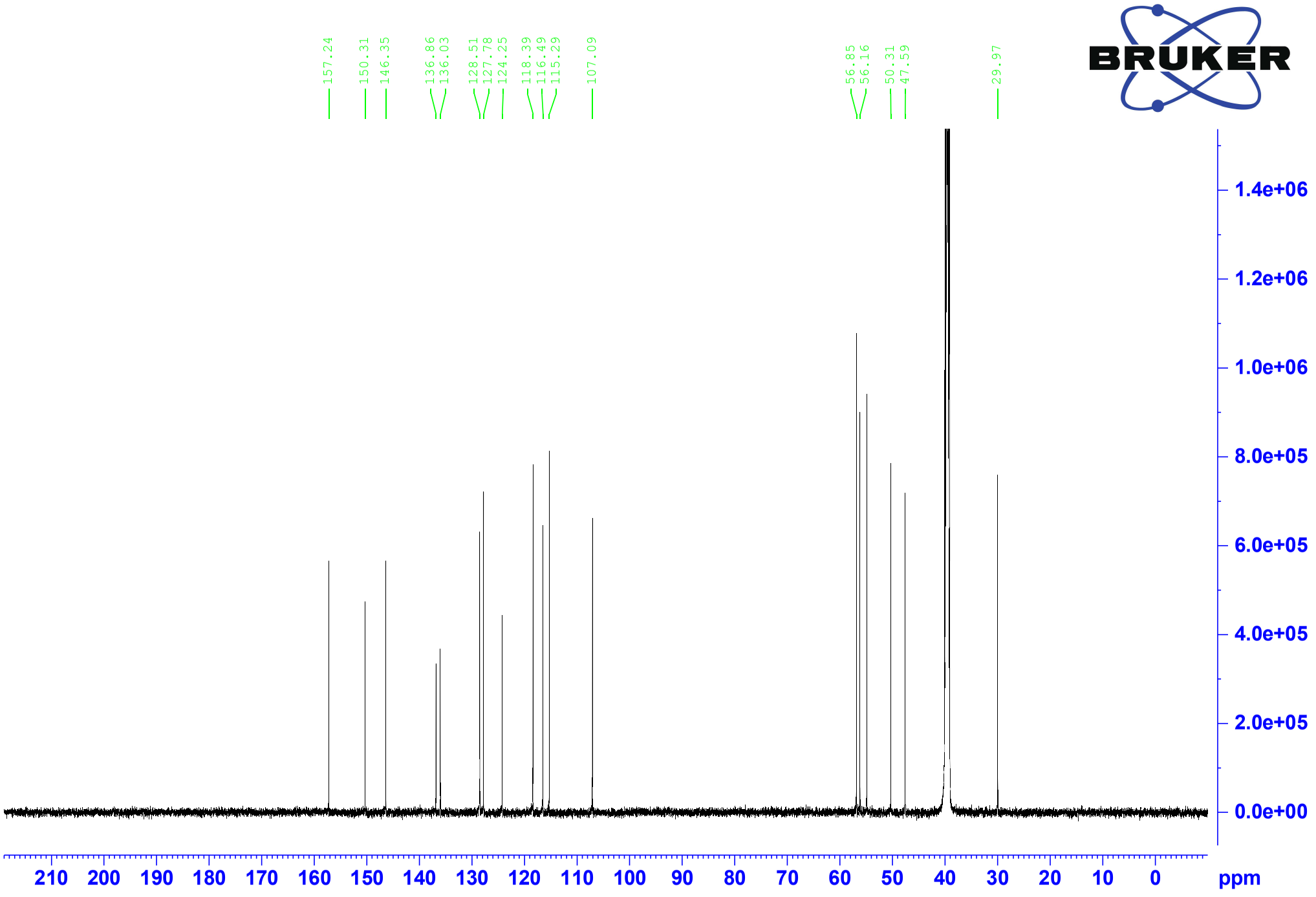
2,5-dimethoxy-4-nitrophenylethylamine hydrochloride (2C-N, 1.8)



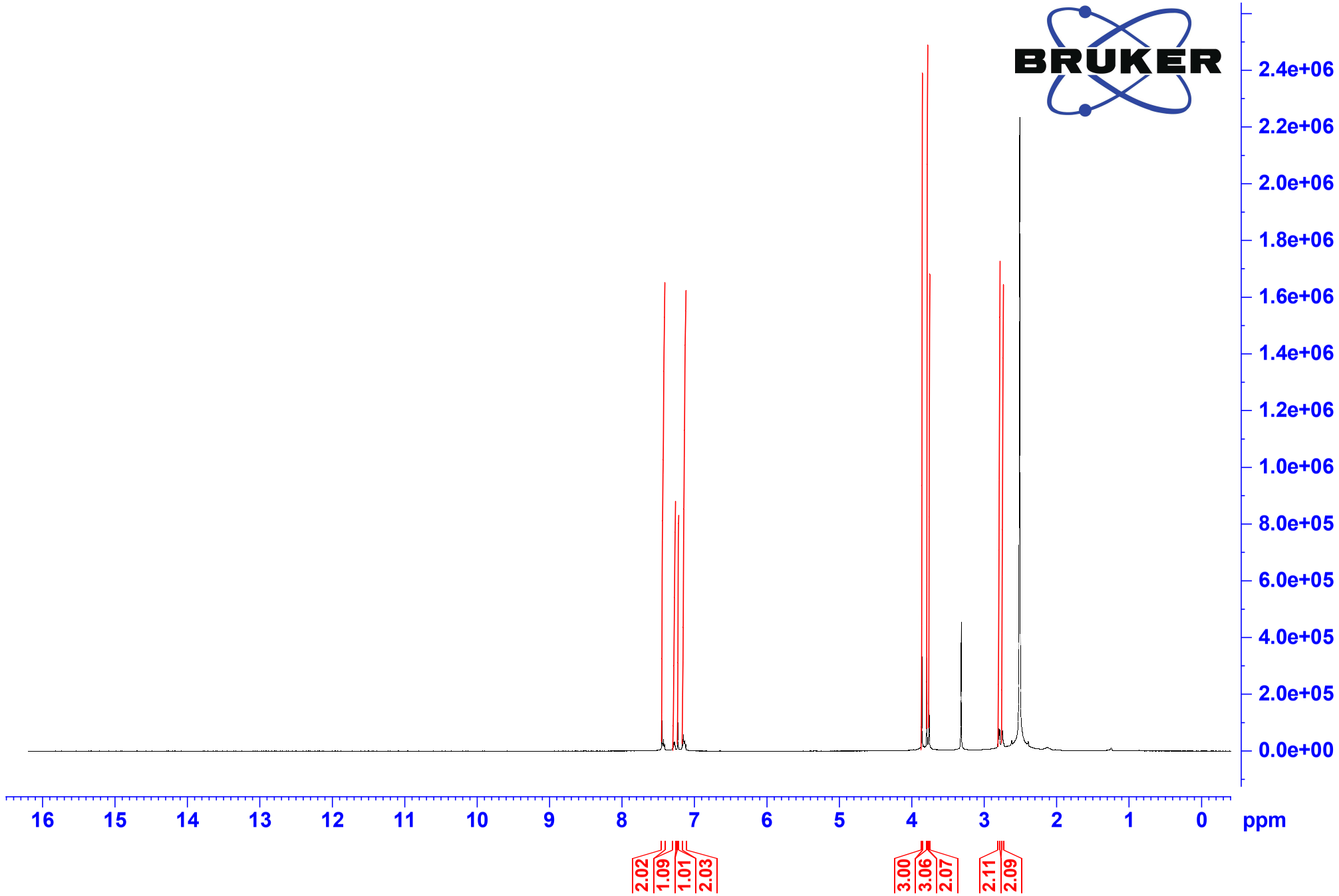
4-nitro-2,5-dimethoxy-N-(2-hydroxybenzyl)phenethylamine hydrochloride (25N-NBOH, 1.81)



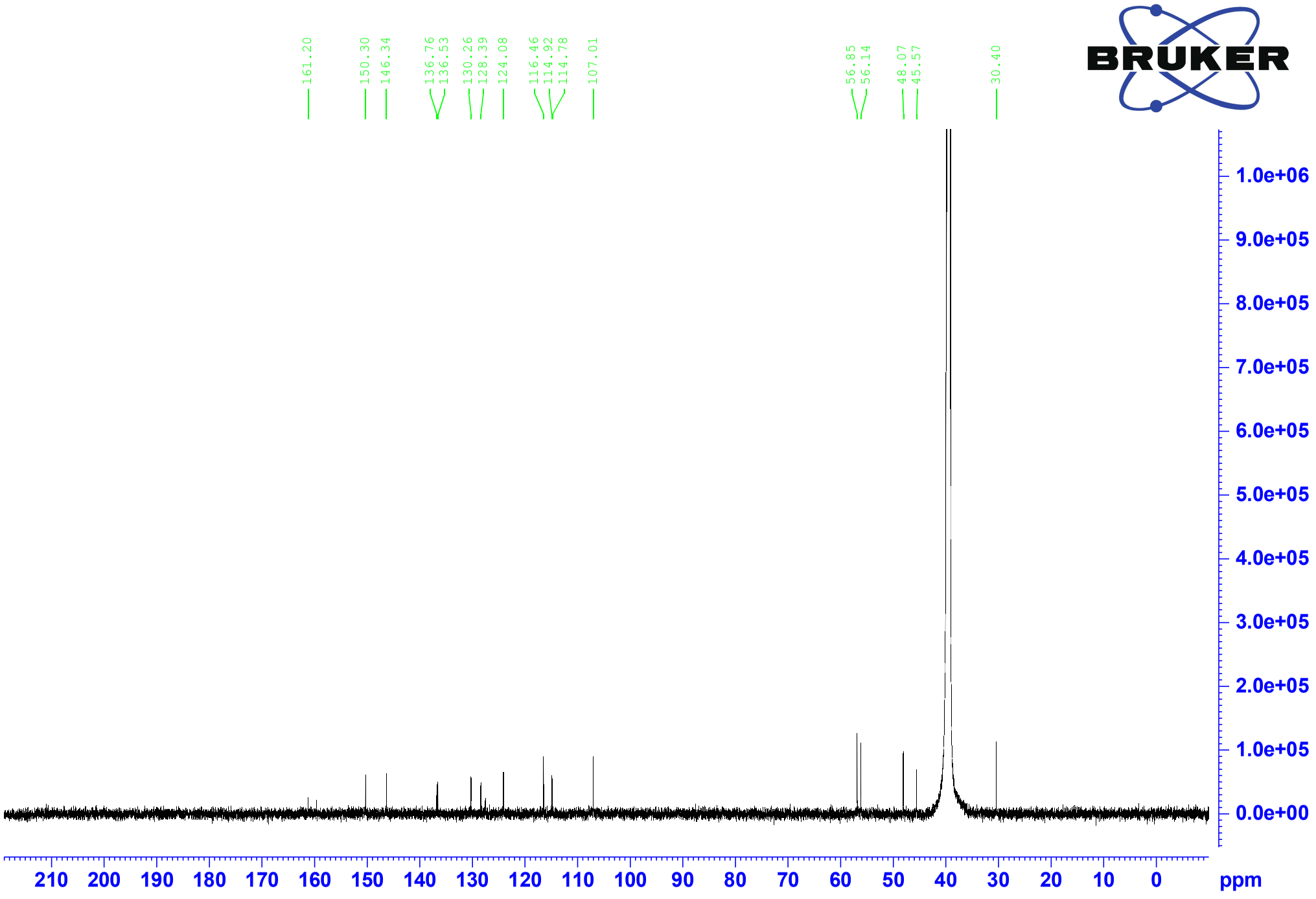
4-nitro-2,5-dimethoxy-N-(2-hydroxybenzyl)phenethylamine hydrochloride (25N-NBOH, 1.81)



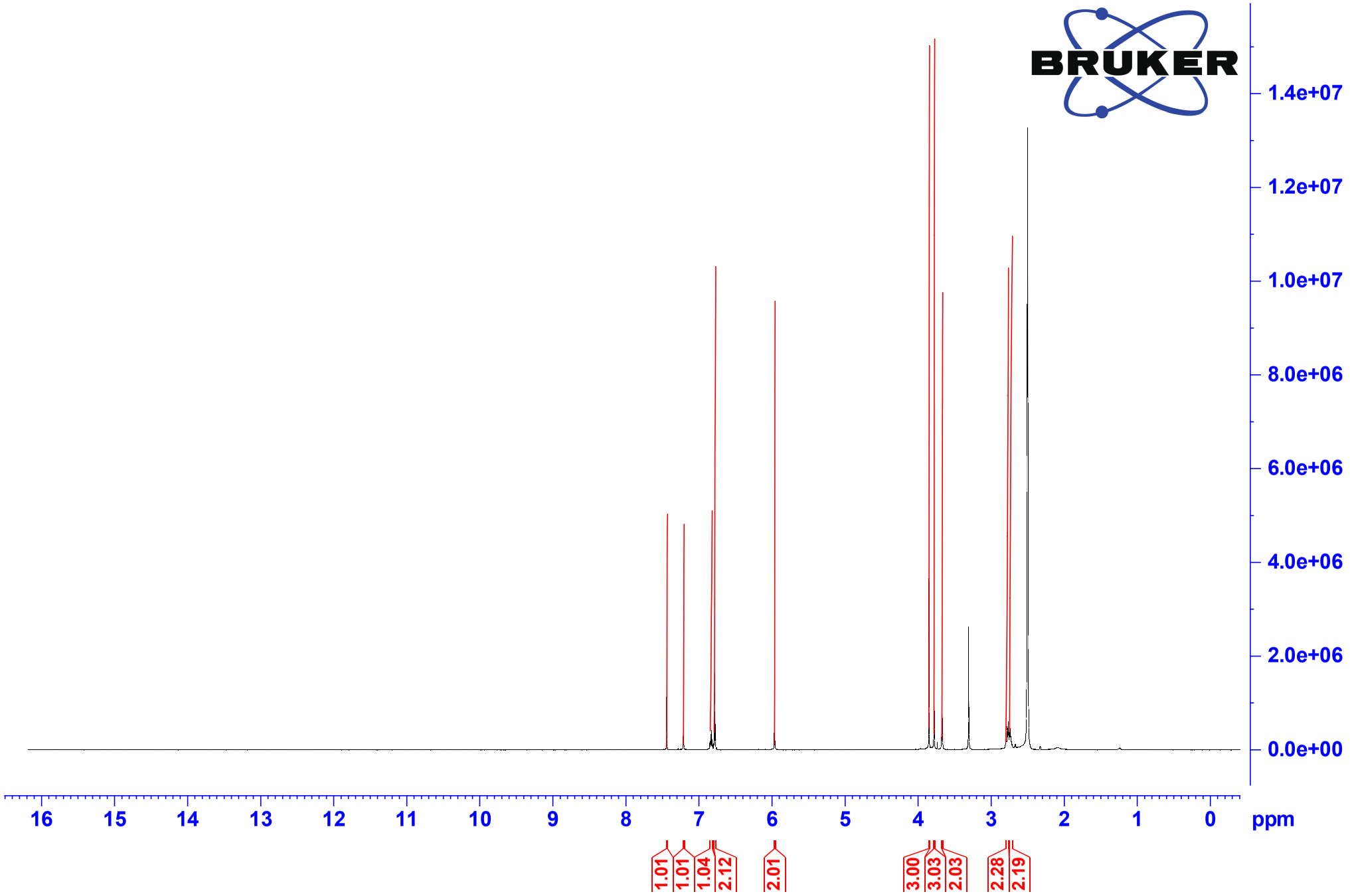
4-nitro-2,5-dimethoxy-N-(2-fluorobenzyl)phenethylamine hydrochloride (25N-NBF, 1.83)



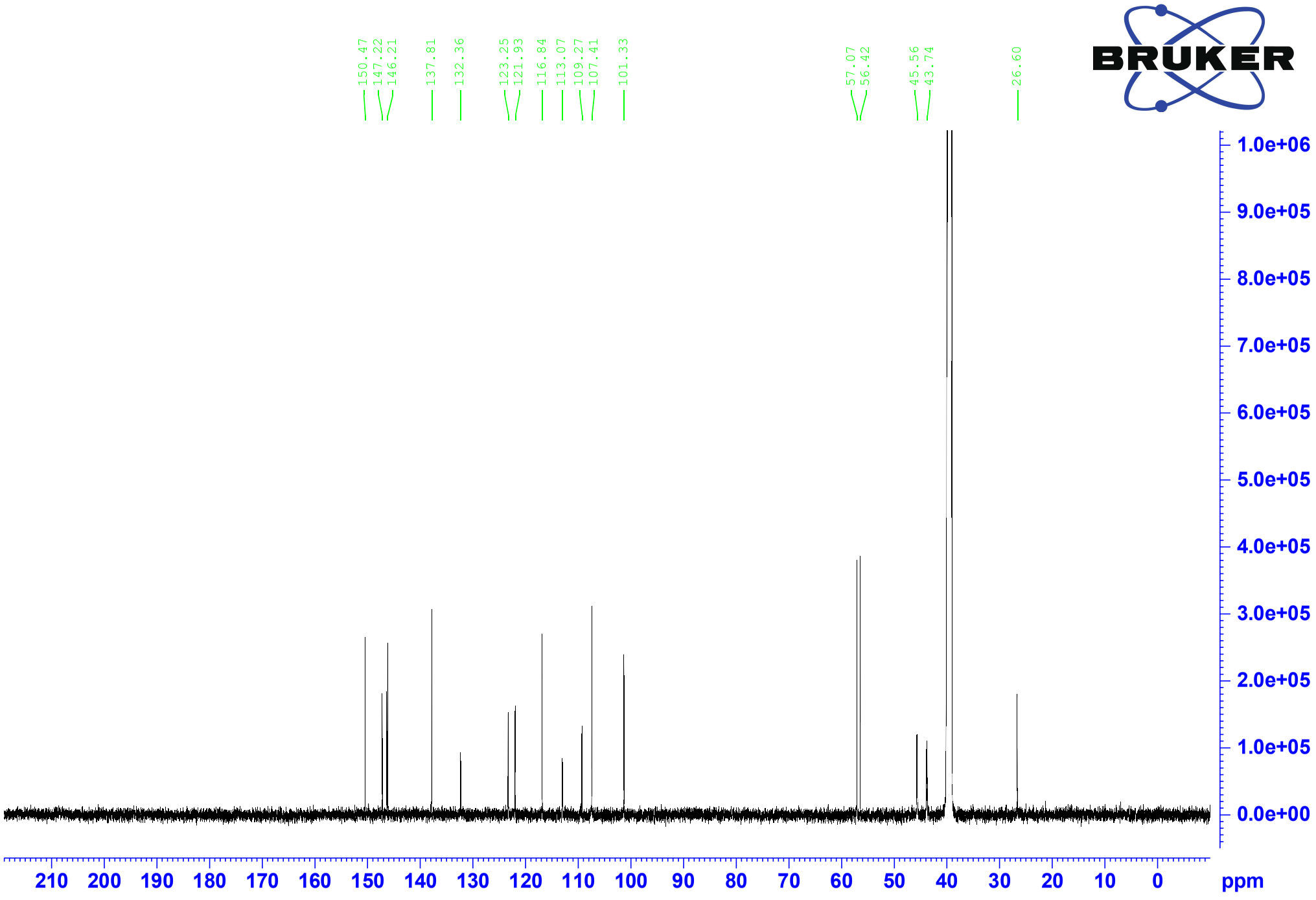
4-nitro-2,5-dimethoxy-N-(2-fluorobenzyl)phenethylamine hydrochloride (25N-NBF, 1.83)



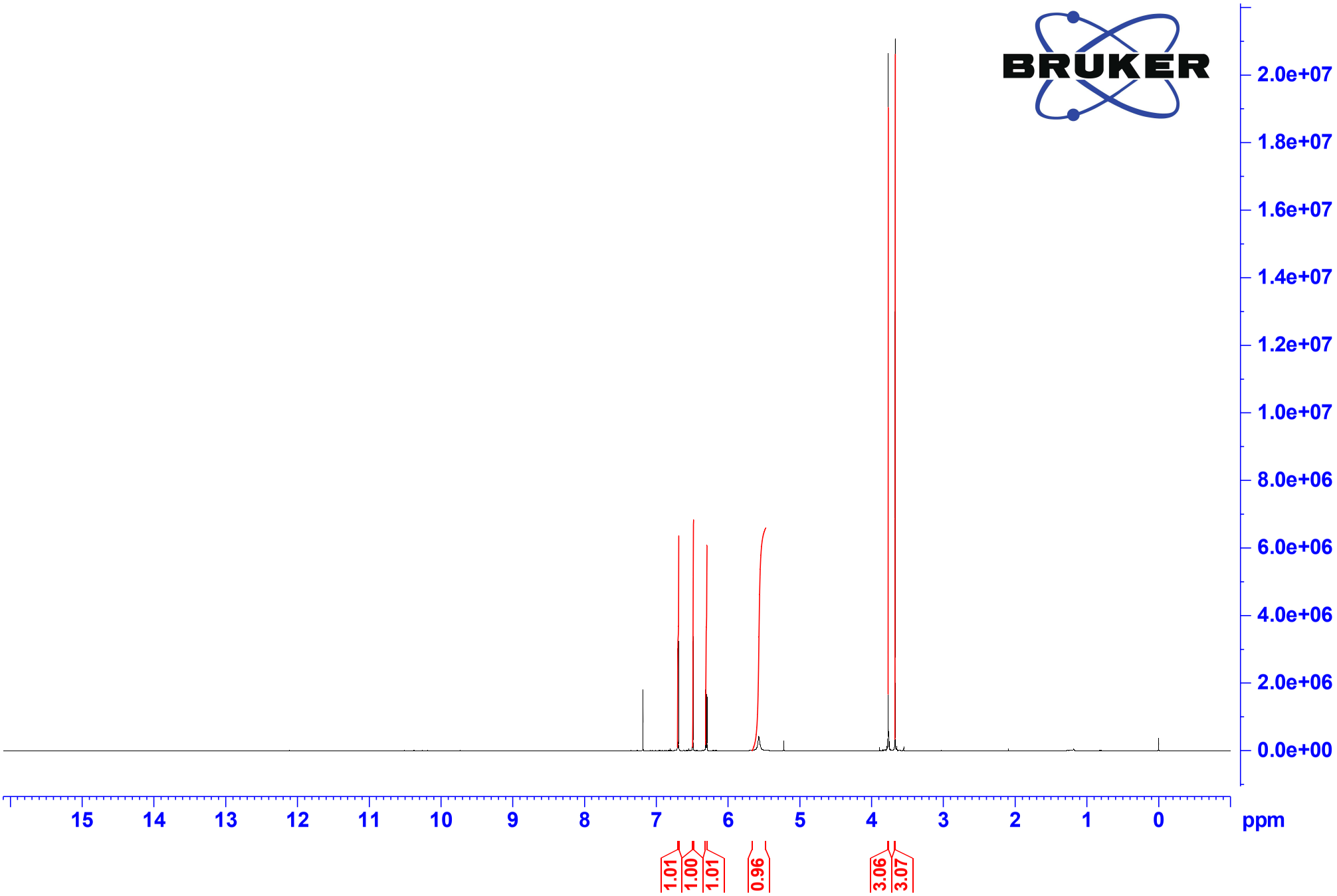
N-(benzo[d][1,3]dioxol-4-ylmethyl)-2-(2,5-dimethoxy-4-nitrophenyl)ethan-1-amine hydrochloride (25N-NBMD, 1.84)



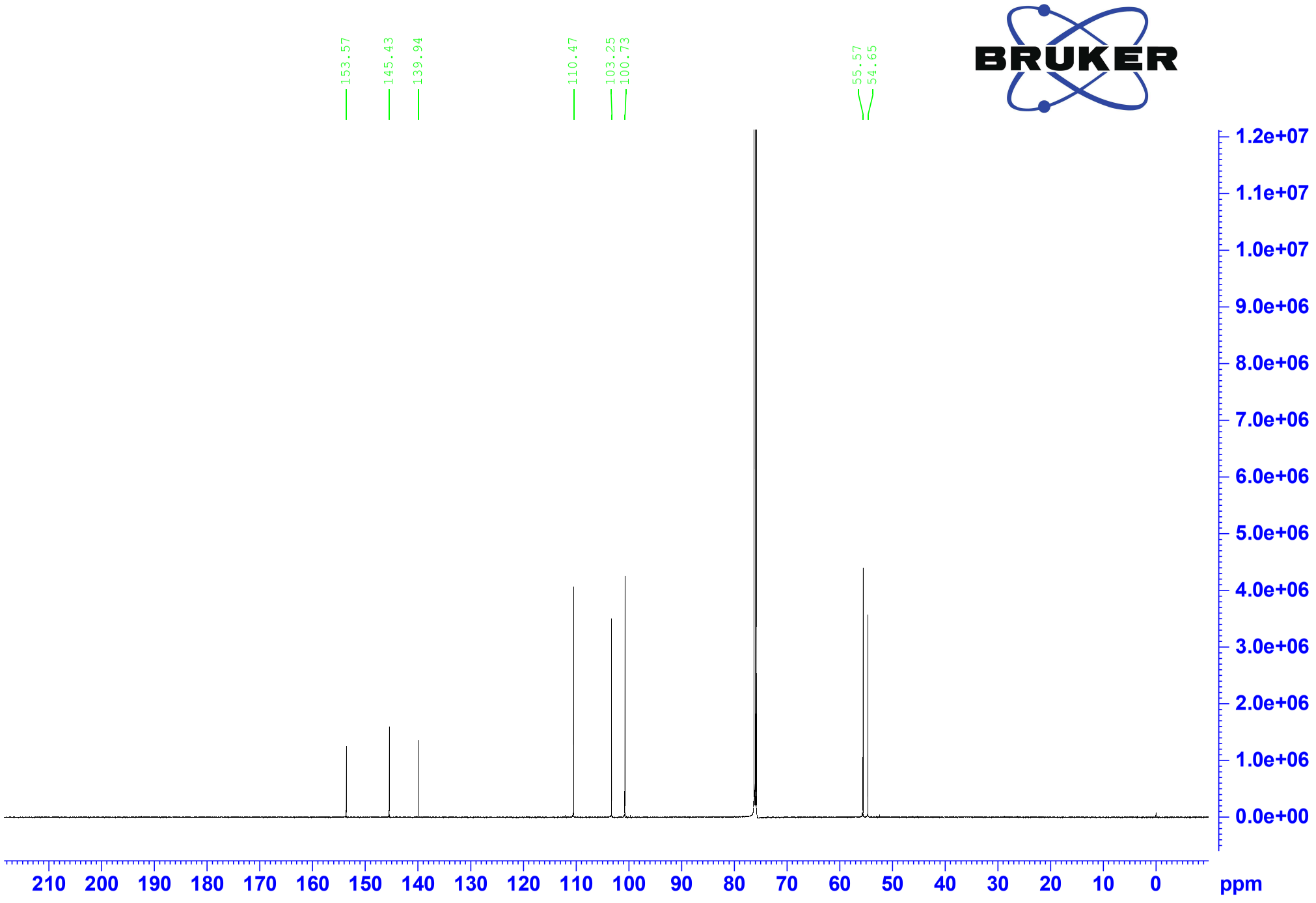
N-(benzo[d][1,3]dioxol-4-ylmethyl)-2-(2,5-dimethoxy-4-nitrophenyl)ethan-1-amine hydrochloride (25N-NBMD, 1.84)



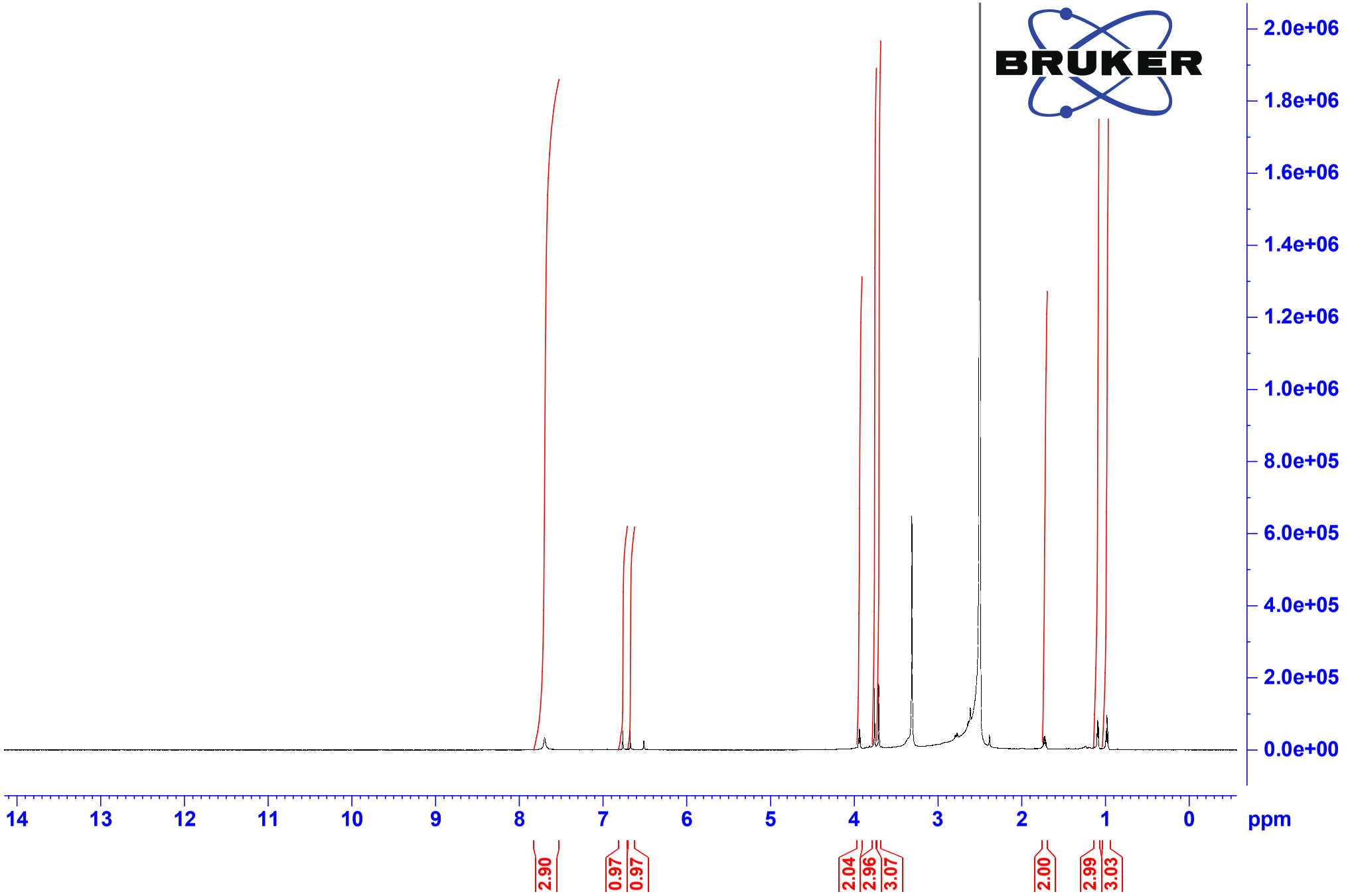
2,5-dimethoxyphenol (4.2)



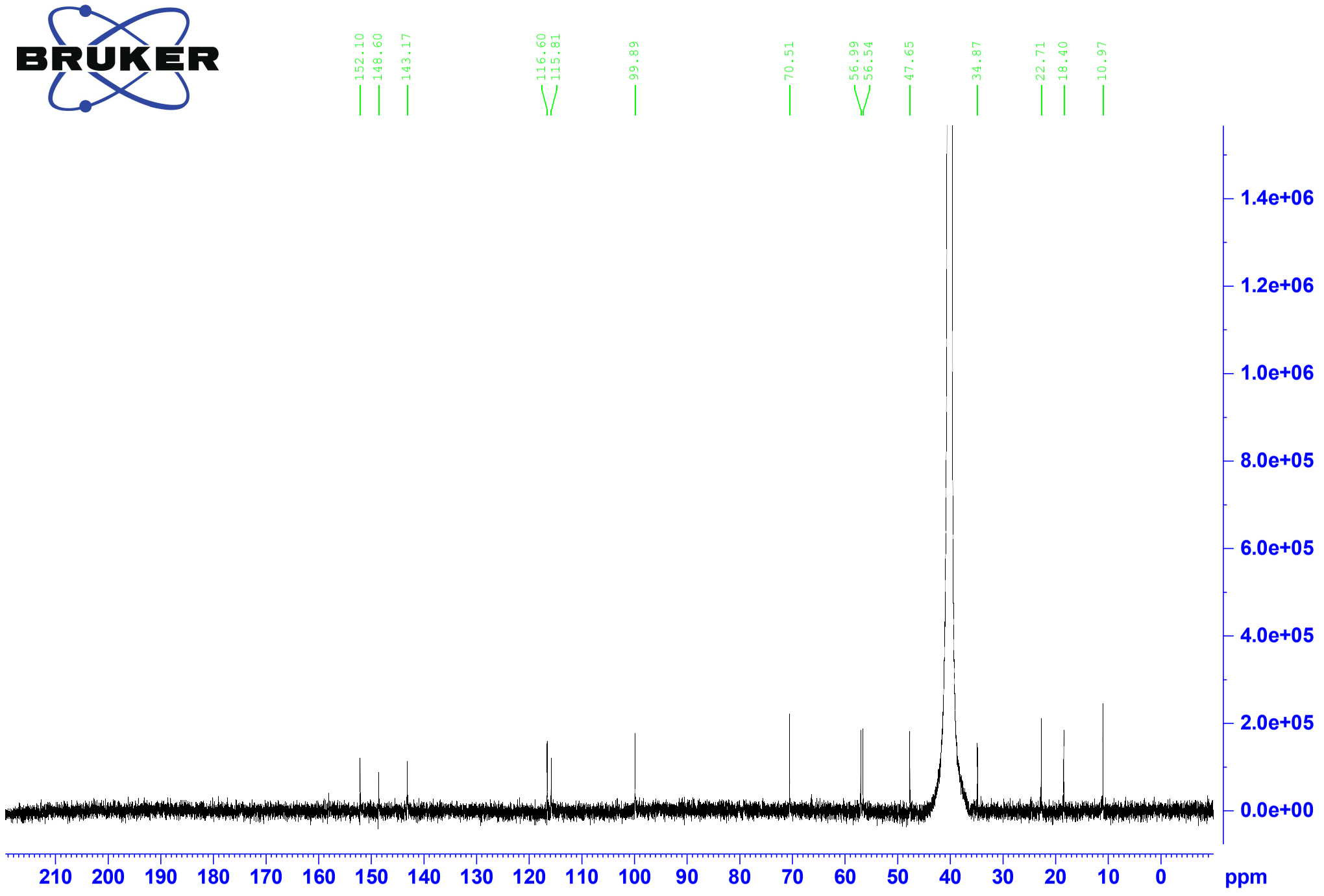
2,5-dimethoxyphenol (4.2)



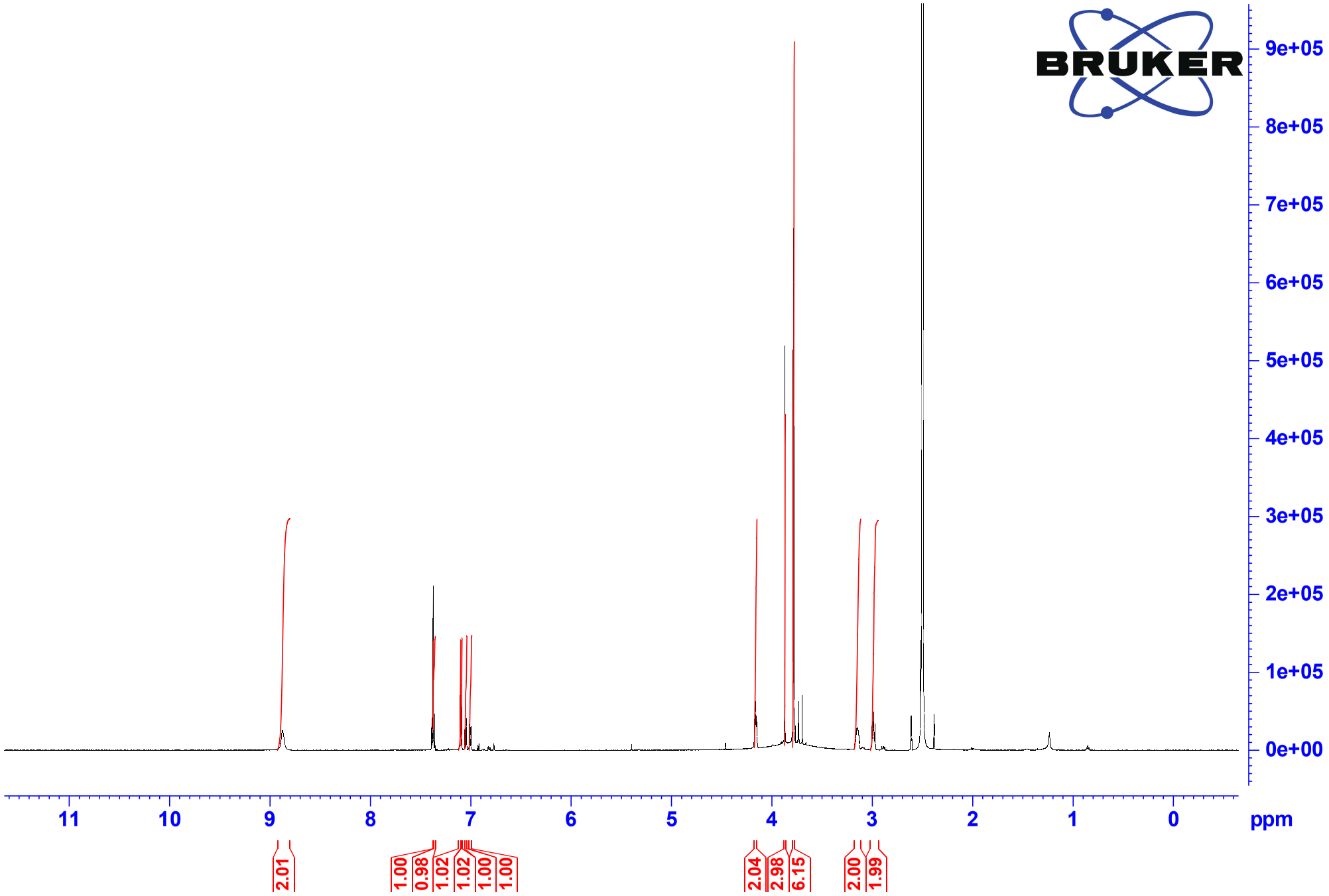
2,5-dimethoxy-4-propoxyamphetamine hydrochloride (MPM, 2.21)



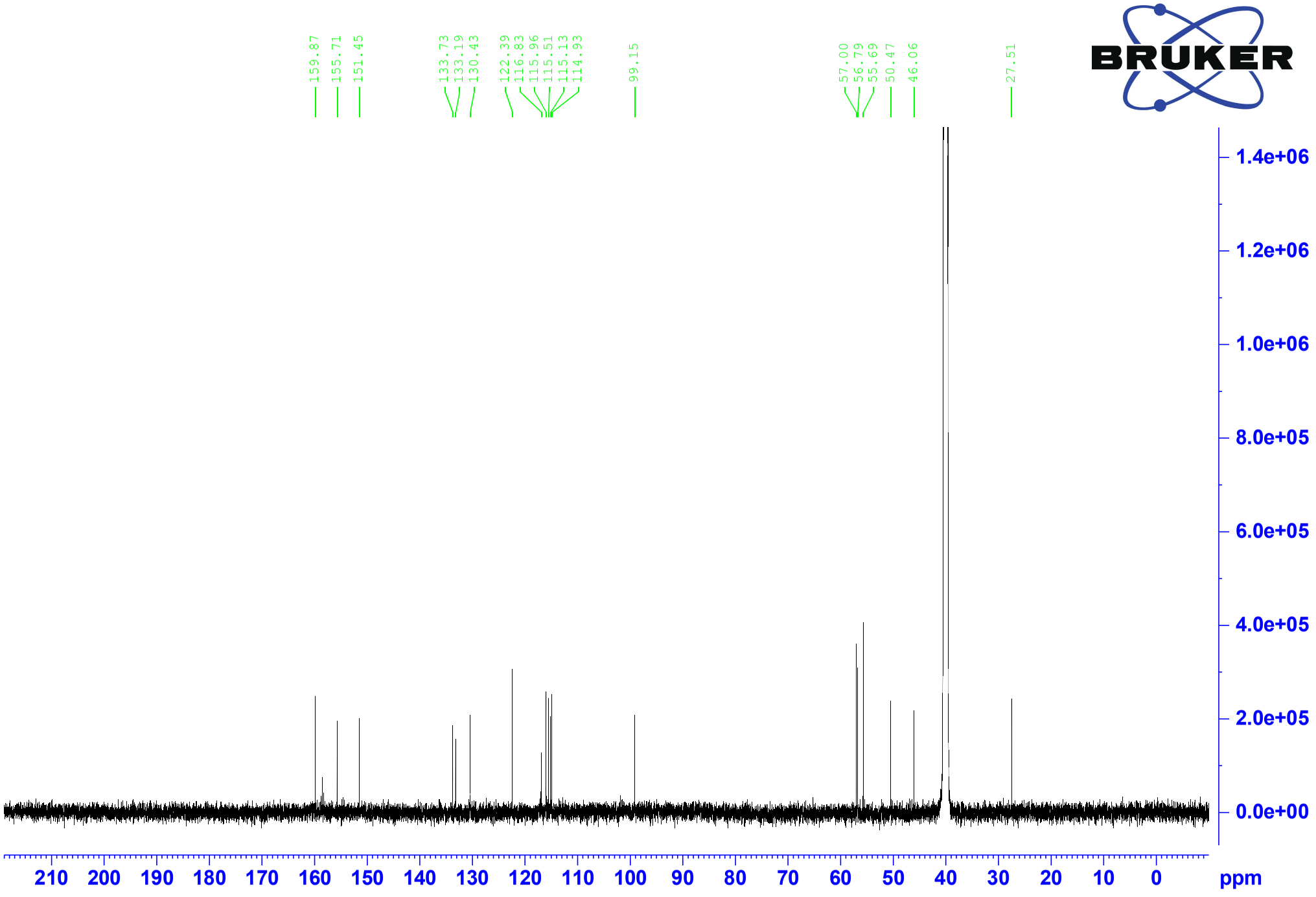
2,5-dimethoxy-4-propoxyamphetamine hydrochloride (MPM, 2.21)



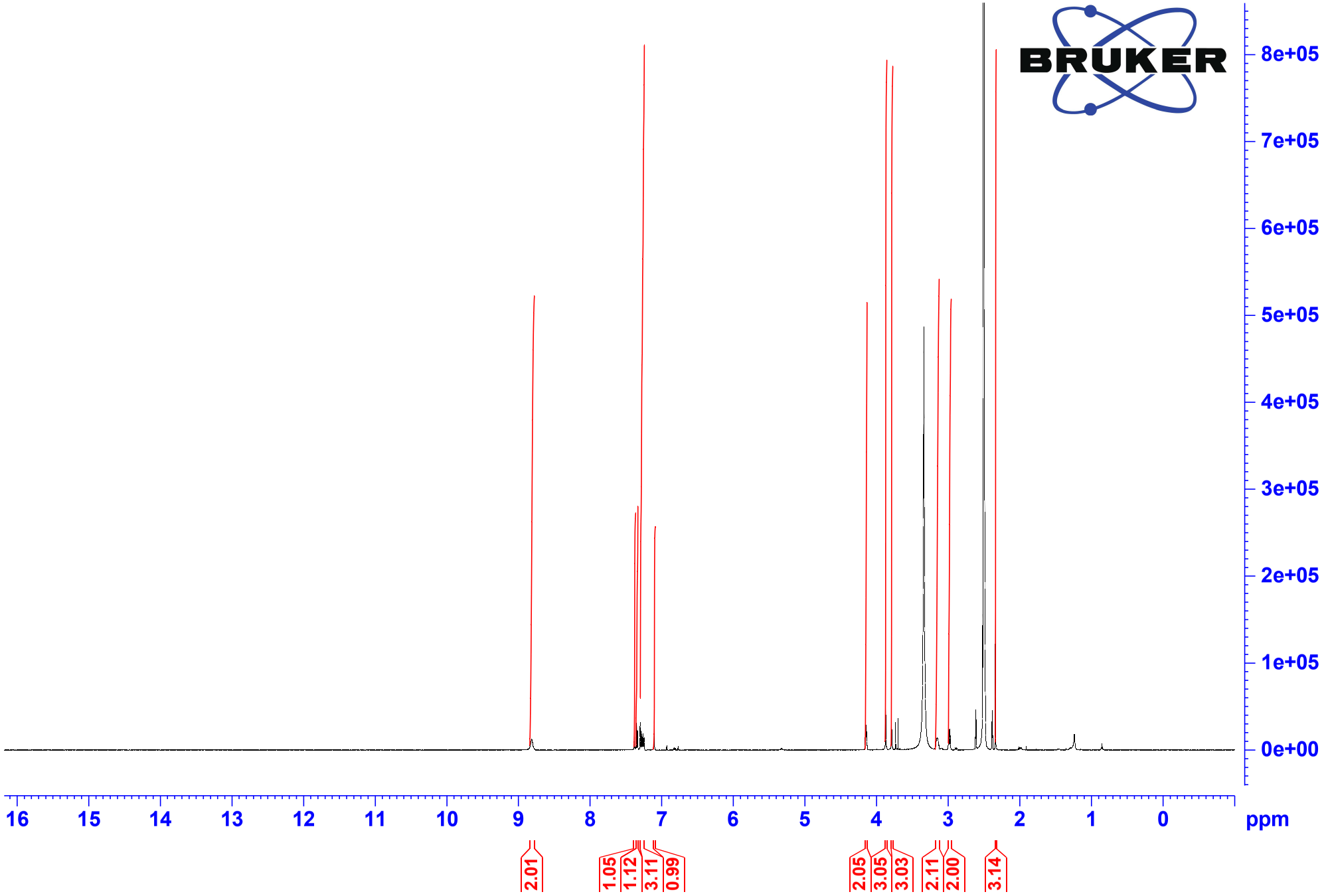
4-cyano-2,5-dimethoxy-N-(3-methoxybenzyl)phenethylamine hydrochloride (1.66)



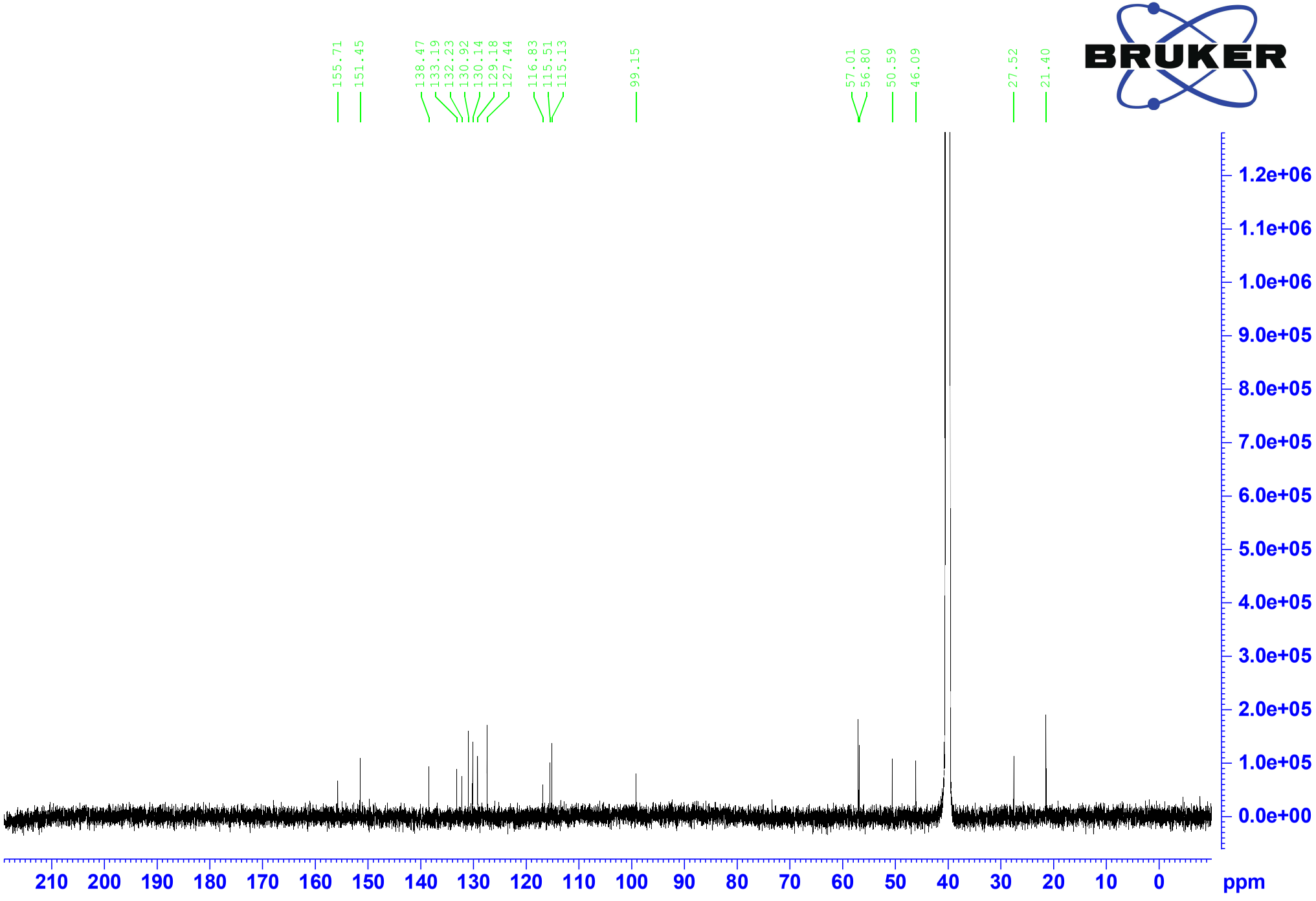
4-cyano-2,5-dimethoxy-N-(3-methoxybenzyl)phenethylamine hydrochloride (1.66)



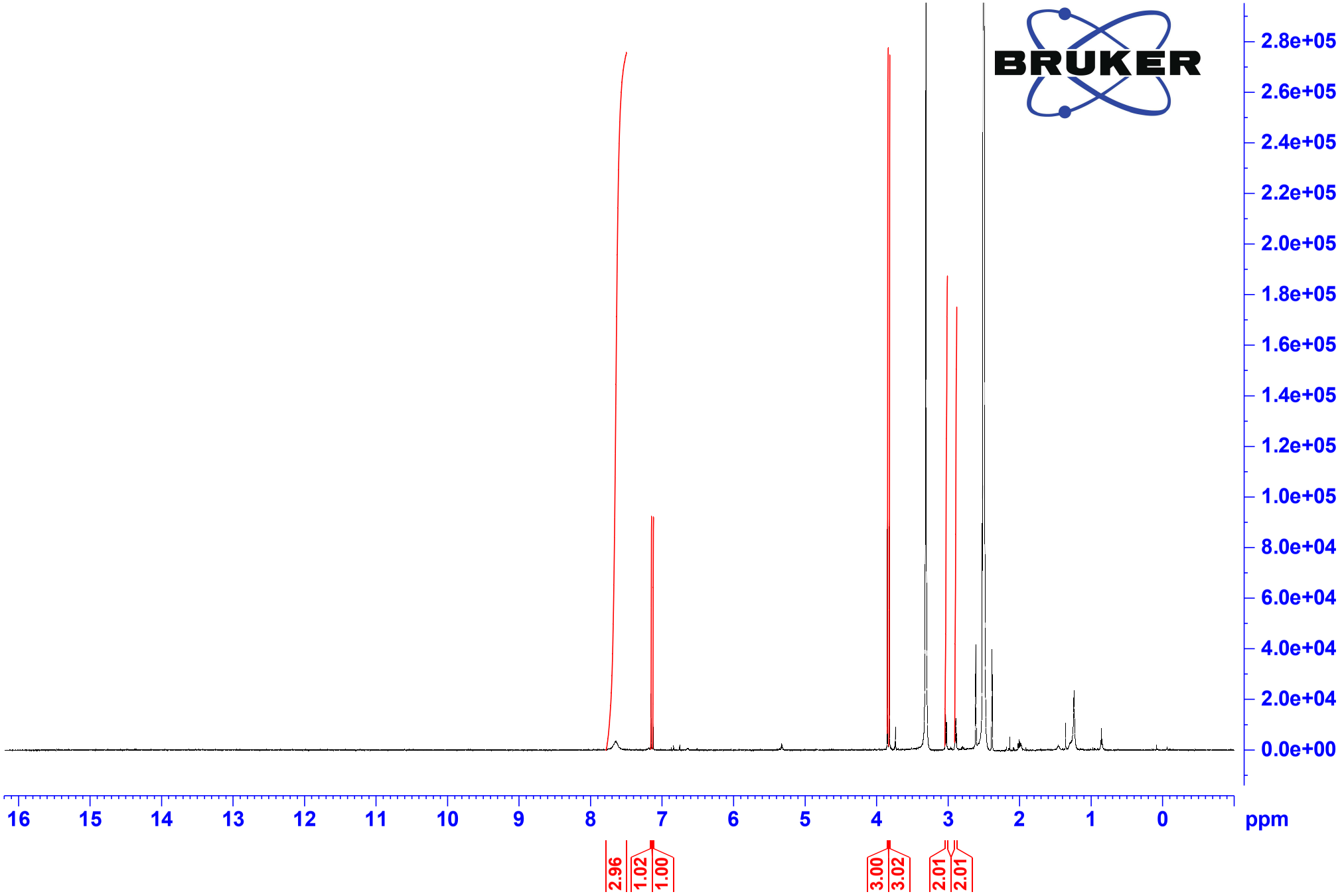
4-cyano-2,5-dimethoxy-N-(3-methylbenzyl)phenethylamine hydrochloride (1.67)



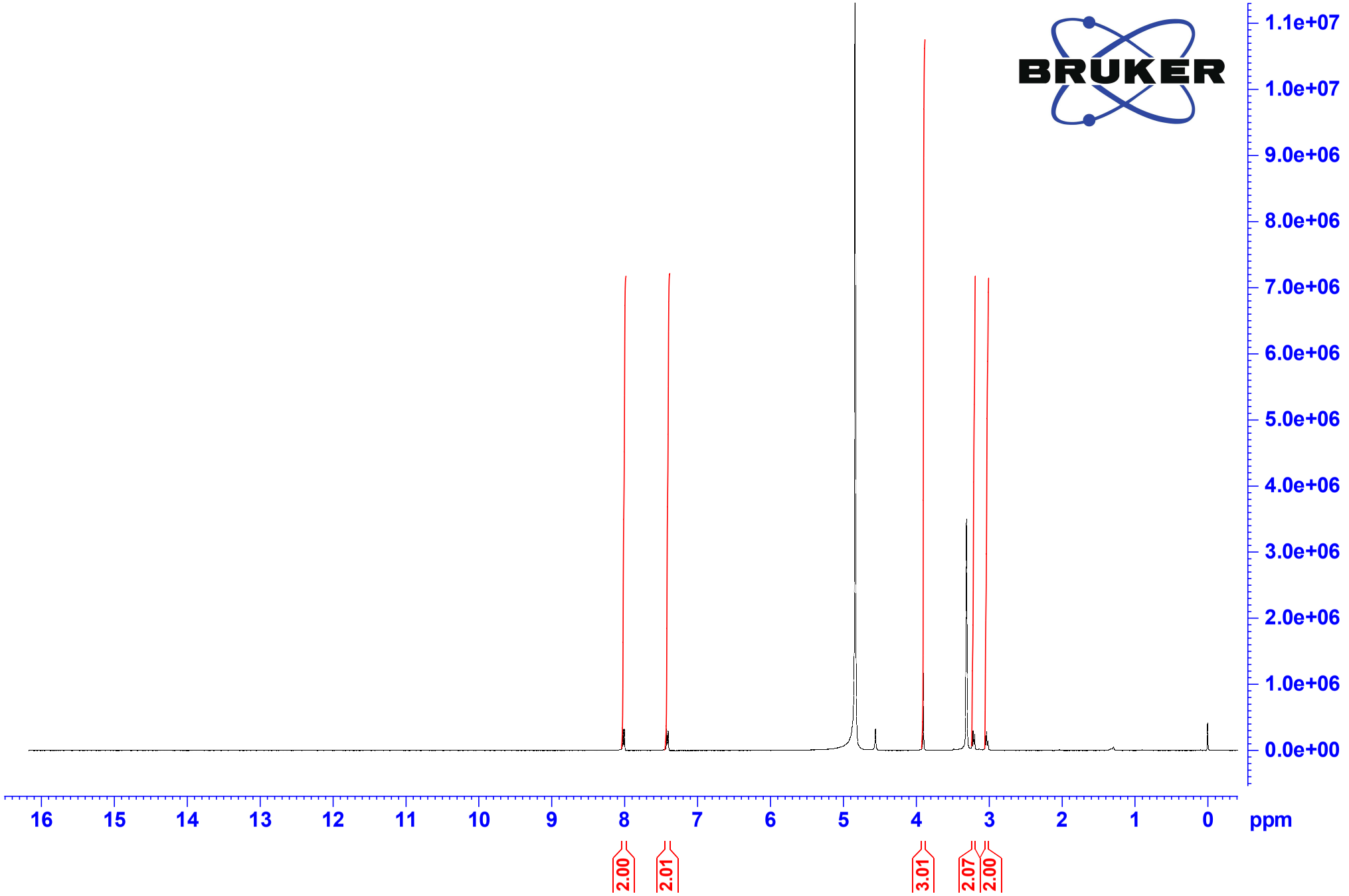
4-cyano-2,5-dimethoxy-N-(3-methylbenzyl)phenethylamine hydrochloride (1.67)



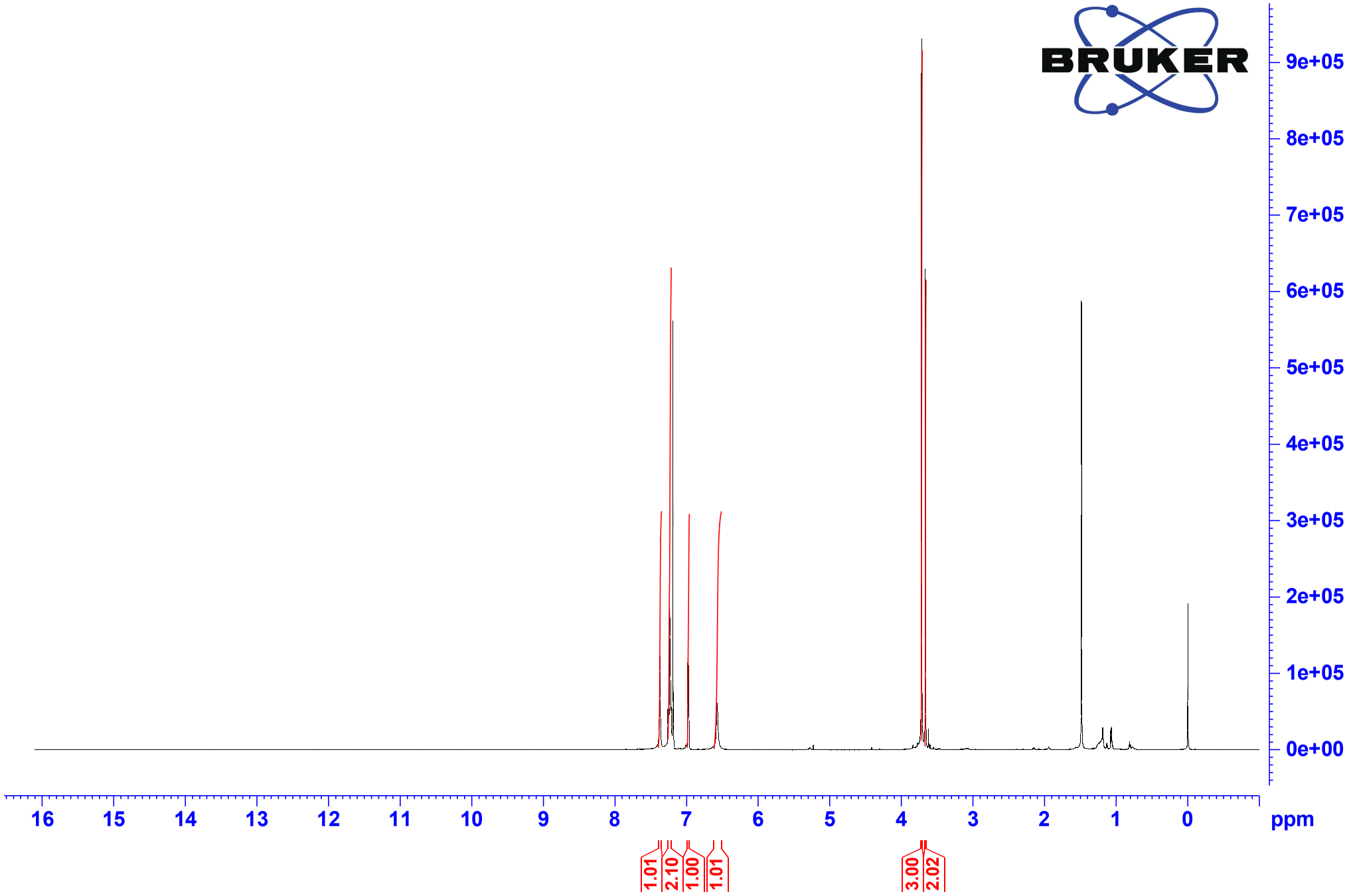
2,5-dimethoxy-4-trifluoromethylphenylethylamine hydrochloride (2C-TFM, 1.12)



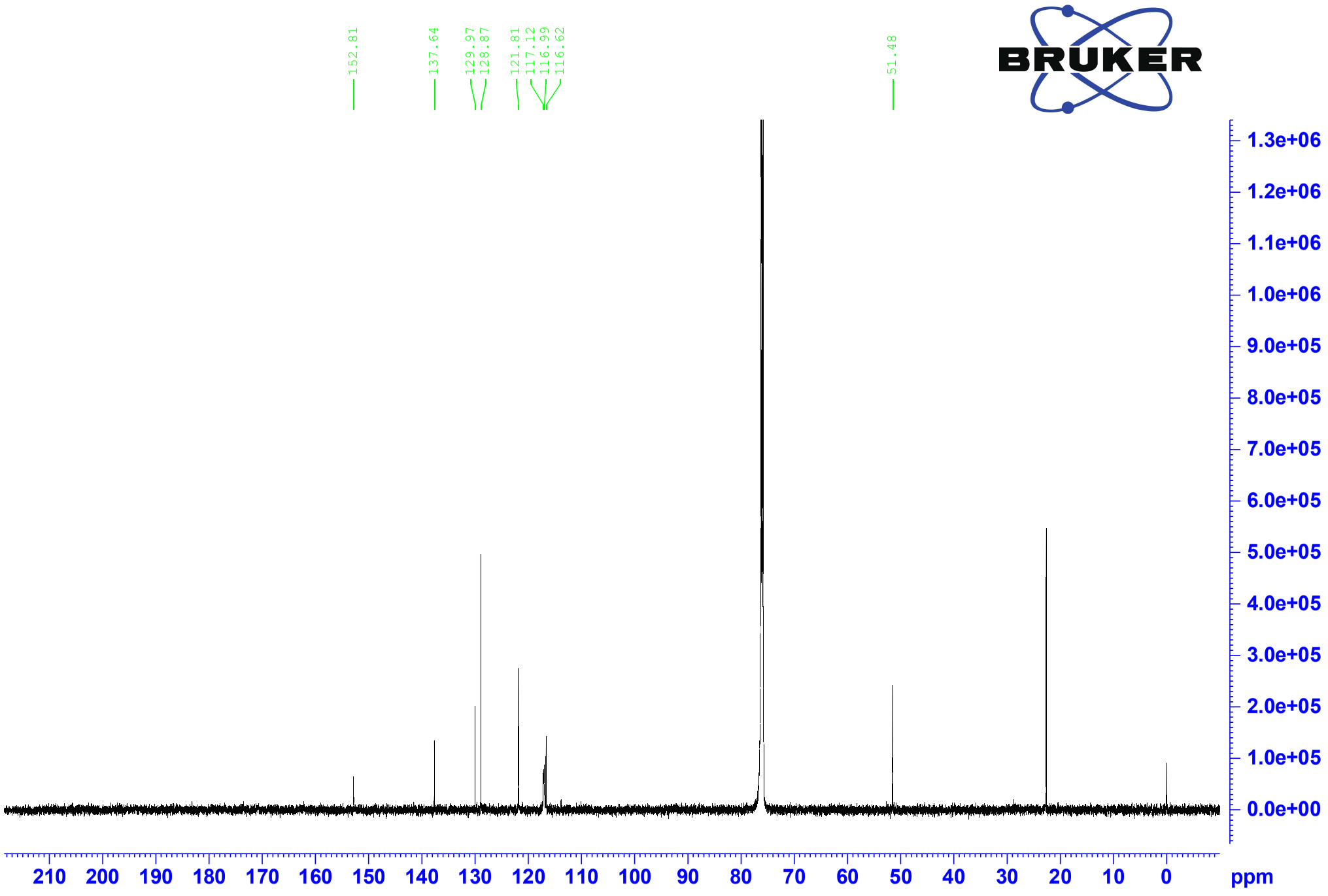
methyl 4-(2-aminoethyl)benzoate hydrochloride (9.2)



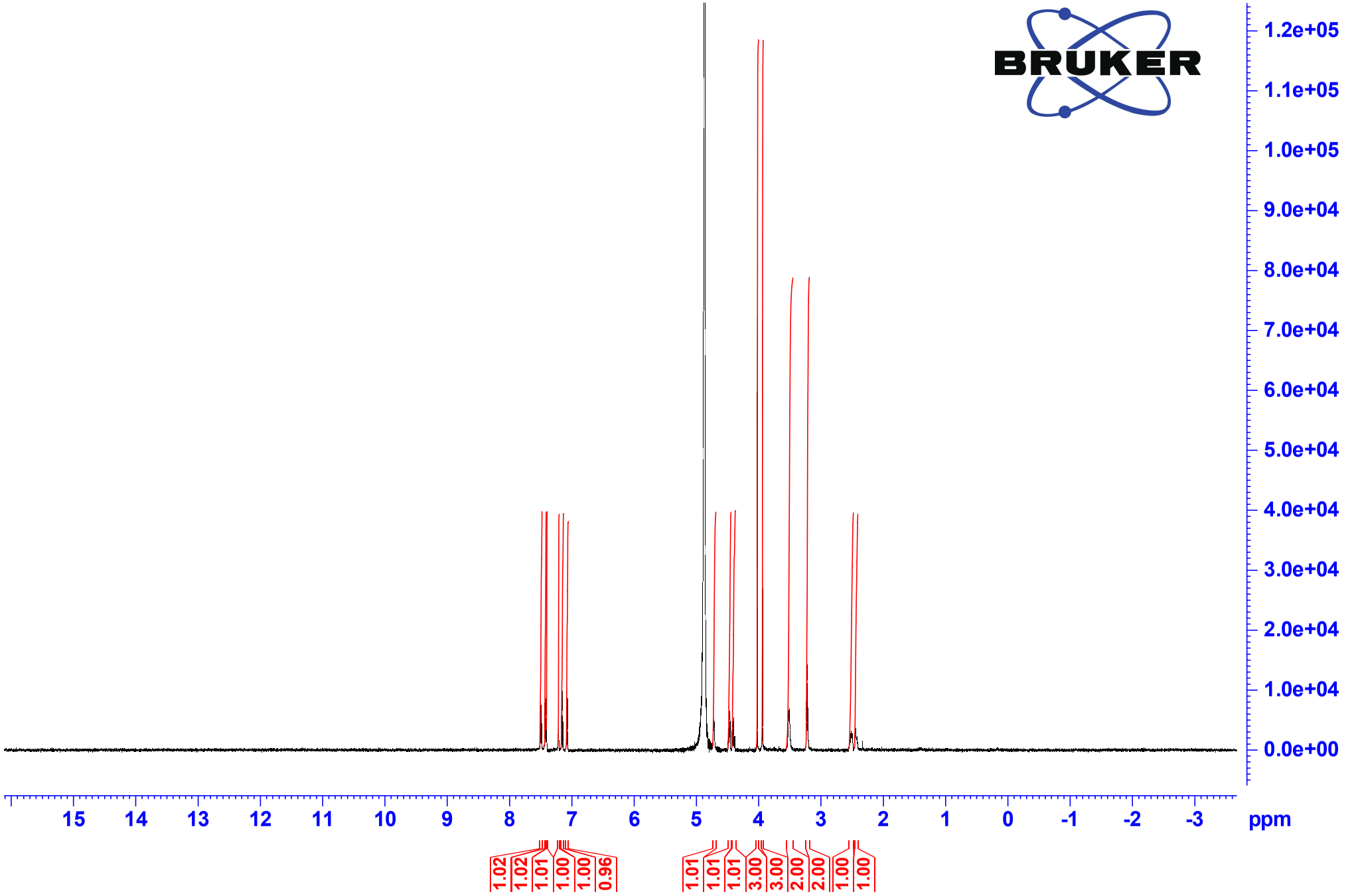
methyl (3-(cyanomethyl)phenyl)carbamate (9.7)



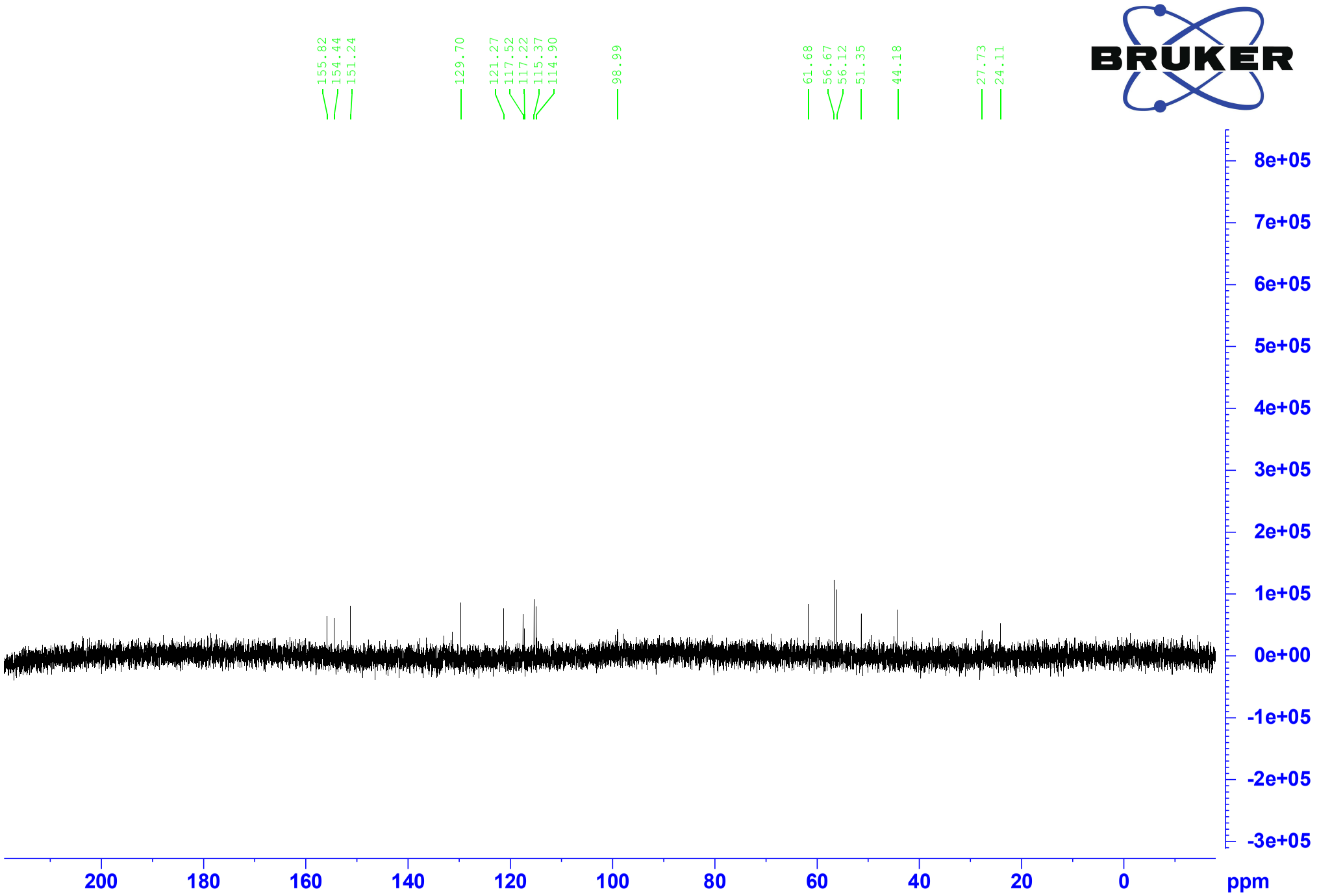
methyl (3-(cyanomethyl)phenyl)carbamate (9.7)



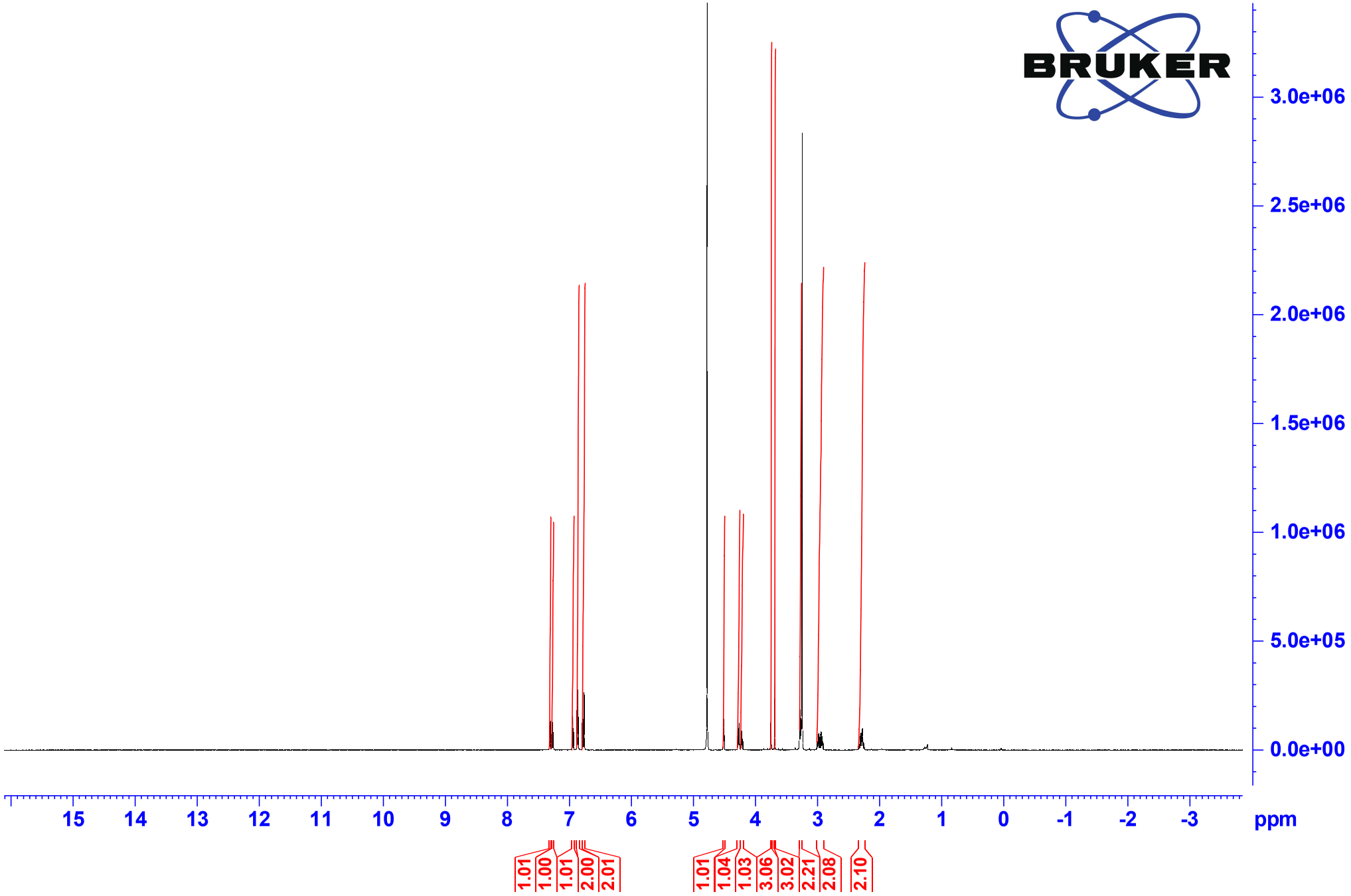
(±)-4-(2-(chroman-4-ylamino)ethyl)-2,5-dimethoxybenzonitrile trifluoroacetate (11.4)



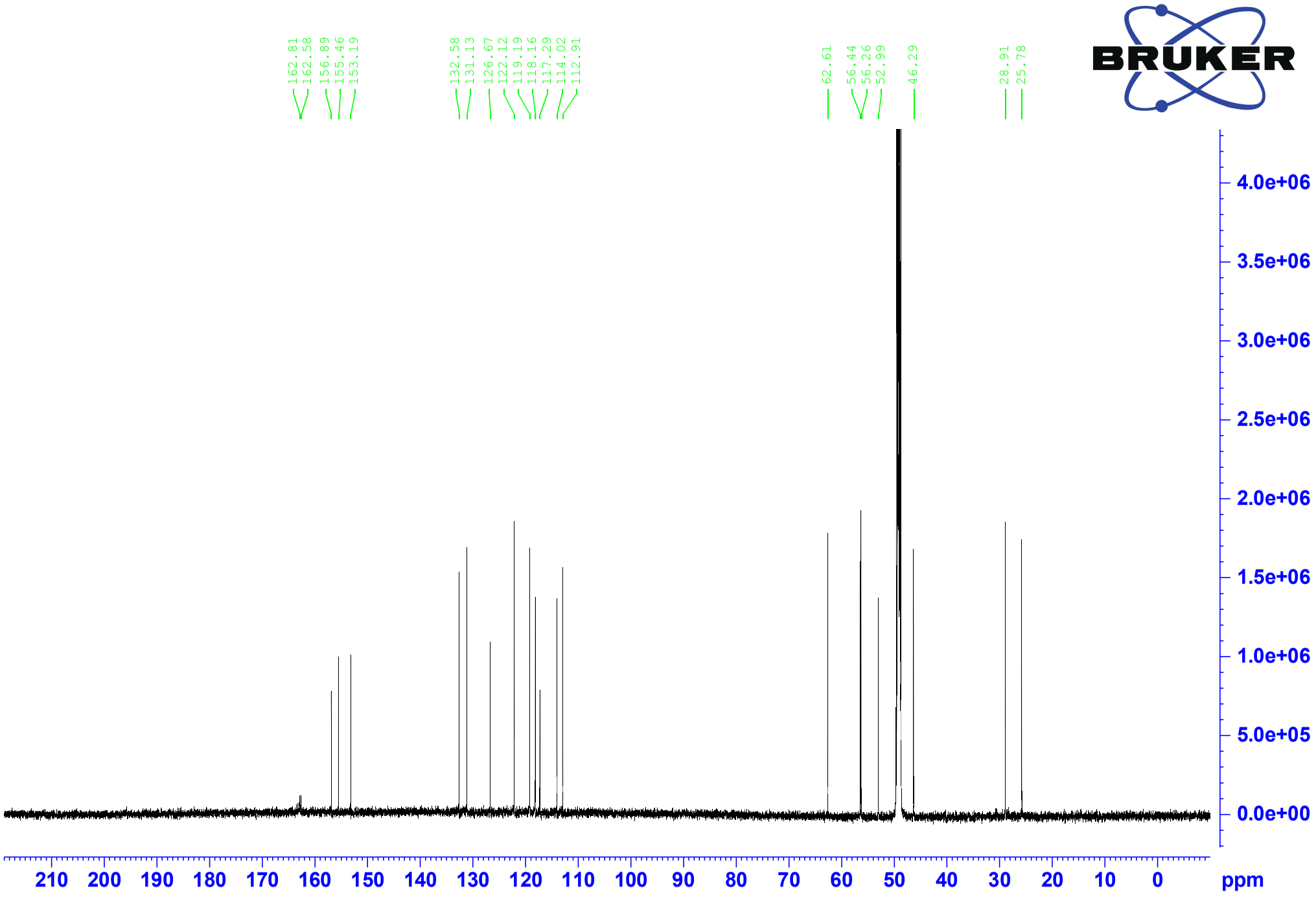
(±)-4-(2-(chroman-4-ylamino)ethyl)-2,5-dimethoxybenzonitrile trifluoroacetate (11.4)



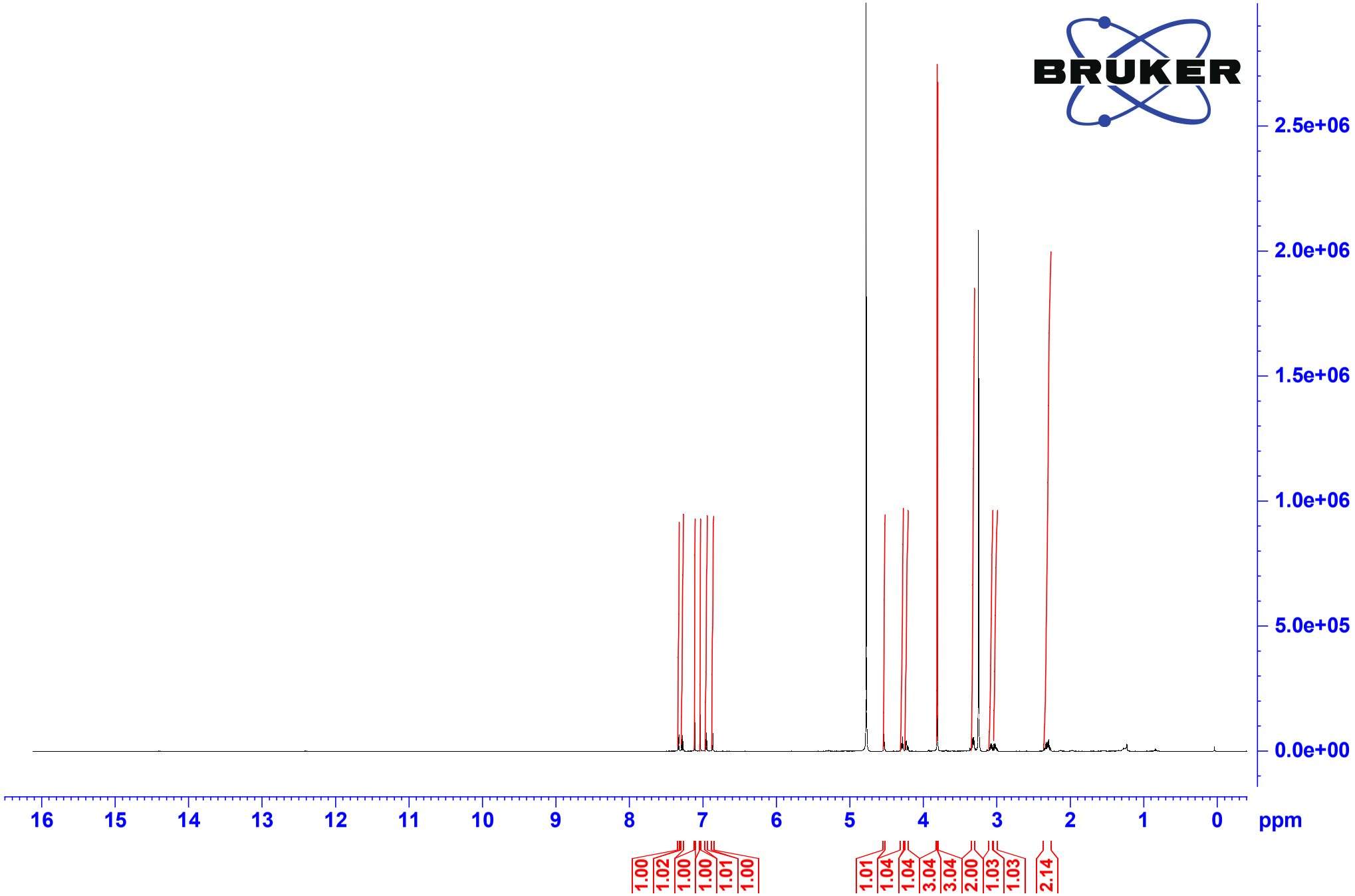
(±)-N-(2,5-dimethoxyphenethyl)chroman-4-amine trifluoroacetate (11.5)



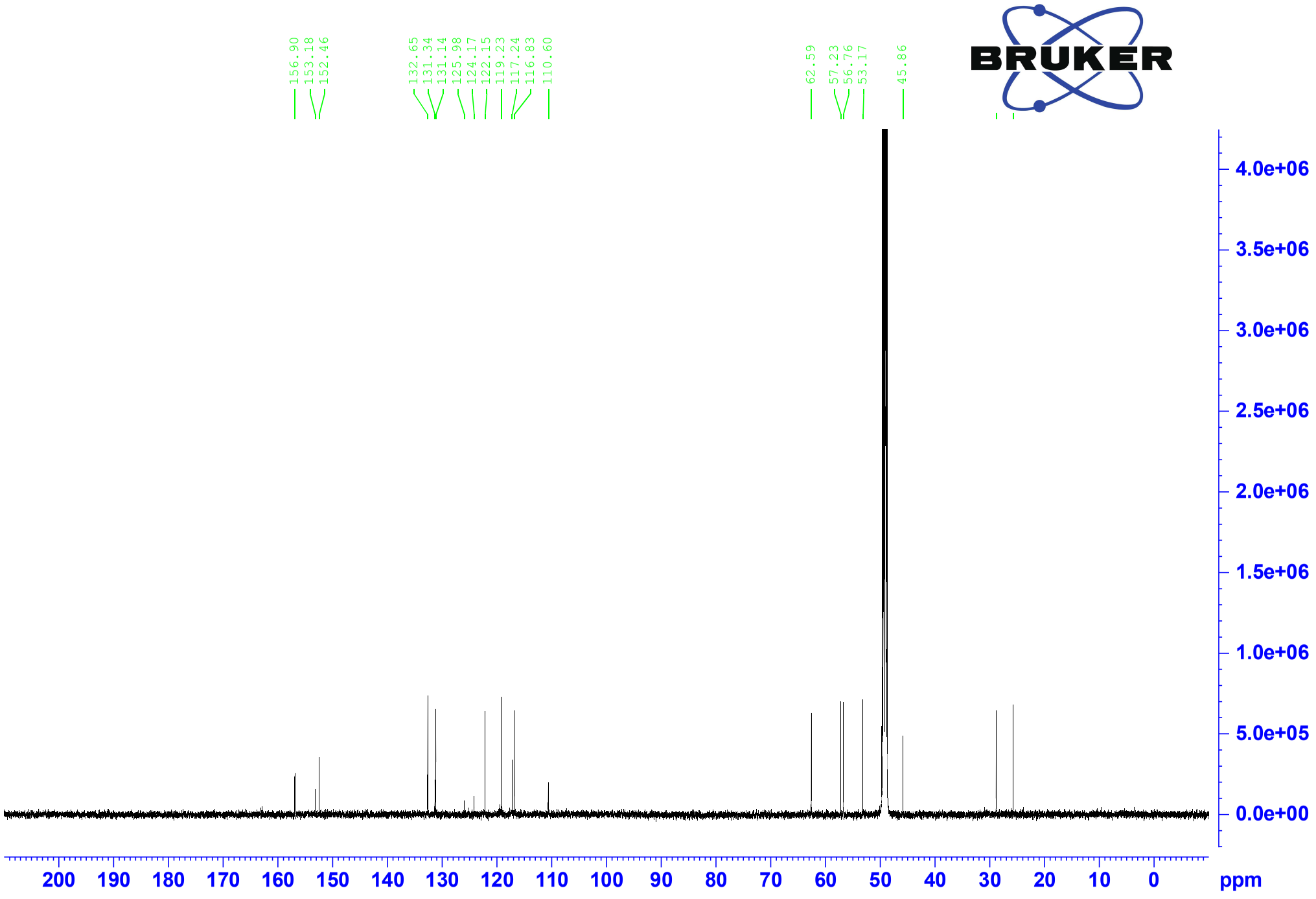
(±)-N-(2,5-dimethoxyphenethyl)chroman-4-amine trifluoroacetate (11.5)



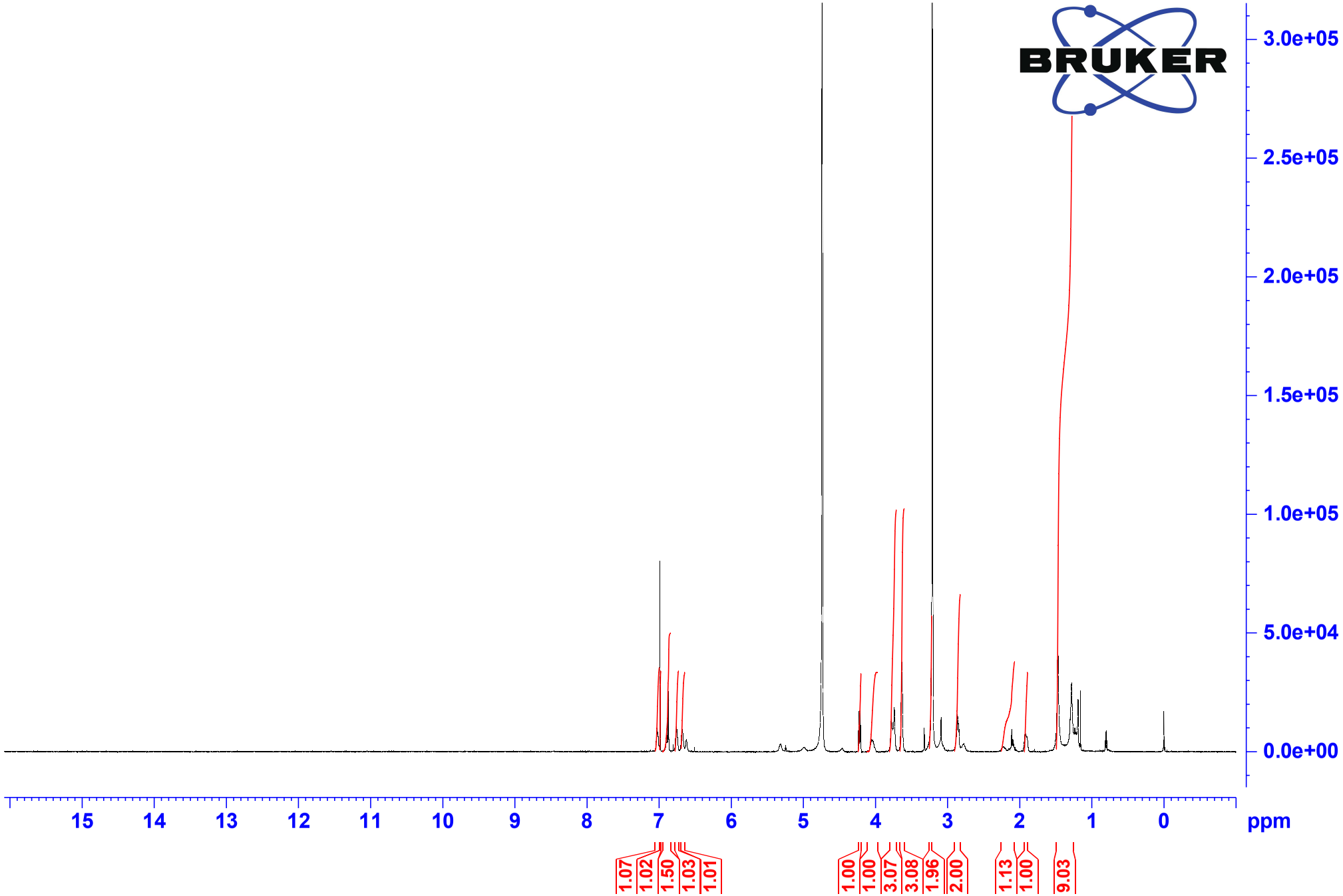
(±)-N-(2,5-dimethoxy-4-(trifluoromethyl)phenethyl)chroman-4-amine trifluoroacetate (11.8)



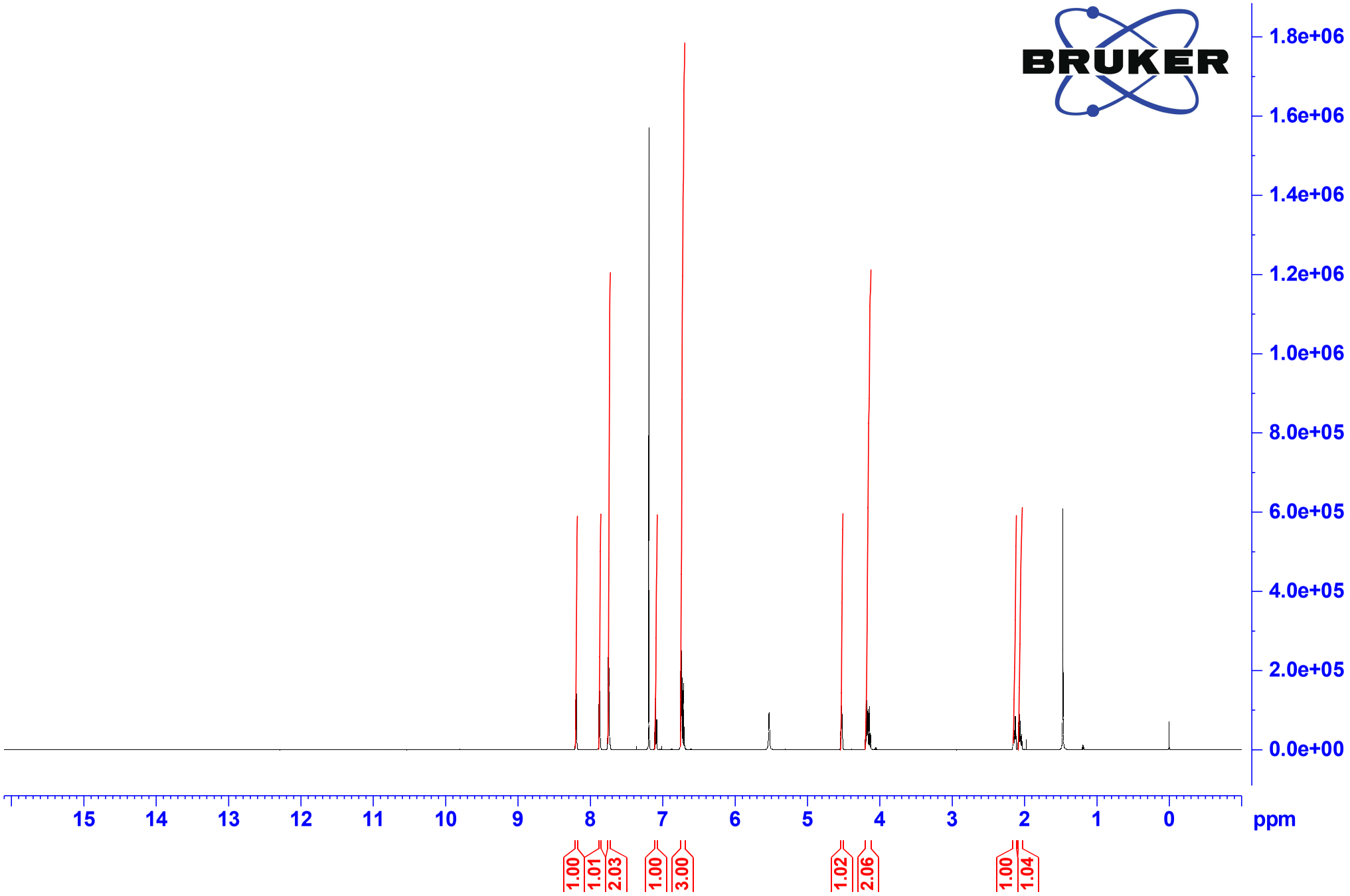
(±)-N-(2,5-dimethoxy-4-(trifluoromethyl)phenethyl)chroman-4-amine trifluoroacetate (11.8)



(±)-tert-butyl chroman-4-yl(4-cyano-2,5-dimethoxyphenethyl)carbamate (12.3)



N-(chroman-4-yl)-2-nitrobenzenesulfonamide (14.2)



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