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RESEARCH ARTICLE

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A Stereocontrolled Total Synthesis of Lipoxin B4 and its Biological Activity as a Pro-Resolving Lipid Mediator of Neuroinflammation

C. Frank Lee,[a] Carla E. Brown,[a] Alexander J. Nielsen,[a] Changmo Kim,[b],[c],[h] Izhar Livne-Bar,[b],[c] Philip J. Parsons,[d] Christophe Boldron,[e] François Autelitano,[e] Donald F. Weaver,[a],[f],[g],[h] Jeremy M. Sivak,[b],[c],[i] Mark A. Reed*[a],[j]

[a] Dr. C. F. Lee, Dr. C. E. Brown, Dr. A. J. Nielsen, Prof. Dr. D. F. Weaver, Prof. Dr. M. A. Reed*
Centre for Medicinal Chemistry and Drug Discovery
Krembil Research Institute, University Health Network
Toronto, Ontario, Canada
E-mail: mark.reed@uhnresearch.ca

[b] C. Kim, Dr. I. Livne-Bar, Prof. Dr. J. M. Sivak
Donald K. Johnson Eye Institute
Krembil Research Institute, University Health Network
Toronto, Ontario, Canada

[c] C. Kim, Dr. I. Livne-Bar, Prof. Dr. J. M. Sivak
Department of Ophthalmology & Vision Science
University of Toronto
Toronto, Ontario, Canada

[d] Prof. Dr. P. J. Parsons
Molecular Sciences Research Hub
White City Campus, Imperial College London
London, United Kingdom

[e] Dr. C. Boldron, Dr. F. Autelitano
Campus Curie
Evotec (France) SAS
Toulouse, France

[f] Prof. Dr. D. F. Weaver
Department of Fundamental Neurobiology
Krembil Research Institute, University Health Network
Toronto, Ontario, Canada

[g] Prof. Dr. D. F. Weaver
Department of Chemistry
University of Toronto
Toronto, Ontario, Canada

[h] Prof. Dr. D. F. Weaver
Department of Medicine
University of Toronto
Toronto, Ontario, Canada

[i] C. Kim, Prof. Dr. J. M. Sivak
Department of Laboratory Medicine & Pathobiology
University of Toronto
Toronto, Ontario, Canada

[j] Prof. Dr. M. A. Reed
Department of Pharmacology & Toxicology
University of Toronto
Toronto, Ontario, Canada

Abstract: Two stereocontrolled, efficient, and modular syntheses of eicosanoid lipoxin B4 (LXB_4) are reported. One features a stereoselective reduction followed by an asymmetric epoxidation sequence to set the vicinal diol stereocentres. The dienyne was installed via a one-pot Wittig olefination and base-mediated epoxide ring opening cascade. The other approach installed the diol through an asymmetric dihydroxylation reaction followed by a Horner-Wadsworth-Emmons olefination to afford the common dienyne intermediate. Finally, a Sonogashira coupling and an alkyne hydrosilylation/proto-desilylation protocol furnished LXB_4 in 25% overall yield in just 10 steps. For the first time, LXB_4 has been fully characterized spectroscopically with its structure confirmed as previously reported. We have demonstrated that the synthesized LXB_4 showed similar biological activity to commercial sources in a cellular neuroprotection model. This synthetic route can be employed to synthesize large quantities of LXB_4, enable synthesis of new analogs, and chemical probes for receptor and pathway characterization.

Introduction

Lipoxin B4 (LXB_4) is a non-classic eicosanoid and member of the specialized pro-resolving mediator (SPM) family of polyunsaturated fatty acids, derived from arachidonic acid (AA) through a series of oxidation steps by 5-lipoxygenase (5-LOX) and 12/15-LOX.[1,2] Extensive chromatographic evidence along with comparisons of biological activities to related eicosanoids led to the structural and configurational assignment of LXB_4[3,4] and related lipoxin A4 (LXA_4) (Figure 1).[5] Both lipoxins have been associated with the promotion of anti-inflammatory and pro-resolution processes since their initial discovery in 1984.[6] Of the two structurally similar

lipoxins, LXA_4 has been well-studied and shown to primarily bind to the G-protein coupled formyl peptide receptor 2 (ALX/FPR2) and GPR32, which initiates downstream anti-inflammatory effects.[7,8] However, endogenous receptors for LXB_4 have so far not been identified. Additionally, spectroscopic analysis to confirm the structure of LXB_4 remains ambiguous, even though it has been the subject of previous total syntheses and is reported to exhibit potent bioactivities.[9–11]

The first total synthesis of LXB_4 was reported by Leblanc and co-workers in 1985 via a chiral pool strategy from 2-deoxy-D-ribose.[12a] LXB_4 and its all-*trans* isomer were reported in order to assign the stereochemistry relative to an authentic natural sample. Shortly thereafter in 1986, Morris and co-workers utilized a very similar strategy to synthesize LXB_4 and its methyl ester for the same purpose.[12b] In that same year, Nicolaou and co-workers independently reported a stereocontrolled total synthesis of LXB_4 and its isomers using Sharpless asymmetric epoxidation and asymmetric reduction strategies to install the stereocentres in the natural product.[12c] Since then, the Depezay and Spur groups have also reported formal and total syntheses of LXB_4.[12d-f] However, previously reported syntheses to date are generally low yielding, together with long synthetic routes and lack full spectral characterization for LXB_4.

Recently, both LXA_4 and LXB_4 have been implicated in the regulation of neuroinflammation and neurodegeneration.[13] Specifically, therapeutic LXB_4 treatment was significantly more potent and efficacious than LXA_4 in promoting direct neuroprotection in a variety of neuronal cell types, and from acute and chronic injury models of the neurodegenerative disease glaucoma.[14] Based on these data, our group has initiated a drug discovery program to study LXB_4 signaling. To enable the identification and deconvolution of LXB_4's putative biological targets, we have developed a novel and efficient total synthesis of the natural product LXB_4. This modular and efficient synthetic approach has been used to synthesize appreciable quantities of LXB_4, allowing us to study its role in neuronal signaling mechanisms and evaluate its therapeutic potential as a treatment for neurodegeneration. Finally, for the first time, we have comprehensively characterized the structure of LXB_4 using a combination of 1D and 2D ^1H and ^{13}C NMR, high resolution mass spectrometry and optical rotation, confirming that the structure of LXB_4 is indeed (5*S*,6*E*,8*Z*,10*E*,12*E*,14*R*,15*S*)-5,14,15-trihydroxyicosa-6,8,10,12-tetraenoic acid (**2**), as shown in Figure 1.

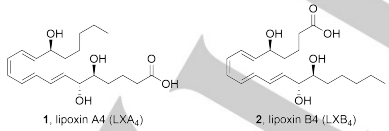
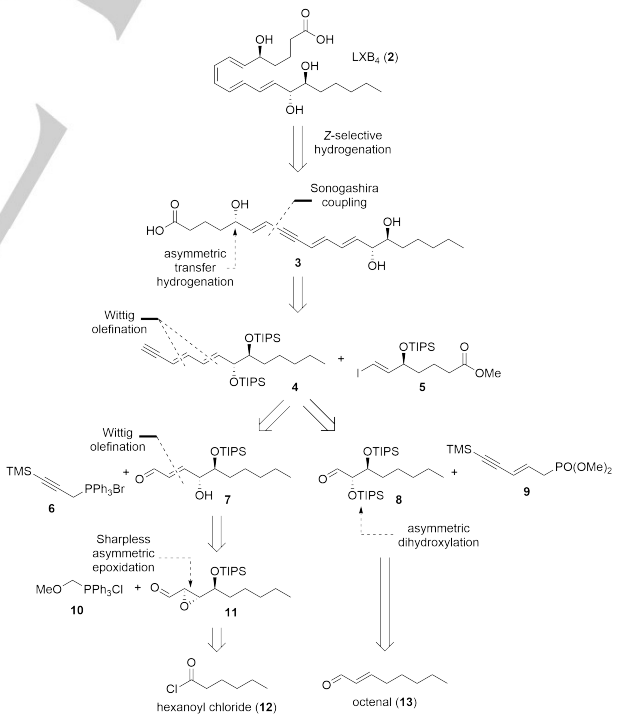


Figure 1. Chemical structures of lipoxins A4 (**1**) and B4 (**2**).

Results and Discussion

Lipoxins differ in their aliphatic sidechains but share a characteristic and synthetically challenging conjugated tetraene backbone with an *E,Z,E,E*-configuration (Figure 1), alongside three key alcohol stereocentres. Formal and total syntheses of LXB_4 have been previously reported using stereospecific,[12c,g] enantioselective[12d,h] and chiral pool synthetic strategies.[12b,e,f,i] However, we required a robust and modular synthesis of LXB_4, to both provide unambiguous spectroscopic evidence for its structure, and to enable the future exploration of synthetic analogs for structure-activity relationship (SAR) studies to probe the biological function of LXB_4 in neuroprotection and neurodegeneration.

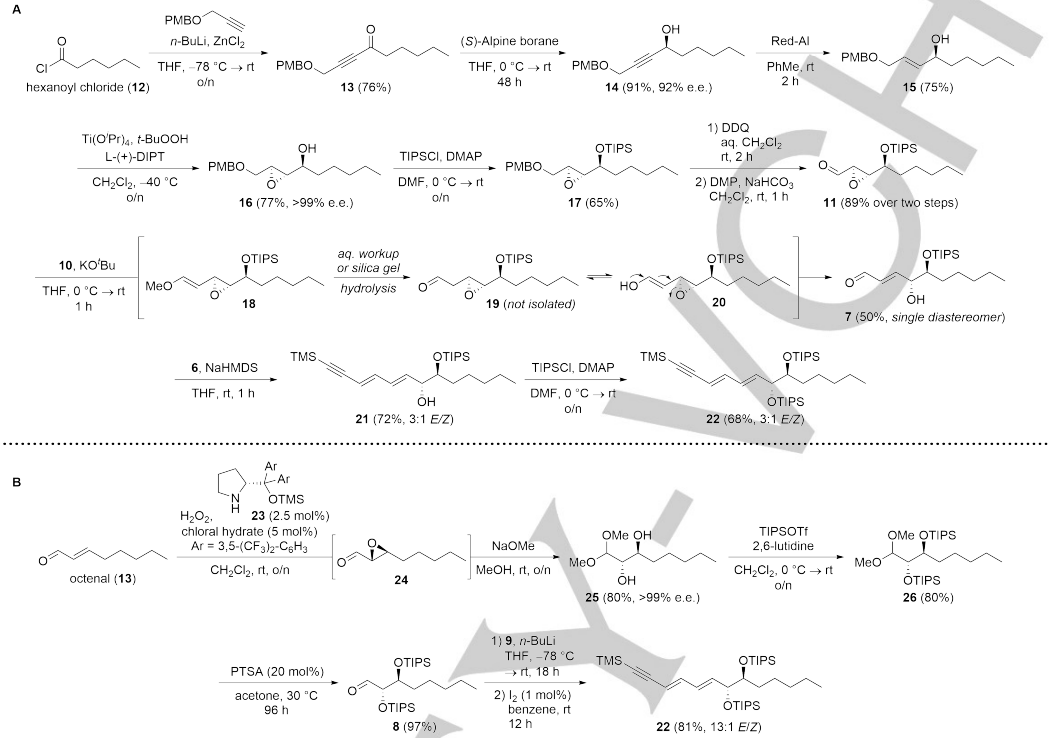
Based on our retrosynthetic analysis, a *Z*-selective semi-hydrogenation of alkyne **3** was envisaged to afford LXB_4 (**2**) as depicted in Scheme 1. Intermediate **3** would be constructed via a Sonogashira coupling of two key fragments, dienyne **4** and vinyl iodide **5**. Two routes relying on carbonyl olefinations of functionalized aldehydes were envisioned to dienyne **4**, whereas vinyl iodide **5** can be synthesized from commercially available starting materials following a literature route.[15] The first route to dienyne **4** depended on a Wittig olefination between phosphonium salt **6** and aldehyde **7**, itself the product of a Wittig one-carbon homologation/isomerization cascade on epoxy aldehyde **11**. We initially elected to define the stereochemistry of the vicinal diol moiety via asymmetric carbonyl reduction and Sharpless asymmetric epoxidation process,[16] and constructed **11** from hexanoyl chloride **12** using an approach similar to that taken by Kobayashi and co-workers in their syntheses of hydroxyeicosatetraenoic acids (HETEs)[17] and resolvins.[18,19] An alternative route to dienyne **4** depended on the Horner-Wadsworth-Emmons (HWE) reaction of phosphonate **9** with aldehyde **8**; similar olefinations have been used in the syntheses of various other triene and tetraene natural products and derivatives.[20a-g] In turn, it was anticipated that aldehyde **8** could be prepared in a few steps from octenal, with the alcohol stereocentres installed in the first step via an organocatalytic asymmetric dihydroxylation.[21]



Scheme 1. Retrosynthetic analysis of LXB_4 (**2**).

The synthesis of advanced intermediate epoxy aldehyde **11** was initially achieved from hexanoyl chloride. Addition of PMB-protected propargyl alcohol to hexanoyl chloride **12** afforded alkynone **13** in 76% yield. The use of readily available acyl chlorides

highlights the modularity of this approach, as any aliphatic acyl chloride can be incorporated for SAR studies. Asymmetric reduction of **13** using (*S*)-alpine borane gave the propargyl alcohol **14** in 91% yield and 92% *e.e.*, which was followed by a selective reduction to the *E*-olefin in 75% yield using Red-Al. The desired epoxy alcohol **16** was then obtained in 77% yield using Sharpless asymmetric epoxidation conditions. The resulting alcohol was protected as the TIPS ether **17**, which was subjected to sequential PMB removal with DDQ followed by a DMP oxidation to the desired epoxy aldehyde **11** in excellent yields.

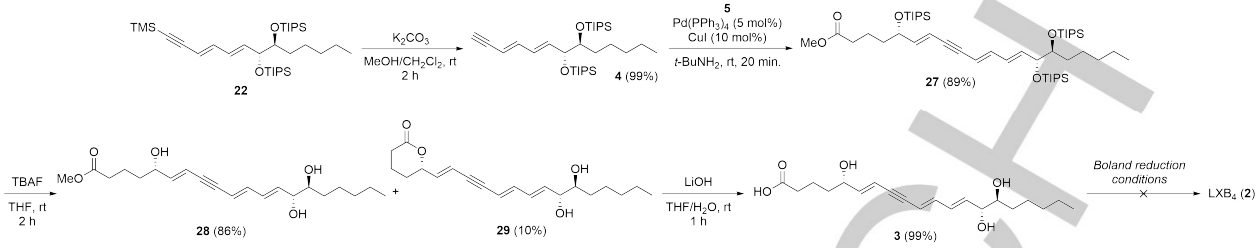


Scheme 2. A) Synthesis of common dienyne intermediate **22** from hexanoyl chloride **12** via an asymmetric reduction with (*S*)-alpine borane followed by a Sharpless asymmetric epoxidation sequence and a proposed one-pot Wittig olefination/base-mediated epoxide ring opening cascade. B) Synthesis of common dienyne intermediate **22** from octenal **13** via an asymmetric dihydroxylation with Jørgensen's catalyst and a Horner-Wadsworth-Emmons reaction. PMB-: *p*-methoxybenzyl-; Bu-: butyl-; THF: tetrahydrofuran; rt: room temperature; o/n: overnight; PhMe: toluene; L-(+)-DIPT: (+)-diisopropyl L-tartrate; TIPS-: triisopropylsilyl-; DMAP: 4-dimethylaminopyridine; DMF: dimethylformamide; DDQ: 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; aq.: aqueous; DMP: Dess-Martin periodinane; TMS-: trimethylsilyl-; Me-: methyl-; PTSA: *p*-toluenesulfonic acid.

With aldehyde **11** in hand, a Wittig olefination with (methoxymethyl)triphenylphosphonium chloride **10** furnished the desired hydroxy enal **7** in 50% isolated yield in one step, instead of yielding the expected homologated epoxy aldehyde **19** (Scheme 2A). We initially proposed to access **7** from a base-mediated epoxide ring-opening of **19**; however, we observed that the enol ether product of the olefination reaction **18** was hydrolyzed during aqueous workup or silica gel chromatography to the corresponding enol **20**, which presumably undergoes an acid or base catalyzed rearrangement, simultaneously forming the enal system and opening the epoxide to fruitfully give **7** as a single diastereomer in moderate yield. Synthesis of the TMS-protected dienyne fragment **22** was completed by a Wittig olefination on hydroxy enal **7** with commercially available ylide **6**, followed by a TIPS protection to furnish the globally protected advanced intermediate as a single diastereomer with a 3:1 *E/Z* ratio (Scheme 2A) (49% over two steps).

While the synthesis of fragment **22** was achieved from hexanoyl chloride in 10 steps, the modest *E/Z* ratio and overall yield (6%) prompted the exploration of an alternative route that would reduce the number of steps, while remaining amenable to the incorporation of varying aliphatic chains for future SAR studies. Thus, a route that hinges on an organocatalytic asymmetric dihydroxylation of readily available enals was designed (Scheme 2B).[21] Starting from octenal **13**, the asymmetric dihydroxylation proceeded via an epoxyaldehyde intermediate **24** that was formed upon treatment of **13** with the second generation Jørgensen catalyst (**23**) and hydrogen peroxide; chloral hydrate acted as a phase transfer catalyst for this first step.[22–24] While epoxide **24** can be isolable, it can also be directly converted to trans diol **25** by the addition of excess sodium methoxide with concurrent protection of the aldehyde as a dimethyl acetal.[21] Although the conversion of **13** to **25** is reported as a one-pot procedure, we decided to add a peroxide quenching step to eliminate the possibility of any base-catalyzed epoxidation of trace/unconverted octenal, which would negatively impact the *e.e.* of **25**. Using this modified procedure, **25** was isolated as a single diastereomer in 80% yield and >99% *e.e.* (see Supporting Information) following simple aqueous workup. Subsequent protection of the diol with excess TIPSOTf gave the globally protected intermediate **26** in 80% yield. Chemoselective transacetalization of the dimethyl acetal to yield aldehyde **8** was achieved using catalytic PTSA in acetone and very mild heating. The completion of the dienye intermediate from aldehyde **8** proceeded via a HWE reaction with phosphonate **9** followed by an isomerization of the resulting protected dienyne using catalytic iodine.[25] Indeed, similar olefination-isomerization strategies have been successfully used in the synthesis of other conjugated polyenes and lipoxin

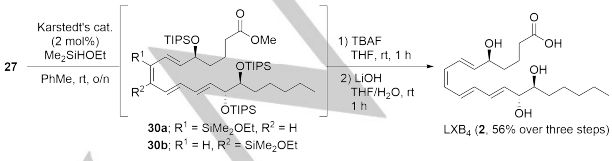
analogues.[12c,20b,e,f,20h–l] In our hands, the HWE reaction of aldehyde **8** yielded the desired dienyne in 11:10 *E*/*Z* ratio, but isomerization using trace iodine in benzene gave **22** in a 13:1 *E*/*Z* ratio and 81% isolated yield over two steps. This alternative route from octenal **13** was achieved in 5 steps with a much improved 50% overall yield.



Scheme 3. Synthesis of triol intermediate **3** via Sonogashira coupling and attempted conversions to LXB_4 via semi-hydrogenation. TBAF: tetra-*n*-butylammonium fluoride.

Having established two viable routes to the desired dienyne intermediate, the carbon backbone of LXB_4 was achieved smoothly with the quantitative removal of the TMS group followed by Sonogashira coupling of enyne **4** with vinyl iodide **5**,[15,20m–q,26] yielding the coupled product **28** in 89% yield (Scheme 5). The TIPS groups were globally removed with TBAF delivering the triol **28** in good yield, followed by hydrolysis of the methyl ester to give the penultimate product **3**. The corresponding lactone **29** was also isolated in low quantities but it can easily be converted back to the methyl ester **28** or directly hydrolyzed to **3**.[12c]

With **3** in-hand, various conditions were explored to the synthesis of LXB_4 via reduction of the internal alkyne (Scheme 5). Unfortunately, various conditions using a Boland reduction of **2** failed to generate the desired *E,Z,E,E*-conjugated tetraene, contrary to previous reports on similar internal alkynes.[20h,k,27–32] In our hands, the Boland reduction conditions led to starting material recovery. Next, we explored semi-hydrogenation to generate the delicate tetraene system. Unfortunately, no reduction was observed when using P–2 Ni with ethylene diamine.[33,34] As well, the use of various poisoned Pd catalysts, including Lindlar's catalyst, consistently produced a mixture of LXB_4, residual starting material, and over-reduced by-products that were difficult to separate. Attempts to tune the reactivity by modifying the Pd source, additives, and solvent sources were unsuccessful; thus, we opted to pursue alternative conditions for installing the tetraene system. We were inspired by the Karstedt alkyne hydrosilylation/proto-desilylation approach used by Hansen and co-workers in their synthesis of resolvin D1 congeners.[20q,35] Treatment of globally protected **27** with Karstedt's catalyst and ethoxy(dimethyl)silane led to a mixture of hydrosilylated regioisomers **30a** & **30b**. The regioisomers were subjected to a global silyl deprotection followed by hydrolysis to furnish LXB_4 in 56% isolated yield over three steps via semi-preparative HPLC purification. (Scheme 4).



Scheme 4. Synthesis of LXB_4 (**2**) via hydrosilylation/proto-desilylation of **27**. Karstedt's catalyst: platinum(0)-1,3-divinyl-1,1,3,3-tetramethyldisiloxane.

Instability of LXB_4 presented a substantial obstacle in its synthesis and purification. LXB_4 is highly unstable to air and light, and rapid degradation was observed when LXB_4 was stored as a solid even at low temperature (–20 °C). Telescoping the final transformations from **27** to **2,** preventing light exposure, and storing all crude intermediates in a methanol solution were required to obtain LXB_4 of sufficient purity. LXB_4 could be stored as a solution in methanol or ethanol at –20 °C for several weeks or at –80 °C for one to three months before substantial degradation was observed. Using this route, we were able to synthesize 100–200 mg (~0.25–0.5 mmol) of LXB_4. However, because of the instability of LXB_4, synthesis of **2** on larger scales is not prudent. Instead, we were able to generate the advanced intermediate **27** on an approximately 1 mmol scale. This intermediate can be stored at –20 °C for at least six months with minimal decomposition and can be used to rapidly generate pure LXB_4.

In conducting a review of the literature around the synthesis of LXB_4, we were surprised to discover that incomplete spectroscopic data have been reported for this natural product. ^{1}H NMR, mass spectrometry, IR, UV, and optical rotation data have been reported for the LXB_4 methyl ester (LXB4-ME), but data provided for the free acid was non-existent. Given the advances in analytical instrumentation since the total synthesis of LXB_4 was first reported, we were able to obtain extensive spectroscopic data (^{1}H, ^{13}C, COSY, HSQC, NOESY, and HMBC), high-resolution mass spectrometry, HPLC, and optical rotation for this natural product. We were gratified to observe that the spectra for the material we obtained were consistent with the proposed structure of LXB_4, and also matched the spectral data previously reported for similar tetraene systems, primarily LXB_4-ME,[12c] LXA_4,[3,12g] resolvin D1,[18] and resolvin D2.[29] Furthermore, we have been able to resolve several NMR peaks at 6.38, 6.28, 5.83, and 5.73 ppm as doublet of doublets which were previously assigned as multiplets in the spectra of LXB_4-ME (see Supporting Information). To further confirm the structure of LXB_4, our synthesized material (UHN) was compared to a commercial sample (Cayman Chemical)[36] and found to be identical by HPLC (mobile phase: acetonitrile + 0.1% TFA and MilliQ water + 0.1% TFA, gradient from 10:90→95:5, retention time 9.97 min for **2**, 9.95 min for commercial sample) and HRMS (ESI HRMS calculated for $C_{20}H_{32}O_5Na$ $[M+Na]^+$, predicted m/z 375.2147, found 375.2152, commercial sample 375.2165).[37]

In order to confirm the bioactivity of this material, we tested its neuroprotective effects in a neuronal injury model. Recent clinical failures of a variety of neuroprotection trials have intensified interest in new treatment strategies. Evidence is accumulating that lipoxins and other lipid mediators can have an important impact on neuroinflammation and neuronal survival.[13,38] We previously

reported that in addition to their known pro-resolution functions, lipoxins demonstrate an exciting novel protective bioactivity directly on neurons.[14] In particular, LXB_4 was surprisingly 20-fold more potent than LXA_4 in an established neuronal injury model,[14,39] and also active in a variety of primary neurons, including cortical and hippocampal cells, and retinal ganglion cells. These broad neuroprotective effects were subsequently correlated with demonstrated LXB_4 efficacy *in vivo* using both acute and chronic models of retinal ganglion cell death associated with the common neurodegenerative disease glaucoma, a leading cause of vision loss and blindness worldwide.[40-42] Yet the lack of efficient syntheses of LXB_4 coupled with the expensive cost from commercial sources (USD$806/100µg, Cayman Chemical)[36] have made this new mechanism of action challenging to modify and study. In this regard with our new synthetic route towards LXB_4, we profiled the neuroprotective activity of newly synthesized LXB_4 (UHN) and compared it against the commercial source.[36] For this assay, HT22 neuronal cells were pre-treated with LXB_4 from both sources (commercial vs. in-house), followed by a glutamate challenge to induce excitotoxic cell death.[14] Cell viability was then measured using an XTT assay. Both commercial and in-house samples of LXB_4 were significantly neuroprotective in this assay, demonstrating a similar three-fold recovery in neuronal survival at 1 µM (Figure 2A). In a parallel dose-response experiment, the EC_{50} of the newly synthesized LXB_4 was determined to be 292.8 nM (Figure 2B).

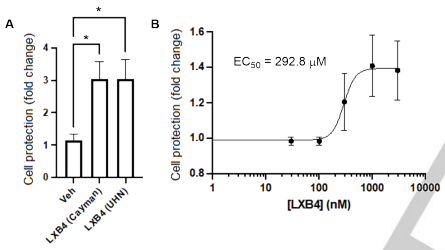
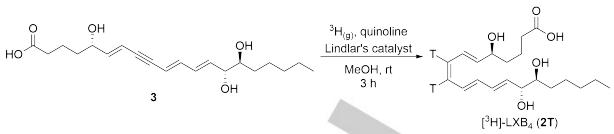


Figure 2. *In vitro* validation studies between synthesized LXB_4 vs. commercial LXB_4 from Cayman Chemical. A) HT22 cells were treated with 1µM of LXB_4 from either a commercial source (Cayman) or internally synthesized (UHN). Both showed significant protection in a glutamate injury model (n=8, *p<0.05, bars are S.E.). B) A dose response curve for the internally synthesized LXB_4 (UHN) in the same assay indicates an EC_{50} of 292.8 nM (n=8, bars are S.E.).

In the interests of further characterization of LXB_4 bioactivity, we are pursuing screening technologies that required the ligand (LXB_4) to be radiolabelled with tritium. We envisaged incorporating tritium in the last synthetic step from advanced intermediate **3** via hydrogenation with tritium gas (Scheme 5). Although the transformation resulted in a mixture of unreacted LXB_4, tritiated-LXB_4, and over-reduced LXB_4, the desired tritiated product **2T** was isolated in small quantities via semi-preparative HPLC purification with 96% radiochemical purity along with trace amounts of over-reduced material (see Supporting Information). Nonetheless, the radiolabelled sample contained sufficient specific activity of 37 Ci/mmol, exceeding the 20 Ci/mmol required to screen >6,200 human plasma membrane monomers, heterodimers, and secreted proteins within the panel.[43] Results of this study, along with target identification and deconvolution using photoaffinity-labelled LXB_4 and aromatic mimics, will be described in subsequent upcoming publications by our group.



Scheme 5. Synthesis of tritiated LXB_4 (**2T**) via hydrogenation with Lindlar's catalyst and tritium gas.

Conclusion

In summary, we have developed a stereocontrolled and modular total synthesis of LXB_4 to facilitate target deconvolution and advancement of SAR studies. The chromatographic data of LXB_4 are consistent with previous reports[1,2,12c,e] and commercial sources.[36] More importantly, we have now reported unambiguous spectroscopic data to confirm the configurational structure of LXB_4 to be (5*S*,6*E*,8*Z*,10*E*,12*E*,14*R*,15*S*)-5,14,15-trihydroxyicosa-6,8,10,12-tetraenoic acid.[37] Two routes to the advanced enyne intermediate **4** were developed, both of which will allow for the preparation of future derivatives with varying aliphatic chains. Coupling of **4** with known vinyl iodide **5** proved an excellent strategy for building the carbon backbone of LXB_4. Moreover, we have explored several strategies for the conversion of the internal alkyne precursors (**3** or **27**) to the sensitive tetraene system of LXB_4. Ultimately, the hydrosilylation/proto-desilylation protocol with Karstedt's catalyst yielded the desired tetraene system of the natural product. Final hydrolysis furnished 100–200 mg of the natural product LXB_4 in 10 steps with an overall yield of 25%, constituting the shortest, most efficient route of this natural product to date. With this new route in hand, LXB_4 analog synthesis, as well as the synthesis of LXB_4-derived chemical biology probes are currently underway to further investigate the biological function of LXB_4 in neuroprotection and neurodegeneration. These analogs will also be used for target receptor and pathway identification purposes, which will be the subject of forthcoming reports.

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Keywords: lipoxins • natural products • pro-resolving lipid mediators • total synthesis • neuroinflammation

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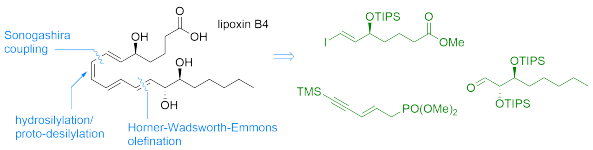
Entry for the Table of Contents



Two stereocontrolled, efficient, and modular syntheses of eicosanoid lipoxin B4 (LXB_4) are reported along with full spectral characterization of the natural product for the first time. Synthetic LXB_4 exhibited significant neuroprotection in a cellular model of retinal ganglion cell survival assay. Our synthetic routes enable syntheses of new analogs and chemical probes for receptor and pathway identification.

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