JOURNAL OF NATURAL PRODUCTS

Note

Cite This: J. Nat. Prod. XXXX, XXX, XXX−XXX

pubs.acs.org/jnp

Phorbol Rearrangements

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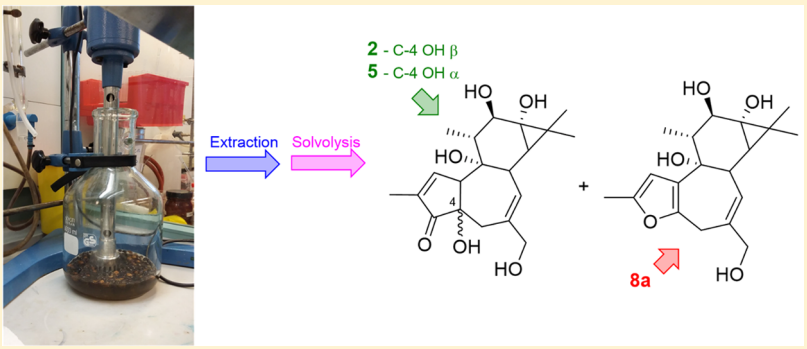
Supporting Information



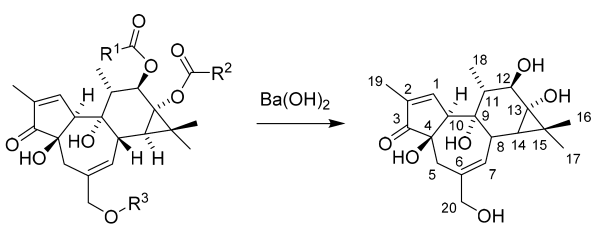
ABSTRACT: An alternative procedure for isolation of 4*β*-phorbol from seeds of *Croton tiglium* has been developed, and an artifact containing a furan ring formed by rearrangement of 12,13,20-*O*-triacylated phorbol derivatives into (6b*S*,7*R*,8*R*,8a*S*)-2-(hydroxymethyl)-5,7,9,9-tetramethyl-3,7,8,9,9a,9b-hexahydrocyclopropa[3′,4′]benzo[1′,2′:3,4]cyclohepta[1,2-*b*]furan-6b,8,8a-triol (**8a**) has been characterized. A mechanism involving an oxidative rearrangement and a decarboxylation for formation of the artifact is proposed.

The oil from the seeds of *Croton tiglium* L. (Euphorbiaceae) was imported from India to Europe around 1812 as a new purgative drug.[1] However, application of the oil on the skin causes severe skin irritation and redness.[2] Except for periorbital facial rejuvenation[3] the use of croton oil has been abandoned because of its proinflammatory and tumor-promoting effects.[4] Due to compound lability, the correct structures of the active phorbol esters were not elucidated until 1967 by X-ray crystallographic analysis,[5] although preliminary structural studies were reported.[6,7] The 12,13-*O*-diacyl derivatives of phorbol (Scheme 1, **1a**) are potent agonists acting on members of the protein kinase C (PKC) enzyme family.[2,8]

The intriguing bioactivities of the phorbols have promoted an interest in development of protocols enabling isolation of the compounds in gram scale from croton oil.[2,9] A number of 12,13-*O*-phorbol diesters only differing in the acyl groups (Scheme 1) are present in croton oil together with some 12,13,20-*O*-triesters.[2,8b] The most used tool in bioassays for investigating the effects of PKC has become 12-*O*-tetradecanoylphorbol 13-acetate (TPA, **1b**), also named phorbol 12-myristate 13-acetate (PMA). In order to avoid a tedious and difficult separation of the ester derivatives, most protocols for gram-scale isolation suggest that the crude oil be saponified by using barium hydroxide as the base, thus providing the pentavalent alcohol, 4*β*-phorbol (**2**) (Scheme 1), which can be readily purified. Subsequently, the acyl groups may be reintroduced.[9]

The use of harsh alkaline conditions on a multifunctional unsaturated molecule containing several rings might be expected to cause side reactions such as degradation and rearrangement. Two such reactions have already been described, namely, oxidative opening of the pentenone ring.[10] A putative mechanism for the oxidative rearrangement has been proposed (Scheme S1, Supporting Information).[10] The second described rearrangement is an epimerization at C-4 (Scheme S2, Supporting Information).[2] This reaction was described to occur when treating **2** with sodium methoxide.[2,11] Compounds **6** and **7**, epimerized at C-10 and C-2, respectively, were observed as minor side products. The mechanism outlined in Scheme S2 may explain how this product mixture may arise.

Scheme 1. Saponification of Esters of 4*β*-Phorbol (1a)



1a: R^1, R^2 = alkyl, R^3 = -H
1b: R^1 = -$(CH_2)_{12}CH_3$, R^2 = -CH_3 R^3 = -H
1c: R^1, R^2, R^3 = *n*$(CH_2)_{10}CH_3$

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Received: July 25, 2018

DOI: 10.1021/acs.jnatprod.8b00607
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Epimerization at C-4 has as a consequence that the corresponding 12,13-*O*-diesters lose affinity for PKC.[12] Instead they possess a high affinity for the vanilloid transient receptor potential (TRP) cation channel subfamily V member 4 (i.e., TRPV4).[13] Esterification at O-20 in phorbol 12,13-*O*-diesters with homovanillic acid also confers an affinity for TRPV4 to the resulting triesters.[14] However, masking of O-20 causes a loss of affinity for PKC.[15] The PKC activation by some 20-acylated phorbols may be explained by nonspecific interactions.[16]

In order to enable the synthesis of peptide-based enzyme-activated prodrugs of **2** similar to those reported for the natural product thapsigargin,[17] gram-scale isolation of phorbol esters **1a** was undertaken by using procedures similar to those previously published.[2,9] However, instead of using croton oil, seeds of *C. tiglium* were selected as the starting material. We had to try to extract *C. tiglium* seeds instead of croton oil, because we were not able to isolate phorbol esters from the purchased croton oil. Our hypothesis is that phorbol esters had been purposely removed, probably due to toxicity issues. Repeated extraction of finely ground seeds with ethyl acetate under sonication at 40 °C yielded upon evaporation a dark yellow tar. As noticed previously,[9] direct solvolysis of the extract with methanolic base was problematic, as 4β-phorbol (**2**) and 4α-phorbol (**5**) proved difficult to separate from the glycerol produced during solvolysis of the triacylglycerols present in the extract. In order to avoid this obstacle, a defatting step was included in the protocol. Thus, the concentrated crude extract was dissolved in MeOH, affording two separable phases, in which undesired lipophilic compounds were retained in the upper phase. A potential loss of a fraction of phorbol triesters during this defatting procedure was accepted since the procedure was fast and afforded the desired 4β-phorbol **2** in good yield. The lower methanolic phase, mainly containing phorbol diesters and triesters, was diluted with MeOH and treated with barium hydroxide. Strong basicity of the solution is very important for efficient quantitative solvolysis. However, if the solvolysis was performed at room temperature, extensive epimerization to **5** occurred. A difficult separation of **2** and **5** causing substantial loss in the yield of **2** rendered these conditions undesirable. Attempts to get a good separation of the two epimers by column chromatography using silica gel or reversed-phase C_{18} silica gel failed if **2** and **5** were present in similar amounts. In this case a substantial number of the fractions contained both compounds. Fortunately, almost no epimerization occurred if the solvolysis was performed at a lower temperature, although a prolonged reaction time was required, as this expectedly made the conversion more sluggish. Indeed, when the solvolysis was performed at 0 °C for 18 h, only a trace amount of epimer **5** was generated.

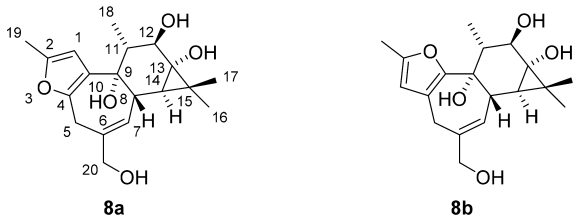


Figure 1. Two possible structures for the rearranged phorbol product containing a fused furan ring.

Besides **2** and a small amount of epimer **5**, an additional spot was observed by thin-layer chromatography (TLC) of the reaction mixture. Visualization by heating the TLC plate when sprayed with 10% sulfuric acid in MeOH revealed a magenta spot, a color very different from the brown color of the spots of the phorbols. The NMR spectra of the purified byproduct were similar to those of the phorbols except for the signals originating from the cyclopentenone moiety, which either had disappeared or were shifted to very different resonance values. A signature signal appeared in the HSQC spectrum at δ_H 6.1,δ_C 108, which is characteristic for H-3,C-3 in a furan ring. Two isomers appeared to be possible considering that the product was derived from a phorbol drivative. Strong correlations in the HMBC spectrum were present between the two protons attached to C-5 and the two carbon atoms attached to the oxygen in the furan ring (i.e., C-2 and C-4). In contrast, no correlations were observed between H-5 and the remaining two carbon atoms of the furan ring (i.e., C-1 and C-10). The signature signal at δ_H 6.1,δ_C 108 could then be assigned to H-1 and C-1. These observations are in agreement with the proposed structure **8a**.

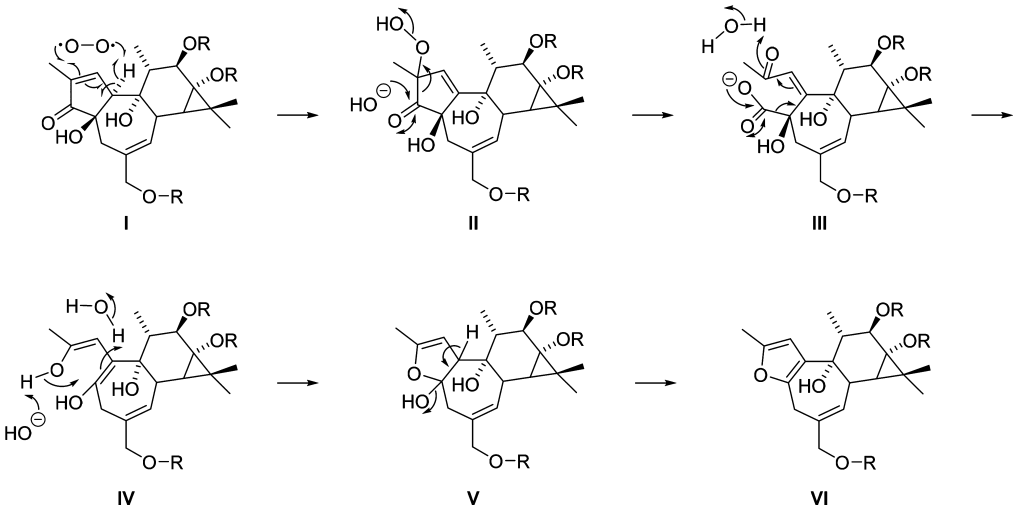
The presence of **8a** in the solvolysis mixture of the seeds of *C. tiglium* extract can be explained by one of two options: (i) compound **8a** or esters of compound **8a** are natural products, which previously have been disregarded in the studies of extracts of croton seeds, or (ii) compound **8a** is an artifact formed by an oxidative rearrangement followed by a decarboxylation of natural products present in the seeds. An HSQC correlation of the crude phorbol ester fraction prior to saponification revealed no signal at δ_H 6.1,δ_C 108, indicating that compound **8a** is an artifact. Since only some furans exhibit signals at this location of HSQC correlation, no interfering weak signals from other molecules would seem to contradict the interpretation that the compound is an artifact.

To elucidate how **8a** may originate from phorbol derivatives found in the seeds of *C. tiglium*, several attempts at rearrangement of 4β-phorbol, 4α-phorbol, and 12,13-*O*-phorbol diesters were performed, but no furan derivatives were formed. In contrast, if 4β-phorbol 12,13,20-*O*-tridodecanoate (**1c**)[18] was treated with barium hydroxide in MeOH under similar conditions to those used for base-catalyzed solvolysis of the methanol extract of *C. tiglium* seeds, compound **8a** was indeed formed. The oxidative opening of the cyclopentenone ring depicted in Scheme S1 also only occurs for phorbol esters masked at O-20.[10] Thus, the presence of 4β-phorbol 12,13,20-*O*-triesters in *C. tiglium*[8b] infers that these derivatives are likely substrates that may be transformed into furan derivatives during the saponification procedure. No attempts were made to identify compounds possessing the skeleton of artifact **4** in the solvolysed mixture, since these are formed from **3** only after a prolonged exposure to sodium methoxide and oxygen (Scheme S1).[10]

Inspired by the suggested mechanism for the oxidative opening of the cyclopentenone ring converting **3** into **4** (Scheme S1, Supporting Information),[10] an oxidative decarboxylative mechanism may be proposed for the formation of **8a** (Scheme 2). Triplet oxygen reacts with a triacylphorbol (**I**) to give allylic hydroperperoxide **II**, which by attack from a hydroxide ion on the C-3 carbonyl and concomitant loss of a hydroxide ion from the hydroperoxide functionality is converted into the vinylogic β-keto carboxylate **III**. After decarboxylation, dienol **IV** is formed, which can cyclize and eliminate water to give furan **VI**, which is solvolysed to give

DOI: 10.1021/acs.jnatprod.8b00607
J. Nat. Prod. XXXX, XXX, XXX−XXX

Scheme 2. Oxidative Decarboxylation of O-20-Masked Phorbol



compound **8a**. In both this rearrangement and the rearrangement described in Scheme S1, a nucleophilic attack on the carbonyl group opens the cyclopentenone ring. The assumption that a free C-20 hydroxy group prohibits this attack would explain why this group has to be masked. Before workup, the reaction mixture was neutralized with sulfuric acid. The last steps in the migration path might occur after the reaction mixture has been made slightly acidic.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Analytical Autopol VI automatic polarimeter. The NMR spectra were recorded on Bruker Advance 400 and 600 MHz spectrometers. ACD NMR manager was used to process NMR spectra. Samples were dissolved in methanol-d_4 or $CDCl_3$ and analyzed at 300 K. The residual solvent peaks were used as internal standards (i.e., methanol-d_4: δ_C 49.00; δ_H 3.31; $CDCl_3$: δ_C 77.00; δ_H 7.26). HRMS were recorded on a Bruker micrOTOF-Q instrument using electrospray ionization in the positive mode. Column chromatography was performed over silica gel 40−63 μm. HPLC was performed on a Water Alliance 2695 apparatus using an Agilent Zorbax XDB C_{18} analytical column (particle size: 3.5 μm, 4.6 × 75 mm), using an aqueous MeOH gradient with 0.1% trifluoroacetic acid (TFA) added (eluent A: H_2O + 0.1% TFA, eluent B: MeOH + 0.1% TFA).

Plant Material. *Croton tiglium* seeds were purchased from Herbalveda UK, East Harrow Middlesex, UK. A sample is deposited at the Pharmacognostic Collection at the Department of Drug Design and Pharmacology under the code PC2018-001.

Extraction and Isolation. Seeds of *C. tiglium* (1493 g) were ground in EtOAc (600 mL) and then sonicated at 40 °C for 1 h. The mixture was filtered, and the ground seeds were sonicated in EtOAc (600 mL) two additional times (for 4 and 1 h). The combined extracts were concentrated. The residue was dissolved in MeOH (500 mL) and shaken in a separation funnel, and then two phases were formed. The lower MeOH layer was isolated, while the upper, mainly triacylglycerol, layer was extracted twice with MeOH (600 mL). The combined MeOH phases were concentrated and chromatographed over silica gel (600 g) starting with heptane−EtOAc (5:1) as an eluent (4 L) and gradually increasing the concentration of EtOAc to heptane−EtOAc (1:1). After concentration, the residue was chromatographed over silica gel (500 g) using gradient elution starting with CH_2Cl_2−MeOH (100:1; 3 L) and gradually increasing the concentration of MeOH to CH_2Cl_2−MeOH (50:1). After concentration of the combined phorbol-containing fractions, the residue (12 g) mainly consisted of phorbol esters.

Solvolysis and Isolation of 4β-Phorbol (2) and Compound 8a. A solution of crude phorbol esters (3.52 g) in MeOH (80 mL) was cooled on ice, and then $Ba(OH)_2{\cdot}8H_2O$ (3.8 g; 12 mmol) was added. The mixture was stirred at 0 °C for 18 h and then neutralized by dropwise addition of 2 M H_2SO_4 until a weakly acidic solution was obtained. After filtration, the filtrate was concentrated and the residue chromatographed over silica gel (500 g) starting with CH_2Cl_2−MeOH (50:1; 5 L) and gradually increasing the concentration of MeOH to CH_2Cl_2−MeOH (12:1). 4β-Phorbol (**2**, 1.06 g) was eluted with CH_2Cl_2−MeOH (11:1), while compound **8a** (174 mg) was eluted with CH_2Cl_2−MeOH (12:1). Both compounds were obtained as a colorless, amorphous powder. The ^{1}H NMR and ^{13}C NMR data for 4β-phorbol (**2**) were identical to those reported.[19] The data for **8a** were identical to those acquired for the sample obtained from solvolysis of 12,13,20-tridodecanoate 4β-phorbol.

12,13,20-Tridodecanoate 4β-phorbol (1c). To a solution of 4β-phorbol (52 mg; 0.142 mmol) in tetrahydrofuran (THF)−CH_2Cl_2 (1:1; 4 mL) were added *N*-ethyl-*N*′-(3-(dimethylamino)propyl)-carbodiimide (108 mg; 0.565 mmol), dodecanoic acid (340 mg; 1.7 mmol), and 4-dimethylaminopyridine (35 mg; 0.287 mmol), and then the mixture was stirred under argon at room temperature for 22 h. On concentration, the residue was dissolved in EtOAc, and upon addition of $NaHCO_3$, the solution was washed twice with water, dried with Na_2SO_4, and concentrated to give a tar. The tar was chromatographed over silica gel (80 g) using hexane−EtOAc (4:1; 500 mL) to obtain 4β-phorbol 12,13,20-tridodecanoate (**1c**, 111 mg; 84.7%): ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.59 (1H, s, H-1) 5.71 (1H, d, J = 4.3 Hz, H-7), 5.40 (1H, d, J = 10.1 Hz, H-12), 4.47 (1H, d, J = 12.3 Hz, H-20a) 4.43 (1H, d, J = 12.3 Hz, H-20b), 3.24 (1H, d, J = 2.3 Hz, H-8), 3.23 (1H, m, H-10) 2.51 (1H, d, J = 18.4 Hz, 1H, H-5a), 2.41 (1H, d, J = 18.4 Hz, H-5b), 2.34−2.24 (6 H, m, 3 × DodecanoylH-2 (DoH-2)), 2.13 (1H, dq, J = 6.3, 5.30 Hz, H-11), 1.76 (3 H, d, J = 1.5 Hz, H-19), 1.67−1.53 (6 H, m, 3 × DoH-3), 1.32−1.23 (17H, m, 3 × DoH-4−11, H-17), 1.20 (3 H, s, H-16), 1.03 (1H, d, J = 5.1 Hz, H-14), 0.86 (12 H, t, J = 6.5 Hz, 3 × DoH-12, H-18); ^{13}C NMR (400 MHz, $CDCl_3$) δ_C 208.8 (C-3), 176.3 (DoC-1), 173.4−173.6 (2 × DoC-1) 160.9 (C-1), 135.7 (C-6), 132.8 (C-2), 132.7 (C-7), 78.1 (C-12), 76.5 (C-9), 73.5 (C-4), 69.1 (C-20), 65.3 (C-13), 56.1 (C-10), 43.0 (C-11), 39.3 (C-8), 38.9 (C-5), 36.2 (C-14), 34.5 (DoC-2), 34.3 (DoC-2′), 34.2 (DoC-2″), 31.9 (3 × DoC-10), 29.7−28.9 (3 × DoC-4-C-9) 25.7 (C-15), 25.2 (DoC-3) 24.9 (DoC-3′), 24.5 (DoC-3″), 23.8 (3 × DoC-11), 22.7 (C-17), 16.8 (C-16), 14.4 (C-18), 14.1 (3 × DoC-12), 10.1 (C-19).

(6b*S*,7*R*,8*R*,8a*S*)-2-(Hydroxymethyl)-5,7,9,9-tetramethyl-3,7,8,9,9a,9b-hexahydrocyclopropa[3′,4′]benzo[1′,2′:3,4]-cyclohepta[1,2-*b*]furan-6b,8,8a-triol (8a). To a solution of 12,13,20-tridodecanoate-4β-phorbol (58 mg; 0.064 mmol) in MeOH (6 mL) was added $Ba(OH)_2{\cdot}8H_2O$ (110 mg; 0.349 mmol)

DOI: 10.1021/acs.jnatprod.8b00607
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at 0 °C, and then the mixture was stirred at 0 °C for 18 h. The reaction mixture was neutralized with 1 M H_2SO_4 and concentrated, and the formed precipitate of $BaSO_4$ removed by filtration. The filtrate was concentrated, and the residue chromatographed over silica gel (60 g) using CH_2Cl_2−MeOH (20:1; 400 mL) gradually increasing the gradient toward CH_2Cl_2−MeOH (12:1) to give compounds **8a** (10 mg, 45%) and **2** (5 mg; 21%), which were obtained as a colorless, amorphous powder. **8a** [(6b*S*,7*R*,8*R*,8a*S*)-2-(hydroxymethyl)-5,7,9,9-tetramethyl-3,7,8,9,9a,9b-hexahydrocyclopropa[3′,4′]benzo-[1′,2′:3,4]cyclohepta[1,2-*b*]furan-6b,8,8a-triol] (10 mg; 45%): $[\alpha]^{21}_{D}$ 64.0 (*c* 1; MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ_H 5.85 (1H, d, *J* = 1.0 Hz, H-1), 5.31 (1H, br d, *J* = 5.1 Hz, H-7), 3.91 (1H, d, *J* = 10.5 Hz, H-12), 3.85 (1H, d, *J* = 13.0 Hz, H-20b), 3.80 (1H, br d, *J* = 13.0 Hz, H-20a), 3.17 (1H, br d, *J* = 16.9 Hz, H-5b), 2.93 (1H, d, *J* = 16.9 Hz, H-5a), 2.27 (1H, t, *J* = 5.0 Hz, H-8), 1.97 (3H, br s, H-19), 1.24 (1H, dq, *J* = 10.4, 6.5 Hz, H-11), 1.08 (3H, s, H-16) 1.04 (3H, d, *J* = 6.4 Hz, H-18), 0.98 (3H, s, H-17), 0.61 (1H, d, *J* = 4.4 Hz, H-14); ^{13}C NMR (400 MHz, CD_3OD) δ_C 148.5 (C-1), 146.8 (C-4), 143.1 (C-6), 130.0 (C-7), 123.1 (C-10), 108.8 (C-1), 79.0 (C-12), 74.6 (C-9), 65.3 (C-20), 61.6 (C-13), 47.8 (C-11, overlapped by signal from CD_3OD), 41.4 (C-8), 36.9 (C-14), 27.4 (C-5), 25.1 (C-15), 23.1 (C-18), 15.9 (C-17), 13.8 C-16), 11.8 (C-19); HRMS *m*/*z* 357.1676 [M + Na]$^+$ (calcd for $C_{19}H_{26}NaO_5^+$ 357.1672). The homogenity was checked by analytical HPLC at a flow of 0.7 mL/min with a gradient method: 10% → 50% B over 25 min. The product **8a** was eluted at 22.7 min with a purity of more than 98%.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00607.

Copies of the ^{1}H, ^{13}C, HCOSY, HSQC, NOESY, and HMBC NMR spectra and HRMS spectrum of compound **8a** and ^{1}H and ^{13}C NMR spectra of 4β-phorbol 12,13,20-tridodecanoate (**1c**); schemes depicting the suggested mechanisms for the oxidative opening of the cyclopentenone ring and epimerization at C-4 (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by a grant from The Czech Republic Technical University to T.Z.

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J. Nat. Prod. XXXX, XXX, XXX−XXX