How to cite: *Angew. Chem. Int. Ed.* **2021**, *60*, 19113–19116
International Edition: doi.org/10.1002/anie.202107785
German Edition: doi.org/10.1002/ange.202107785

Natural Products

Revision of the Unstable Picrotoxinin Hydrolysis Product

*Guanghu Tong and Ryan A. Shenvi**

Abstract: *The plant metabolite picrotoxinin (PXN) is a widely used tool in neuroscience for the identification of GABAergic signaling. Its hydrolysis in weakly alkaline media has been observed for over a century and the structure of the unstable hydrolysis intermediate was assigned by analogy to the degradation product picrotoxic acid. Here we show this assignment to be in error and we revise the structure of the hydrolysis product by spectroscopic characterization in situ. Counterintuitively, hydrolysis occurs at a lactone that remains closed in the major isolable degradation product, which accounts for the longstanding mistake in the literature.*

Following its isolation from *Anamerta cocculus* (*nom. alt. Menispermum cocculus*) by Boullay in 1811, the history of picrotoxin (PTX)[1] became interwoven with two centuries of advances in chemistry, neuroscience and medicine.[2,3] Fifty-five commercial vendors currently list PTX; publications that report its use number in the thousands.[4] PTX antagonizes ionotropic *gamma*-amino butyric acid receptors ($GABA_A$Rs) in mammals and, in addition to the alkaloid bicuculline,[5] serves to identify GABAergic signaling (Figure 1).[6]

Eighty-years after its isolation, Barth and Kretschy argued that PTX comprised not one pure substance, but a mixture of two components, picrotoxinin (PXN, **1**) and picrotin (PTN),[7] both dilactones nearly identical in structure and both reactive to alkaline solutions. Observations in 1916 by Horrmann[8,9] and further degradation studies spanning at least 47 papers[10] (including one as recently as 2020),[11,12] have implicated the electrophilic C15 carbon as the source of picrotoxinin instability to base. Degradation products from alcoholysis and hydrolysis, especially picrotoxic acid (**2a**) and its methyl ester (**2b**),[8] were used to support this assignment (Scheme 1).[11a]

Hydrolysis occurs in a two-step process that is pH-dependent.[13] Between pH 7 and 9, picrotoxinin converts to an unstable carboxylate anion (assumed to be **4**). Higher pH causes irreversible degradation, purportedly through intramolecular ring opening of the 8,9-epoxide (**2a**) by the C3 alkoxide or by an irreversible second lactone hydrolysis (**3a**). Decay of picrotoxinin has taken on new significance with the recent discovery that pH 7.4 buffer supports only a 45-minute half-life at room temperature.[13–15] Whereas picrotoxinin is a potent $GABA_A$ receptor antagonist, its hydrolysis products **2a** and **3a** lack activity, so care must be taken in the preparation and storage of picrotoxinin solutions.[13,14] Hydrolysis occurs even faster in vivo (mouse half-life near 15 min),[14] accounting for large variation in seizure-induction depending on the route of administration.[14] This is crucial information given the widespread use of PTX in neuroscience,[4] and the potential to misidentify GABAergic signaling with decomposed solutions.

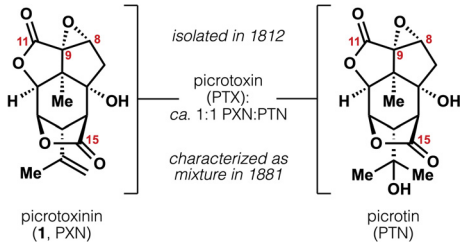
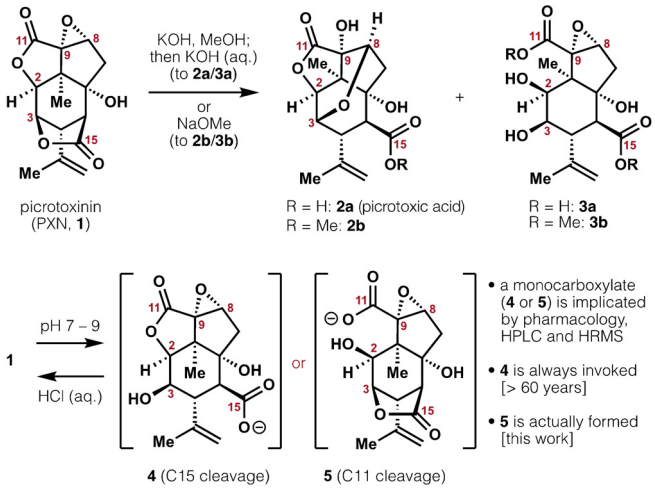


Figure 1. The substance picrotoxin (PTX) is a 1:1 mixture of picrotoxinin (PXN, **1**, highly toxic) and picrotin (PTN, less toxic).



Scheme 1. Alkaline solvolysis of PXN forms picrotoxic acid/ester (**2a**/**2b**) and picrotoxinin dicarboxylic acid/ester (**3a**/**3b**) irreversibly. At weakly basic pH, reversible hydrolysis occurs to form a monocarboxylate assigned as **4**, which is devoid of the toxicity associated with $GABA_A$ receptor antagonism. This structure, however, is incorrect, as shown here.

The structures of hydrolysis (**2a**/**3a**) and methanolysis products (**2b**/**3b**) repeatedly prompted the proposal[11a,13–15,20] that the initial, unstable carboxylate corresponds to structure **4**: at pH 7–9, this carboxylate would persist, but further basification would cause transannular cyclization to **2a**. On the face of it, this proposal makes sense since both isolated products lack the C15 lactone. There are not, however, any spectroscopic data whatsoever on the proposed structure **4**. A claim that the conjugate acid of **4** can be isolated from *Anamirta cocculus* and independently synthesized[16] is con-

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Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under:
https://doi.org/10.1002/anie.202107785.

tradicted by spectroscopic data of the material, which instead matches that of **2a** (see Supporting Information). The isolation of **4** as its conjugate acid would be unusual since, according to HPLC analysis, acidification of pH 9 solutions of PXN that contain the unstable carboxylate anion return PXN.[14] Furthermore, no experimental data over two centuries support the existence of **4** as a stable hydrolysis product, even in solution.

The pH-dependent, stepwise C15 lactone cleavage/8,9-epoxide opening proposal might be countered with an alternative hypothesis that the C11 lactone cleaves reversibly at weakly basic pH, but higher pH promotes irreversible C15 lactone cleavage/8,9-epoxide opening. Both proposals fit experimental data in the absence of in situ characterization of the unstable carboxylate intermediate, although the latter hypothesis might fit better (vide infra). Here we report in situ degradation of PXN and spectroscopic characterization of the elusive carboxylate (including full peak assignment) and find that it corresponds to C11 lactone cleavage: structure **5**, not **4**. To our surprise, the busy 209-year literature history of picrotoxinin had answered this question incorrectly.

We recently reported a synthesis of PXN and a close analog, 5-methyl-PXN.[17] The methyl group enabled quick access to picrotoxinin chemical space (9 steps, 8% overall yield) and retained potent antagonism of rat $GABA_A$ receptors. Furthermore, 5-methyl-PXN hydrolyzed more slowly: about 40% the rate of PXN using pH 8.0 phosphate buffer in D_2O (8.2 pH*).[17,18] We were curious to understand the effect of the 5-methyl substituent on rate and sought to establish a competent ^1H NMR assay for PXN itself, to better characterize the intermediates.

Addition of 1 equiv NaOH to a 0.02 M solution of PXN in D_2O generates a major new set of signals, in addition to at least two minor products (15% and 7% of major, Figure 2). If carried out in pure H_2O, the 0.02 M aqueous PXN solution[19] starts at a pH of 5.57 and, after addition of 1 equiv NaOH, reaches a final pH of 10.54. Prolonged storage of this solution at 22°C has no effect on the distribution of products. However, addition of 1.5 equiv DCl immediately returns picrotoxinin (**1**) and picrotoxic acid (**2a**) (≈85:15). These spectroscopic observations corresponded to prior pharmacological observations: the dose of PXN required to counteract barbiturate poisoning increases with solutions of increasing pH,[13] responses of mice to PXN injection decreased with basification of the initial solutions to pH 9.5,20 and incubation of PXN with rat serum or pH 9 buffer generated a new peak by HPLC corresponding to a monocarboxylate by mass spectroscopy (HRMS, ESI $m/z = 309.0979$, $(M-H)^-$; $C_{15}H_{17}O_7^-$) that returns PXN upon acidification to pH 2.3.[14] In each of these cases, cleavage of the C15 lactone to the monocarboxylate **4** was suggested as the hydrolysis pathway. The 15% minor product clearly corresponded to the sodium carboxylate of picrotoxic acid (**2a**), which was confirmed by treatment of **2a** with 1 equiv NaOH and alignment of these peaks with the in situ hydrolysis spectrum (see Figure 2, lower two spectra). The major product from PXN, however, could not be clearly identified from the ^1H spectrum alone.

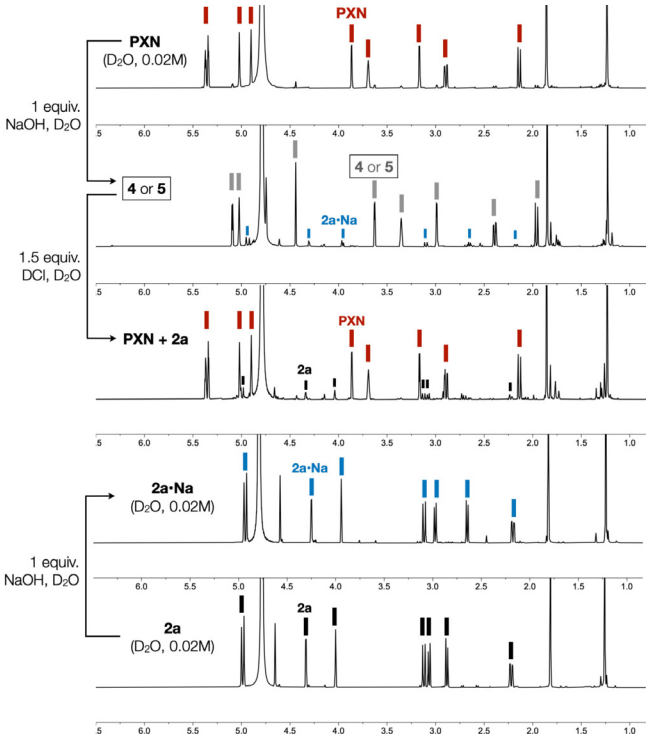

Figure 2. Addition of NaOH (1 equiv) to PXN (**1**) generates one major monocarboxylate (**4** or **5**) and a small byproduct, assigned independently as sodium picrotoxate (**2a·Na**) the conjugate base of picrotoxic acid (**2a**).

If **4** were the correct intermediate, as previously suggested, then its persistence in basic media would be strange: a small conformational relaxation from the initial half-chair to twist-boat would allow the isopropene to rotate out of its axial and position the C3 alcohol near the C8-O σ*. In fact, the small (ca. 15%) amount of sodium picrotoxate (**2a**) observed does not change over time, consistent with its *competitive* formation, not as a product of the monocarboxylate (Figure 3). Addition of 1 more equivalent of NaOH does not increase **2a·Na** but instead leads to a complex mixture of products (see Supporting Information). If, instead, the alternative carboxylate **5** were formed as the major product, then perhaps **4** forms competitively and instantly transforms into **2a** (Figure 4).

Given the structural similarity between **1**, **4** and **5**, and the line broadening associated with dissolution in D_2O, differentiation was not obvious. Careful probe tuning allowed observation of peak splitting patterns and greater signal to noise ratios. Characterization of the major monocarboxylate

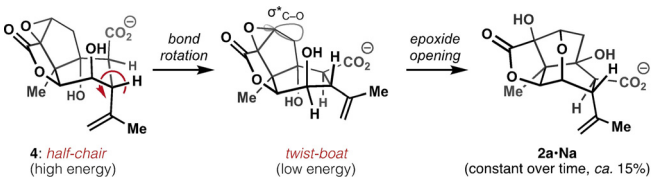

Figure 3. The unchanging ratio of monocarboxylate:**2a·Na** over time is inconsistent with **4** as the major product.

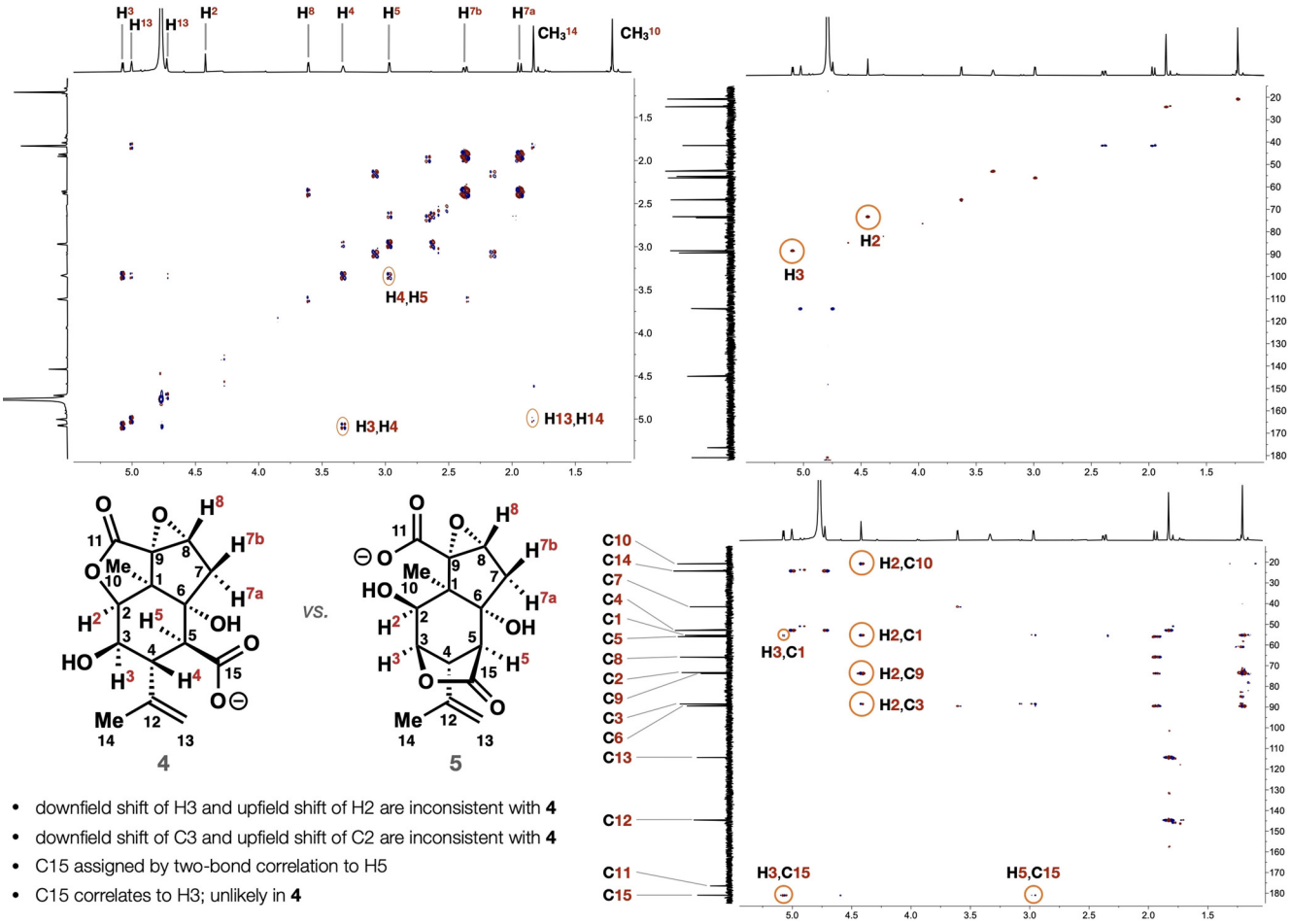

Figure 4. The consistently-proposed C15 carboxylate **4** does not fit the spectroscopic data obtained in situ (1 equiv NaOH, D_2O), whereas the alternative C11 carboxylate **5** fits. Key signals are circled in orange and labelled.

in D_2O using a full suite of NMR experiments (^{1}H NMR, ^{13}C NMR, HMQC, HMBC, DQF-COSY and NOESY), however, excluded proposed structure **4** on the basis of the following data. Proton–proton correlation assigned the four most downfield peaks as H3 ($\delta = 5.07$, d, $J = 4.9$ Hz), the two olefinic protons at C13 ($\delta = 5.00$, s; $\delta = 4.73$, s) and H2 ($\delta = 4.42$, s). All other ^{1}H NMR peaks also could be assigned, but H2 and H3 proved crucial since protons adjacent to alcohols tend to shift upfield relative to their ester counterparts. Compared to **1**, H2 had shifted far upfield ($\delta = 5.32$ to 4.42), whereas H3 remained roughly in place ($\delta = 5.35$ to 5.07), inconsistent with lactone hydrolysis adjacent to H3 and the first indication that structure **4** was incorrect. HSQC identified the carbons (C2 and C3) attached to H2 and H3, which also reflected a large upfield shift for C2 ($\delta = 83.9$ to 73.3) and a large downfield shift for C3 ($\delta = 81.1$ to 88.5). Like the proton peak movements, these carbon shifts indicated a conjugated oxygen bound to C3 but an unconjugated oxygen bound to C2. Furthermore, HMBC correlations supported these H2/H3 assignments (e.g. 3-bond correlation between H2 and C10) and allowed full assignment of ^{13}C peaks. These signals clearly differentiated **4** and **5**. Carbonyl carbon C15 could be identified on the basis of 2-bond correlation to bridgehead proton H5, impossible for alternative carbonyl carbon C11. A cross-peak between C15 and H3 then corresponded to a reasonable 3-bond correlation in **5** through a γ-lactone, whereas **4** would require an unlikely 4-bond correlation through a (non-conjugated) γ-hydroxy-carboxylate.

Taken together, these data exclude the often-proposed monocarboxylate **4** and instead match the alternative structure **5** (Figure 5).[21] To the best of our knowledge, this is the only data in the literature to disprove **4** as the direct hydrolysis product of **1**. Remarkably, these data indicate that the degradation products **2** and **3** derive from a slow, secondary solvolysis event at C15, not the rapid, primary hydrolysis at C11: a counterintuitive preference that misled

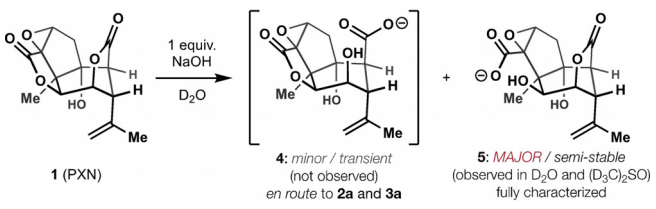

Figure 5. The initial solvolysis product of PXN is **5**, not **4**, as previously supposed.

the community for years. Establishment of **5** answers the longstanding question posed by Conroy[11a] as to whether such a compound might exist and whether the C11 lactone remains intact in the production of **2a**; here we answer both affirmatively. This is crucial information to better understand structure–activity relationships of the picrotoxane series, since **5** is now known to be inactive, whereas **4** was assumed to be the inactive structure. Design of PXN analogs could rely on this information to retard C11 hydrolysis and allow persistence in the bloodstream so that non-mammalian LGICs associated with infectious invertebrates might be targeted efficiently.[23]

Conclusion: Here we have revised the initial, unstable hydrolysis product of picrotoxinin (PXN), a longstanding assumption in the literature that has lacked spectroscopic support. By in situ NMR analysis, we find the kinetic site of hydrolysis is C11 leading to **5**, versus the speculated site of C15 leading to **4**. Whereas hydrolysis of C11 is reversible (**5** + HCl → **1**), solvolysis at C15 (**1** + NaOH → **2a**) can be viewed as a "point of no return" that allows relaxation of the half-chair cyclohexane to either of two twist boats, leading to irreversible epoxide opening (**2a**) or double lactone solvolysis (**3a**).[22] Picrotoxinin antagonizes $GABA_A$ receptors potently, and also binds the related ligand-gated ion channels (LGICs) of invertebrates: RDL and GluCl receptors.[23] This pan-selective binding among the extensive LGIC phylogenetic tree suggests the opportunity for analogs to select for individual members and find use as, for example, antiparasitics or insecticides. However, the rapid solvolysis and/or metabolism of **1** limits its potential applications. Characterization of **5** as the unstable PXN hydrolysis product will be useful in the development of stabilized analogs of **1**.

Acknowledgements

Support was provided by the National Institute of Health (R35 GM122606), and Jiangsu Industrial Technology Research Institute (JITRI Fellowship to G.T.). We thank Dr. L. Pasternack and Dr. D.-H. Huang for NMR assistance and Dr. J. Chen and Brittany Sanchez for HRMS measurements.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: GABA · hydrolysis · natural products · NMR spectroscopy · picrotoxin

[1] For a comprehensive review, see: L. A. Porter, *Chem. Rev.* **1967**, *67*, 441.
[2] A Web of Science search covering only 1904–2021 (i.e. excluding significant early work) identifies 3612 papers dealing with picrotoxin, the majority of which are sorted in the overlapping categorizes of neurosciences (1904), pharmacology (976), physiology (537) and biochemistry molecular biology (241) and behavioral sciences (196) (retrieved May 8, 2021). The alternatively spelled "picrotoxine" turns up 25 references.
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Manuscript received: June 11, 2021
Accepted manuscript online: July 8, 2021
Version of record online: July 20, 2021

