



8-16-2022

Synthesis and Characterization of *d*₅-Barbarin for Use in Barbarin-Related Research

Sucheta Kudrimoti

University of Kentucky, kusucheta@uky.edu

Jacob Machin

University of Kentucky, jacob.machin@uky.edu

Adedamola S. Arojojoye

University of Kentucky, adedamolaarojojoye@uky.edu

Samuel G. Awuah

University of Kentucky, awuah@uky.edu

Rodney Eisenberg

Frontier BioPharm, LLC.

Follow this and additional works at: https://uknowledge.uky.edu/gerc_facpub

See next page for additional authors

 Part of the [Chemistry Commons](#), [Large or Food Animal and Equine Medicine Commons](#), and the [Pharmacy and Pharmaceutical Sciences Commons](#)

[Right click to open a feedback form in a new tab to let us know how this document benefits you.](#)

Repository Citation

Kudrimoti, Sucheta; Machin, Jacob; Arojojoye, Adedamola S.; Awuah, Samuel G.; Eisenberg, Rodney; Fenger, Clara; Maylin, George; Lehner, Andreas F.; and Tobin, Thomas, "Synthesis and Characterization of *d*₅-Barbarin for Use in Barbarin-Related Research" (2022). *Maxwell H. Gluck Equine Research Center Faculty Publications*. 67.

https://uknowledge.uky.edu/gerc_facpub/67

This Article is brought to you for free and open access by the Maxwell H. Gluck Equine Research Center at UKnowledge. It has been accepted for inclusion in Maxwell H. Gluck Equine Research Center Faculty Publications by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.

Synthesis and Characterization of *d*₅-Barbarin for Use in Barbarin-Related Research

Digital Object Identifier (DOI)

<https://doi.org/10.1002/dta.3357>

Notes/Citation Information

Published in *Drug Testing and Analysis*.

© 2022 The Authors. *Drug Testing and Analysis* published by John Wiley & Sons Ltd.

This is an open access article under the terms of the [Creative Commons Attribution](#) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.



Authors

Sucheta Kudrimoti, Jacob Machin, Adedamola S. Arojojoye, Samuel G. Awuah, Rodney Eisenberg, Clara Fenger, George Maylin, Andreas F. Lehner, and Thomas Tobin

RESEARCH ARTICLE

WILEY

Synthesis and characterization of d_5 -barbarin for use in barbarin-related research

Sucheta Kudrimoti¹ | Jacob Machin¹  | Adedamola S. Arojojoye² | Samuel G. Awuah^{2,3} | Rodney Eisenberg⁴ | Clara Fenger⁵ | George Maylin⁶ | Andreas F. Lehner⁷ | Thomas Tobin¹ 

¹The Department of Veterinary Science and the Maxwell H. Gluck Equine Research Center and the Department of Toxicology and Cancer Biology, University of Kentucky, Lexington, Kentucky, USA

²Department of Chemistry, University of Kentucky, Lexington, Kentucky, USA

³Center for Pharmaceutical Research and Innovation, College of Pharmacy and Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky, USA

⁴Frontier BioPharm, LLC, Richmond, Kentucky, USA

⁵Equine Integrated Medicine, Georgetown, Kentucky, USA

⁶New York Drug Testing and Research Program, Ithaca, New York, USA

⁷Veterinary Diagnostic Lab Section of Toxicology, Michigan State University, Lansing, Michigan, USA

Correspondence

Thomas Tobin, The Department of Veterinary Science and the Maxwell H. Gluck Equine Research Center and the Department of Toxicology and Cancer Biology, University of Kentucky, Lexington, KY 40546, USA.
Email: ttobin@uky.edu

Funding information

Funding sources are as noted in the Acknowledgements section. Funding sources provided no role in the design of the study nor in the collection, analysis, and interpretation of these data.

Abstract

Based on structural similarities and equine administration experiments, Barbarin, 5-phenyl-2-oxazolidinethione from *Brassicaceae* plants, is a possible source of equine urinary identifications of aminorex, (*R,S*)-5-phenyl-4,5-dihydro-1,3-oxazol-2-amine, an amphetamine-related US Drug Enforcement Administration (DEA) controlled substance considered illegal in sport horses. We now report the synthesis and certification of d_5 -barbarin to facilitate research on the relationship between plant barbarin and such aminorex identifications. D_5 -barbarin synthesis commenced with production of d_5 -2-oxo-2-phenylacetaldehyde oxime (d_5 -oxime) from d_5 -acetophenone via butylnitrite in an ethoxide/ethanol solution. This d_5 -oxime was then reduced with lithium aluminum hydride (LiAlH_4) to produce the corresponding d_5 -2-amino-1-phenylethan-1-ol (d_5 -phenylethanolamine). Final ring closure of the d_5 -phenylethanolamine was performed by the addition of carbon disulfide (CS_2) with pyridine. The reaction product was purified by recrystallization and presented as a stable white crystalline powder. Proton NMR spectroscopy revealed a triplet at 5.88 ppm for one proton, a double doublet at 3.71 ppm for one proton, and double doublet at 4.11 ppm for one proton, confirming d_5 -barbarin as the product. Further characterization by high resolution mass spectrometry supports the successful synthesis of d_5 -barbarin. Purity of the recrystallized product was ascertained by High Performance Liquid Chromatography (HPLC) to be greater than 98%. Together, we have developed the synthesis and full characterization of d_5 -barbarin for use as an internal standard in barbarin-related and equine forensic research.

KEYWORDS

aminorex, *Barbarea vulgaris*, d_5 -barbarin, equine forensic science, internal standard

Sucheta Kudrimoti, Jacob Machin, and Adedamola S. Arojojoye shared first authorship.

TT conceived and directed the project, and TT, CF of the North American Association of Racetrack Veterinarians (NAARV), and GAM, Director of the New York Drug Testing and Research Program, contributed to the data interpretation and analysis and reviewed and approved the proposed interim SLOD from an equine practitioner and regulatory scientist's point of view. TT, RE, SK, and JJM designed the basic chemical synthesis reactions, AFL, RE, SK, JJM, GM, ASA, and SGA reviewed the NMR and mass spectral data and advised and contributed to manuscript drafting and interpretation. TT coordinated, organized, and edited all drafts of this manuscript with ongoing contributions from all authors, and all authors reviewed approved the final manuscript submitted for publication.

This is an open access article under the terms of the [Creative Commons Attribution](https://creativecommons.org/licenses/by/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2022 The Authors. *Drug Testing and Analysis* published by John Wiley & Sons Ltd.

1 | INTRODUCTION

Based on structural similarities and equine administration experiments, barbarin, 5-phenyl-2-oxazolidinethione from *Brassicaceae* plants, is a possible source of aminorex (*R,S*)-5-phenyl-4,5-dihydro-1,3-oxazol-2-amine, (Figure 1) identifications in race and sport horse urines. Aminorex is an amphetamine-related (Hofmaier et al, 2013⁴) US Drug Enforcement Administration (DEA) controlled substance considered illegal in racing and sport horses. Aminorex is also an Association of Racing Commissioners International [ARCI] Class 1, Penalty class A foreign substance, so identifications of aminorex in equine samples can give rise to significant penalties for horsemen (ARCI Uniform Classification Guidelines for Foreign Substances January 2018 (V.13.4),² the suggested penalties being in the order of a 1-year suspension and a \$10,000 fine.

This relationship between plant barbarin and equine urinary aminorex identifications was first suggested by Teale and Biddle (2018),³ who had identified aminorex in English sport horse plasma samples with no known exposure to aminorex or levamisole, levamisole being an equine anthelmintic, and immune stimulant known to metabolize to aminorex.^{4,5} Reviewing their aminorex identifications, the absence of any known sources of aminorex, the presence of a number of small plant-related molecules in their equine plasma samples and the lack of presence of pemoline or rexamino, known metabolites of

levamisole,^{6,7} Teale and Biddle³ proposed that the likely source of their aminorex identifications was glucobarbarin, a barbarin precursor found in *Brassicaceae* plants.

Plants of the genus *Barbarea Brassicaceae* family contain glucobarbarin, a barbarin precursor. Structural damage in these plants triggers hydrolysis of glucobarbarin by myrosinase to an intermediate which spontaneously cyclizes to barbarin, Figure 2, which then functions as an insect repellent or attractant.⁸ As set forth above, barbarin is related structurally to aminorex, and consumption of *Brassicaceae* plant fragments in equine feed is therefore a possible source of unexplained aminorex identifications, as demonstrated in our recently published research.⁹

While this equine administration research⁹ links consumption of the *Brassicaceae* plant *Barbarea vulgaris* to urinary aminorex identifications, it does not unequivocally identify barbarin as the proximate chemical source of these identifications. To address this matter, we have streamlined the synthesis, purified, and characterized *d*₅-barbarin, the availability of which will allow more definitive identification of the relationship between plant barbarin and equine consumption of such plant material being associated with equine aminorex identifications. *D*₅-barbarin was synthesized by a variant of previously described barbarin synthesis methods¹⁰⁻¹⁴ as follows.

2 | *D*₅-BARBARIN SYNTHESIS

Synthesis of *d*₅-barbarin was achieved in three steps, Figure 3 below, a modification of our previously described synthetic methodology. Briefly, *d*₅-acetophenone was subjected to a butylnitrite-mediated transformation to *d*₅-2-oxo-2-phenylacetaldehyde oxime (*d*₅-oxime) in good yield. Reduction of *d*₅-oxime using LiAlH₄ afforded the hydroxylamine, *d*₅-2-amino-1-phenylethan-1-ol (*d*₅-phenylethanolamine). The final ring closure step to form *d*₅-barbarin is an atom-economy transformation that utilizes carbon disulfide in pyridine as described in

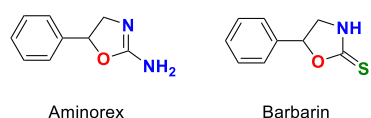


FIGURE 1 Structures of aminorex and barbarin. Aminorex, (*R,S*)-5-phenyl-4,5-dihydro-1,3-oxazol-2-amine, molar mass, 162.19 g/mol (left), and barbarin, 5-phenyl-2-oxazolidinethione, molar mass, 179.24 g/mol (right) [Colour figure can be viewed at wileyonlinelibrary.com]

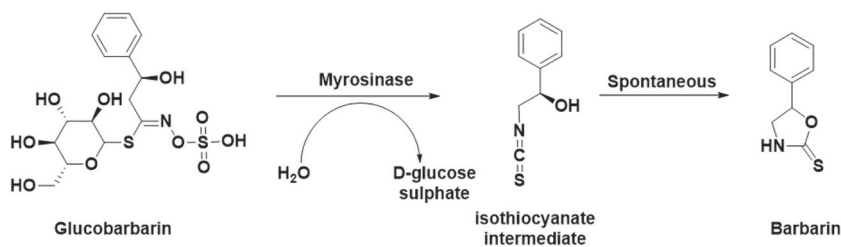


FIGURE 2 In plants, glucobarbarin is hydrolyzed by the enzyme myrosinase to the isothiocyanate intermediate, above center, which spontaneously cyclizes to barbarin.

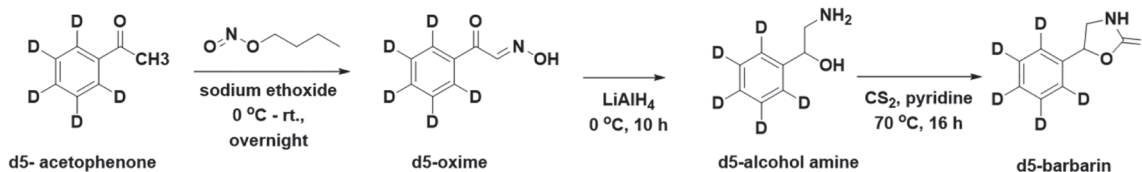


FIGURE 3 Overall reaction scheme for synthesis of *d*₅-barbarin, starting with *d*₅-acetophenone, butylnitrite, and sodium ethoxide, yielding *d*₅-oxime, followed by reduction with lithium aluminum hydride to give *d*₅-phenylethanolamine, which was then reacted with carbon disulfide in the presence of pyridine to yield *d*₅-barbarin.

our previous reports on the synthesis of unlabeled barbarin.^{13,14} Details on the synthesis are provided in the supporting information.

3 | EXPERIMENTAL

Synthesis of d_5 -oxime: Butyl nitrite (0.96 ml, 8.2 mmol) in ice-cold ethanol (50 ml) was added to sodium ethoxide (0.565 g, 8.2 mmol) in

a reaction vessel. To this solution, d_5 -acetophenone (1 g, 8.0 mmol) dissolved in 10 ml of ethanol was added dropwise over 30 min at 0°C, and the reaction warmed to room temperature overnight. The precipitate formed was filtered, washed with ether, dissolved in a minimum quantity of water acidified with glacial acetic acid, and the resulting off-white solid filtered and recrystallized from ethanol (0.56 g, 45% yield). The recovered d_5 -oxime material was characterized by ^1H NMR

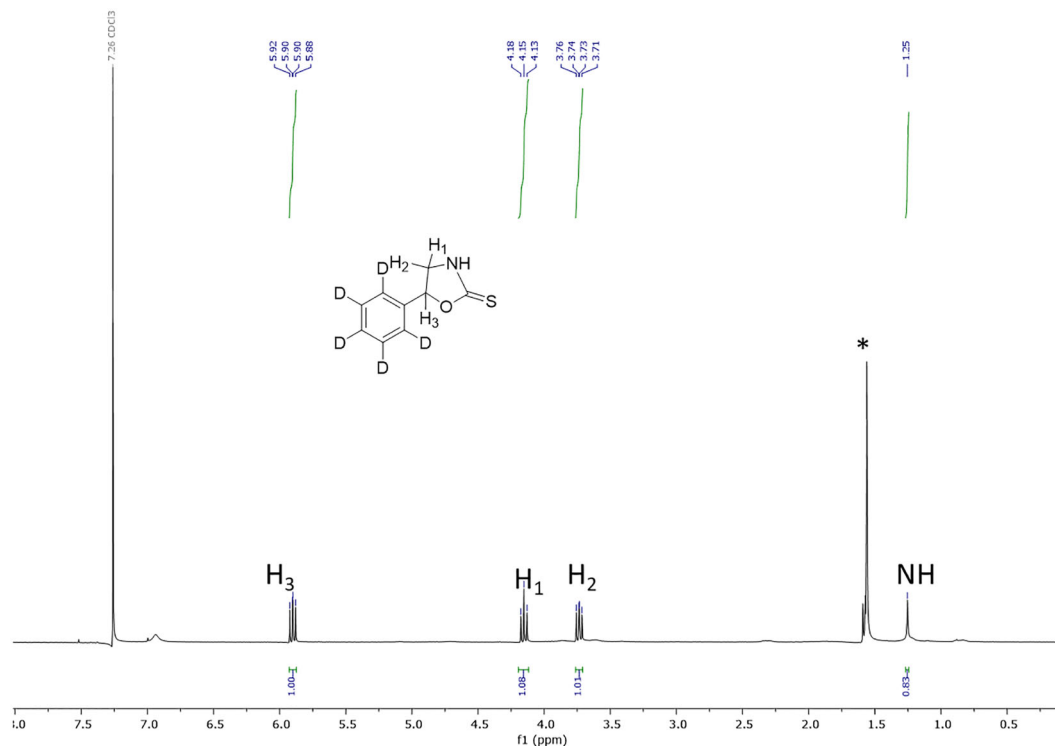


FIGURE 4 Proton NMR of d_5 -barbarin. * = water [Colour figure can be viewed at wileyonlinelibrary.com]

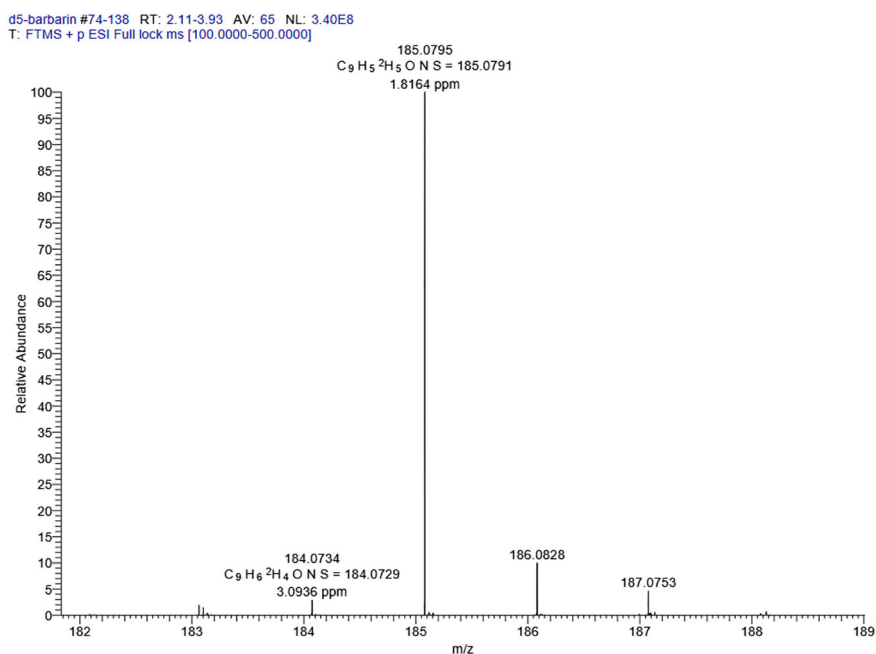


FIGURE 5 Mass spectrum of d_5 -barbarin by high-resolution electrospray ionization-mass spectrometry $[\text{M} + \text{H}]^+$ for $\text{C}_9\text{H}_4\text{D}_5\text{NOS}$, m/z 185.0795, as above, matching an expected value of 185.0791 [Colour figure can be viewed at wileyonlinelibrary.com]

and mass spectrometry as appropriate for d_5 -oxime, m/z 154.0791, $^1\text{H NMR}$ ($\text{DMSO-}d_6$, 400 MHz) δ (ppm): ^1H (s, 8.0, 1H), as in Figure S1 in the supporting information.

Synthesis of d_5 -phenylethanolamine: To a round bottomed flask charged with argon and fitted with a dropping funnel and stir bar was added LiAlH_4 (0.115 g, 3.04 mmol 4 equivalent) in anhydrous ether (30 ml) and the LiAlH_4 /ether slurry was stirred at 0°C . Then, d_5 -oxime (0.117 g, 0.76 mmol 1 eq), dissolved in anhydrous ether (10 ml), was added dropwise, and the mixture stirred and refluxed for 10 h. Excess hydride was hydrolyzed by water and ether and the white precipitate formed filtered off. The ethereal filtrate was dried over anhydrous Na_2SO_4 , concentrated to yield a yellow solid (75 mg, 70% yield). The recovered d_5 -phenylethanolamine was characterized by $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ (ppm): 4.87 (t, 1H), 3.10 (dd, 1H) and 2.85 (dd, 1H), 4.80 (1H, OH), 1.98 (2H, NH_2) as in Figure S2 in the supporting information, HRMS: Found m/z : 142.115. Calculated: 142.12.

Synthesis of d_5 -barbarin: d_5 -phenylethanolamine (0.075 g, 1 eq) in THF (20 ml) was added to excess carbon disulfide (1 ml) in the presence of 1 equivalent of pyridine. The mixture was refluxed at 70°C for 16 h and the reaction monitored by TLC. Upon completion, the reaction was cooled to room temperature, concentrated, and washed with 1 N HCl, water, and the aqueous layer extracted with DCM. The resulting organic layers were combined, dried with Na_2SO_4 , and the yellow solid recrystallized twice from DCM/hexane solvent system (45 mg, 50% yield). $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ (ppm): 5.88 (t, 1H, $J = 8$ Hz), 4.13 (t, 1H, $J = 8$ Hz), 3.71 (dd, 1H, $J = 4$ Hz, 8 Hz), 1.25 (s, 1H) (Figure 4), and HRMS: Found (m/z): $[\text{M} + \text{H}]^+$ for $\text{C}_9\text{H}_4\text{D}_5\text{NOS}$ 185.0795. Calculated: 185.0791 (Figure 5). Purity was determined to be >98% by RP-HPLC: $R_f = 4.58$ min using the

following method: Flow rate: 1 ml/min; $\lambda = 280$ nm; eluent A = DI water with 0.1% trifluoroacetic acid; eluent B = acetonitrile with 0.05% formic acid; solvent gradient: 0–16 min (0:100 H_2O : ACN), 16 min until end of run (100:0 H_2O : ACN).

3.1 | D_5 -barbarin characterization

The white crystalline material obtained following purification was characterized as follows: Analysis by proton nuclear mass resonance yielded the following, $^1\text{H NMR}$ (CDCl_3 , 400 MHz) δ (ppm): 5.88 (t, 1H, $J = 8$ Hz), 4.13 (t, 1H, $J = 8$ Hz), 3.71 (dd, 1H, $J = 4$ Hz, 8 Hz), 1.25 (s, 1H) as shown in Figure 4. High-resolution mass spectrometry presented $[\text{M} + \text{H}]^+$ for $\text{C}_9\text{H}_4\text{D}_5\text{NOS}$ at m/z 185.0795, as in Figure 5, and mass spectral product ions (Figure 6) could be interpreted as listed in Table 1. Isotopic abundance analysis also provided excellent agreement with expected m/z values and expected relative abundances in high-resolution mass spectrometry experiments (supporting information Table S2). Based on NMR, HRMS, and HPLC, we have characterized the final product as d_5 -barbarin for use in barbarin-related research.

4 | DISCUSSION

D_5 -barbarin synthesis commenced with production of d_5 -oxime from d_5 -acetophenone via butylnitrite in an ethoxide/ethanol solution as described by Norman et al. in 1962.¹⁰ This oxime product was obtained in good yield and reduced with lithium aluminum hydride,¹¹ producing the corresponding d_5 -phenylethanolamine, again in good yield. Final ring closure of the d_5 -phenylethanolamine was performed by addition of carbon disulfide in the presence of pyridine, as previously described.^{13,14} The d_5 -barbarin reaction product was presented as a stable white crystalline powder, which was obtained in sufficient yield, purified by recrystallization, and chemically characterized as d_5 -barbarin by proton NMR, HPLC, and ESI-mass spectrometry and prepared for use as an internal standard in barbarin-related research.

The research requirement for d_5 -barbarin comes from the apparent ability of *Brassicaceae* plants consumed by horses at pasture in Kentucky, New York, and elsewhere to give rise to low-concentration urinary identifications of aminorex, a US DEA schedule 1 controlled substance prohibited in racing and sport horses. A previous unexpected source of aminorex identifications was levamisole, a veterinary

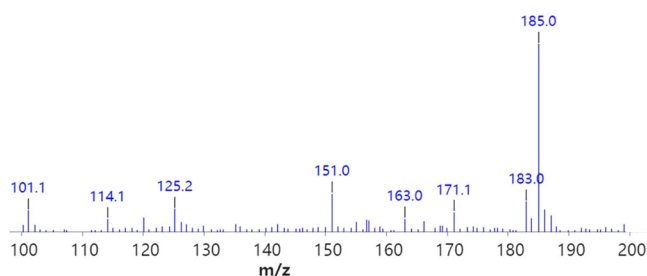


FIGURE 6 ESI-MS of d_5 -barbarin [Colour figure can be viewed at wileyonlinelibrary.com]

Product ion seen in d_5 -barbarin, m/z	Interpretation	Corresponding product ion in d_0 -barbarin, m/z^a
125	$[\text{M} + \text{H}]^+ - \text{carbon oxide sulfide (S=C=O)}$	120
151	$[\text{M} + \text{H}]^+ - \text{hydrogen sulfide (SH}_2\text{)}$	146
185	$[\text{M} + \text{H}]^+$	180

TABLE 1 Interpretation of d_5 -barbarin ESI-MS mass spectral product ions

Note: The corresponding product ions from nondeuterated d_0 -barbarin are listed for comparison.

^aExtracted from ESI-MS of d_0 -barbarin. See Figure S4 in the supporting information.

anthelmintic, and immune stimulant at one time not infrequently prescribed in racing and sport horses. Identification of levamisole administration as a source of aminorex identifications⁴ led to a marked reduction in the number of aminorex identifications in racing horses but not to their complete elimination, as noted by Teale and Biddle in 2018.³

5 | CONCLUSIONS

In closing, *d*₅-barbarin has now been synthesized, purified, and characterized. *D*₅-barbarin presents as a stable white crystalline substance and is available as a stable isotope internal standard for analytical, forensic, or toxicological research. Additionally, if required, this *d*₅-barbarin is available in larger quantities such as may be required for in vitro or in vivo research on the role of barbarin and related plant substances relevant to possible plant sources of aminorex or plant precursors of aminorex giving rise to aminorex identifications in equine drug testing samples.

ACKNOWLEDGEMENTS

This research was made possible by research support from The Equine Health and Welfare Alliance, Inc, Versailles, Kentucky, and the US Trotting Association, Columbus, OH. Further support came from the National Institute of Food and Agriculture, US Department of Agriculture, Hatch Program under project Accession Number 7001029. Other support includes research support from the National Horsemen's Benevolent and Protective Association and the Alabama, Arizona, Arkansas, Ontario, Canada; Charles Town, WV; Florida, Indiana, Iowa, Kentucky, Louisiana, Michigan, Minnesota, Nebraska, Ohio, Oklahoma, Oregon, Pennsylvania, Tampa Bay Downs, Florida, Texas, Washington State, and West Virginia Horsemen's Benevolent, and Protective Associations.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Jacob Machin  <https://orcid.org/0000-0002-9795-3689>

Thomas Tobin  <https://orcid.org/0000-0001-8506-3147>

REFERENCES

- Hofmaier T, Luf A, Seddik A, et al. Aminorex, a metabolite of the cocaine adulterant levamisole, exerts amphetamine like actions at monoamine transporters. *Neurochem Int*. 2014;73:32-41. doi:10.1016/j.neuint.2013.11.010
- ARCI Uniform Classification Guidelines for Foreign Substances January 2018 (V.13.4).
- Teale P, Biddle S. Possible Plant Sources of Aminorex and Potential markers of Their Ingestion in the Horse. *Proceeds of the 22nd International Conference of Racing Analysts and Veterinarians*. Dubai, UAE p 140-148. 2018.
- Barker SA. The formation of Aminorex in racehorses following levamisole administration. A quantitative and chiral analysis following synthetic Aminorex or levamisole administration vs. Aminorex-positive samples from the field: a preliminary report. *J Vet Pharmacol Ther*. 2009;32(2):160-166. doi:10.1111/j.1365-2885.2008.01015.x
- Eiden C, Peyrière H, Diot C, Mathieu O. Prevalence of levamisole and Aminorex in patients tested positive for cocaine in a French University Hospital. *Clin Toxicol*. 2015;53(7):604-608. doi:10.3109/15563650.2015.1054499
- Gutierrez J, Eisenberg RL, Koval NJ, et al. Pemoline and tetramisole 'positives' in English racehorses following levamisole administration. *Ir Vet J*. 2010;63(8):498-500. doi:10.1186/2046-0481-63-8-498
- Ho ENM, Leung DKK, Leung GNW, et al. Aminorex and rexamino as metabolites of levamisole in the horse. *Anal Chim Acta*. 2009;638(1):58-68. doi:10.1016/j.aca.2009.02.033
- Kjaer A, Gmelin R. IsoThiocyanates. XXVII. A new Isothiocyanate glucoside (GlucoBarbarin) furnishing (–)-5-phenyl-2-oxazolidinethione upon enzymic hydrolysis. *L Acta Chem Scand*. 1957;11:906-907. doi:10.3891/acta.chem.scand.11-0906
- Maylin G, Fenger C, Machin J, et al. Aminorex identified in horse urine following consumption of Barbarea vulgaris, a preliminary report. *Ir Vet J*. 2019;72(1):15. doi:10.1186/s13620-019-0153-5
- Norman JJ, Heggie RM, Larose JB. Oximes: 1.The synthesis of some substituted 2-oximinoacetophenones Canadian. *J Chem*. 1962;40(8):1547-1553. doi:10.1139/v62-233
- Walter CR Jr. Preparation of primary amines by reduction of oximes with lithium aluminum hydride and by the Leuckart reaction. *J Am Chem Soc*. 1952;74(20):5185-5187. doi:10.1021/ja01140a060
- Santoro R, Warren R, Roberts G. Spontaneous formation of 4-methyl-5-phenyloxazolidine-2-thione from phenylpropanolamine. *J Chromatogr a*. 1976;117(2):375-382. doi:10.1016/0021-9673(76)80014-3
- Machin J, Kudrimoti S, Eisenberg R, et al. "Synthesis, Characterization and Certification of Barbarin, a Possible Botanical Source of Aminorex Identifications". Submitted for presentation and publication, the Society of Forensic Toxicology (SOFT) Annual Meeting, Oct 07-12, 2018. Minneapolis, Minnesota, p 92. 2018.
- Machin J, Kudrimoti S, Eisenberg R, et al. Synthesis, characterization and certification of Barbarin, a possible botanical source of Aminorex identifications. *Drug Test Anal*. 2020;12:1477-1482. doi:10.1002/dta.2883

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Kudrimoti S, Machin J, Arojojoye AS, et al. Synthesis and characterization of *d*₅-barbarin for use in barbarin-related research. *Drug Test Anal*. 2022;1-5. doi:10.1002/dta.3357