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Total Synthesis and Detection of the Bilirubin Oxidation Product (*Z*)-2-(3-Ethenyl-4-methyl-5-oxo-1,5-dihydro-2*H*-pyrrol-2-ylidene)ethanamide (*Z*-BOX A)

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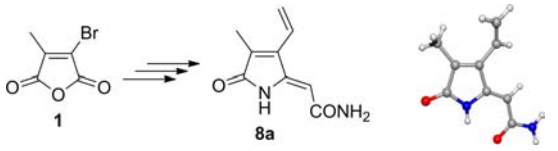
ABSTRACT



The selective total synthesis of the pure *Z*-isomer of BOX A (8a), a product of oxidative heme degradation with significant physiological impact, was achieved in four to six steps starting from 3-bromo-4-methylfuran-2,5-dione (1). *Z*-BOX A forms a strong hydrogen bridge framework in the crystalline state. LC-MS techniques allow identification and characterization of isomeric forms of BOX A.

Heme and hemoglobin degradation proceeds *via* bilirubin and biliverdin which are further degraded oxidatively finally leading to 4-methyl-3-vinyl-maleimide (MVM) as well as to the bilirubin oxidation end products (BOXes) 2-(3-ethenyl-4-methyl-5-oxo-1,5-dihydro-2*H*-pyrrol-2-ylidene)-ethanamide (BOX A) and 2-(4-ethenyl-3-methyl-5-oxo-1,5-dihydro-2*H*-pyrrol-2-ylidene)ethanamide (BOX B) (Scheme 1).[1] The constitutional isomers BOX A and BOX B can also form *Z* and *E* isomers at the exocyclic C=C double bond.

Hemoglobin and its degradation products (HDPs) are considered responsible for cerebral vasospasm,[2,3] which is the most frequent serious complication in survivors of aneurismal subarachnoid hemorrhage. This stroke subtype

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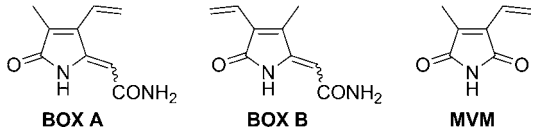
affects about 1 of 10.000 persons leading to severe spasm of the cerebral arteries 4–9 days after the stroke. Approximately 10–15% of them suffer severe permanent neurological dysfunction or die.[4] In contrast to MVM which shows only a weak activity in biological systems, a mixture of these BOXes is highly active already in very low concentrations. Heme[5,6] and its degradation products[7] bind to potassium channels and cause structural alterations. However, there are only very few investigations regarding the mode of action of BOXes due to the fact that the availability of these bilirubin degradation end products is very limited.

Scheme 1. Heme Degradation End Products (HDPs)

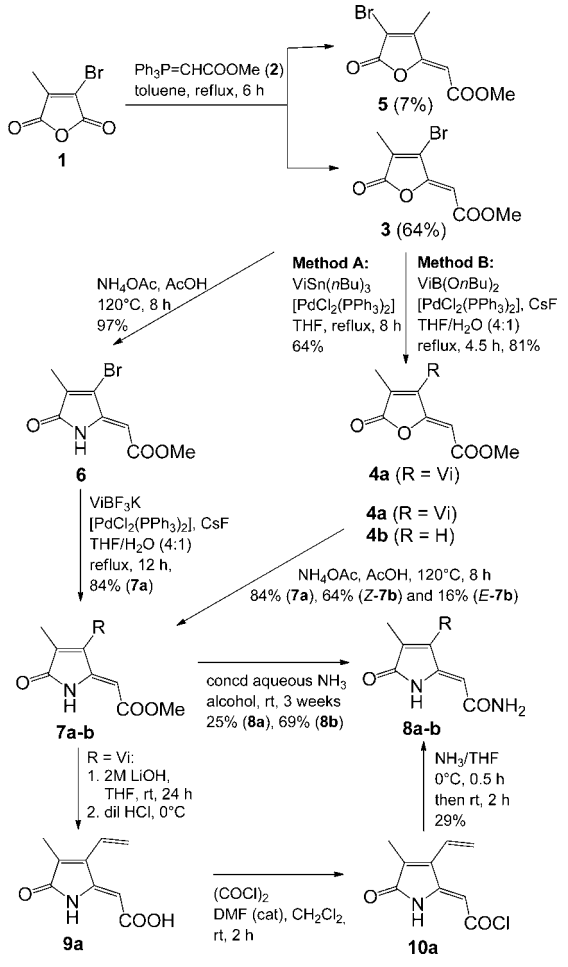


Clark and co-workers[8] described a procedure that yields a crude mixture of BOXes and other degradation products *via* the oxidative break-up of bilirubin or biliverdin with H_2O_2 in a sodium chloride solution. The amounts of accessible breakdown products by this method are very small, and the purification is tedious. Oxidation of bilirubin also succeeds with cytochrome oxidase, but this also fails to yield any pure compounds for biological testing as well.[9]

In all studies investigating the mode of action of BOXes, mixtures of bilirubin degradation products were applied. Separation of BOX A and BOX B was reported,[8] but an assignment of the exocyclic C=C double bond was not possible on the basis of NMR experiments. Due to the tremendous importance of BOXes in medicinal studies a total synthesis of BOX A was developed representing an important step in order to evaluate the specific activity of BOXes. Parallel to these preparative investigations an LC-MS-based analytical procedure was developed to detect HDPs at low concentrations.

For the total synthesis of *E*- and *Z*-BOX A 3-bromo-4-methylfuran-2,5-dione (**1**) was reacted with the phosphorane $Ph_3P{=}CHCOOMe$ (**2**)[10] according to procedures well-known for citraconic anhydride transformations.[11–14] Kayser and co-workers[15,16] showed that alkyl and bromo substituents in the 3-position drastically favor a Wittig reaction at the 2-oxo functionality. However, the total yield of this reaction was rather low for 3-bromo-4-methylfuran-2,5-dione (**1**) due to polymerization of the product.[16] The Wittig reaction of 3-bromo-4-methylfuran-2,5-dione (**1**)[17] with $Ph_3P{=}CHCOOMe$ (**2**) yielded the isomers methyl (*Z*)-(3-bromo-4-methyl-5-oxofuran-2(5*H*)-ylidene)-ethanoate (**3**) and methyl (*Z*)-(4-bromo-3-methyl-5-oxofuran-2(5*H*)-ylidene)ethanoate (**5**) with a ratio of 6:1 (**3**:**5**) on the basis of the integration of ^{1}H NMR spectra (Scheme 2).

Scheme 2. Synthesis of **8a** (BOX A) and **8b**



The purified major product **3** was vinylated with vinylboronic acid dibutyl ester giving pale yellow crystals of methyl (*Z*)-(3-ethenyl-4-methyl-5-oxofuran-2(5*H*)-ylidene)-ethanoate (**4a**) (yield: 81%). A similar procedure with highly toxic tributyl(vinyl)stannane gave lower yields of 64%. Ring transformation of oxofuran **4a** to oxopyrrole **7a**[18] succeeded with ammonium acetate in glacial acetic

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acid without amide formation. Therefore the resulting methyl ester **7a** was treated with an aqueous ammonia solution yielding *Z*-BOX A (**8a**) with a yield of 25%. An alternative route for the conversion of the oxofuran **3** to oxopyrrole **7a** involves ring transformation by ammonia (yielding **6**) followed by a Suzuki coupling. Due to the fact that the vinyl group shows no conjugative interaction with the oxopyrrole ring, a similar reaction sequence allowed the isolation of **8b** from **4b**[19] *via* oxopyrrole **7b**. For the synthesis of **8a**, two additional transformations to the carbonic acid **9a** and to the corresponding chloride **10a** followed by ammonolysis is advantageous due to significantly shorter reaction times.

Crystal structure determinations[20] and NOE experiments clarify the isomerism at the exocyclic C=C double bond because an unambiguous assignment was not possible solely on the basis of NMR experiments by Kranc et al.[8] The (*E*/*Z*)-isomerism of the exocyclic C=C double bond in the 2-position is influenced by steric repulsion and the formation of intramolecular N−H···O bridges in the pyrrole derivatives both leading to a preference for the *Z*-isomers. Only methyl (*E*)-(4-methyl-5-oxofuran-2(5*H*)-ylidene)ethanoate (**4b**) crystallized as an *E*-isomer at this exocyclic C=C double bond.

Taking bond lengths of 148 and 134 pm as reference values for isolated C−C single and C=C double bonds between sp^2 hybridized carbon atoms, a significant delocalization within the conjugated π-system can safely be excluded (see Supporting Information). In addition, the exo-C=O bonds show characteristic C=O double bond values with a slight elongation for the oxopyrrole derivatives. The exocyclic C2=C21 bonds also exhibit a typical double bond distance.

Despite the fact that there exists no significant charge delocalization within the π-system (which would weaken the C=C double bonds) the exchange of the endo-oxygen atom O1 in **4b** by a N1−H functionality with ammonium acetate yielding **7b** leads to a change of the isomerism at the exocyclic C2=C21 bond. Substitution of O1 by a NH group requires a ring opening thus facilitating enhanced flexibility and enabling the formation of an N−H···O bridge. Since the N1−C2 and N1−C5 bonds are shorter than the O1−C2 and O1−C5 bonds, the C2−N1−C5 bond angle becomes larger and the nitrogen atom moves more toward the ring center. Large differences between proximal and distal angles to the exocyclic groups are a consequence of steric strain.

BOX A (**8a**) crystallizes with a chain structure via formation of intermolecular N−H···O bridges (see Supporting Information). The intramolecular N1−H···O23 bridge is the reason for an almost planar molecule despite the fact that the rotation around C21−C22 would reduce the steric hindrance between the C21 methine moiety and the NH_2 fragment of the amide group. Sparse solubility of **8a** in common organic solvents and moderate solubility in protic solvents is due to the formation of larger aggregates via hydrogen bridges. Aggregation is even enhanced by the connection of these zigzag chains by additional (weaker) hydrogen bridges. A similar behavior was observed for **8b**, verifying that the vinyl moiety does not interfere with the hydrogen bridge framework which strongly favors the *Z*-isomers.

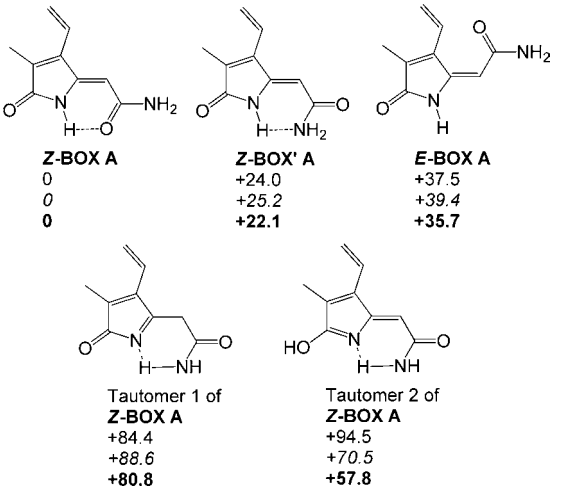

Figure 1. Isomeric representations and relative energy values of BOX A (**8a**). Relative energy values are results on the basis of MP2/def2-TZVPP (bold), B97-D/def2-TZVP (italic), and B3LYP/6-311++G(d,p) functionals (roman).

In agreement with the structural data which verify the lack of significant charge delocalization, NMR data show almost no significant dependency on the substitution pattern. The exchange of O1 by N1 leads to a low field shift of C5 and C22 resonances whereas C2 experiences a high field shift. Chemical shifts of the vinyl substituent show almost no dependency on the substitution pattern of the heterocycle, supporting that this group is not involved in any delocalization processes. The hydrogen bridge framework hinders free rotation of the NH_2 group around the C−N bond. Therefore, two resonances were observed for the protons of this group. Temperature-dependent NMR experiments allow the estimation of the energy barrier for this exchange process which involves break-up of the hydrogen bridges and free rotation around the C−N bond. A value of 68.4 kJ mol^{-1} was calculated for this exchange process (see Supporting Information). In addition, irradiation of *Z*-BOX A (**8a**) with a xenon lamp led to *Z*/*E*-isomerization and the formation of a mixture of both isomers which were separated by LC methods, thus enabling the assignment of the NMR parameters also of the *E*-isomer (see Supporting Information). Further structural information on the *Z*-BOX A isomer was

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(20) Crystallographic information (excluding structure factors) has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication CCDC-830394 for **3**, CCDC-830396 for **4a**, CCDC-830397 for **4b**, CCDC-830395 for **5**, CCDC-948683 for **6**, CCDC-830398 for **7b**, CCDC-948684 for **8a**, and CCDC-830399 for **8b**. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K. [E-mail: deposit@ccdc.cam.ac.uk].

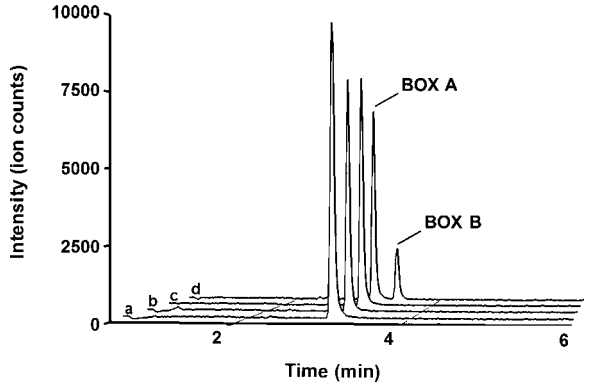

Figure 2. UPLC-MS plots of ion traces $m/z = 179.1$: (a) purified BOX A from bilirubin degradation; (b) 1:1 mixture of purified BOX A from bilirubin degradation and synthetic product, no difference in peak shape; (c) synthetically produced *Z*-BOX A with consistent retention time; (d) *in vitro* preparation of the oxidative degradation of bilirubin resolving the two regioisomers BOX A and BOX B.

obtained by monitoring the characteristic fragmentation via ESI MS/MS (see Supporting Information). The sequence of fragmentation begins with the neutral loss of ammonia and CO from the amide side chain. After rearrangement a further loss of CO as well as methyl and vinyl moieties can be observed. The neutral loss of ethylene instead of CO could be excluded by comparing the mass differences between the fragments with the exact masses for C_2H_4 and CO ($m/z = 28.0313$ vs 27.9949).

Quantum chemical calculations verify that monomeric *Z*-BOX A (**8a**) is energetically favored in comparison to the *E*-isomer by 35.7 kJ mol^{-1} (MP2/def2-TZVPP, Figure 1). In order to investigate the dependence of the energy differences on the quantum chemical method we supplement the *ab initio* MP2/def2-TZVPP data (bold) by density functional results obtained with two different functionals, namely with B3LYP/6-311++G(d,p) (roman) and B97-D/def2-TZVP (italic; see the Supporting Information for technical details). In addition, these calculations also show the preference of a N−H···O bridge in Z-BOX A in comparison to a N−H···N bridge in Z-BOX′ A. Tautomeric forms due to a 1,3-H shift contain exocyclic C−C (tautomer 1) or C−O single bonds (tautomer 2) and are significantly higher in energy than *Z*-BOX A (**8a**). Still, the energy differences are only slightly above the energy of hydrogen bonds between solvent molecules (e.g., about 20 kJ/mol in the water dimer) and one may speculate that thermally induced isomerization is thus thermodynamically feasible. Dimerization of *Z*-BOX A (**8a**) via two intermolecular N−H···O bridges between the amide moieties (as found in the solid state) is energetically favored by 61.8 kJ mol^{-1} (MP2/def2-TZVPP) compared to two isolated molecules.

An ultraperformance liquid chromatography (UPLC) ESI ToF MS method allows separation and detection of products from an *in vitro* oxidation of bilirubin.[8] A multistep gradient of water and acetonitrile, both containing 0.1% formic acid, with a total analysis time of 8.25 min was used to resolve the most relevant oxidation products BOX A and BOX B. The identity of synthetic BOX A and the product derived from the bilirubin breakdown was proven by a series of experiments (Figure 2). The retention times for BOX A derived from the *in vitro* bilirubin degradation and the synthesized product are identical. Furthermore, the injection of a 1:1 mixture of both BOX A preparations did not show any differences in the peak shape (e.g., peak splitting). Results from this study thus unambiguously prove that bilirubin degradation leads to the *Z*-BOX A isomer.

In conclusion, a selective synthesis of (*Z*)-2-(3-ethenyl-4-methyl-5-oxo-1,5-dihydro-2*H*-pyrrol-2-ylidene)ethanamide (BOX A, **8a**) was achieved, now permitting specific studies with respect to cerebral vasospasm. In the past medicinal studies were dependent on partially purified mixtures of BOXes of unknown isomerism. Due to the preparation of both isomers of BOX A the isomers could unambiguously be identified and characterized. On the basis of this preparative protocol a synthetic access of BOX A is introduced which is not based on an oxidative degradation of bilirubin. Starting from commercially available citraconic anhydride purification of the isomer methyl (*Z*)-(3-bromo-4-methyl-5-oxofuran-2(5*H*)-ylidene)ethanoate (**3**) from the minor component methyl (*Z*)-4-bromo-3-methyl-5-oxofuran-2(5*H*)-ylidene)-ethanoate (**5**) enables a selective synthesis of BOX A. Several subsequent conversions finally yield *Z*-BOX A (**8a**) with a four- or six-step synthesis, each reaction step with moderate to good yields. By utilizing the synthetic BOX A isomers in LC-MS experiments, the product of the bilirubin degradation was identified to be *Z*-BOX A (**8a**).

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Supporting Information Available. Experimental procedures and spectral data for all new compounds are provided. In addition, molecular structures of **3**, **4a**, **4b**, **5**, **6**, **7b**, **8a**, and **8b** as well as structural parameters are given. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.