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SYNTHESIS OF A FLUORESCENT LABELED THROMBOXANE A_2 RECEPTOR ANTAGONIST

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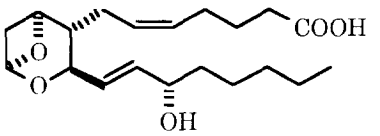
Summary: The synthesis and biological evaluation of a fluorescent labeled probe for the thromboxane A_2 receptor is described.

Since the discovery of thromboxane A_2 (TXA_2, **1**), an unstable metabolite of the arachidonic acid cascade,[4] a variety of stable analogs, each with a modified ring system, have been prepared and their biological characteristics have been evaluated.[5,6] TXA_2 is a powerful inducer of platelet aggregation and vascular and respiratory smooth muscle constriction.[4,7,8] The *in vivo* formation of TXA_2 is thought to play a key role in the pathogenesis of various circulatory and renal disorders.[9-11] Considerable efforts have been directed towards the synthesis of therapeutic agents that can either inhibit TXA_2 biosynthesis or antagonize its binding at the receptor level. Such compounds could potentially have value for the treatment for of a variety of pathophysiological disorders.[12-15]



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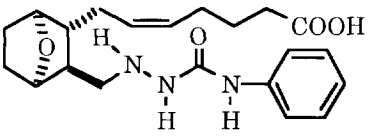
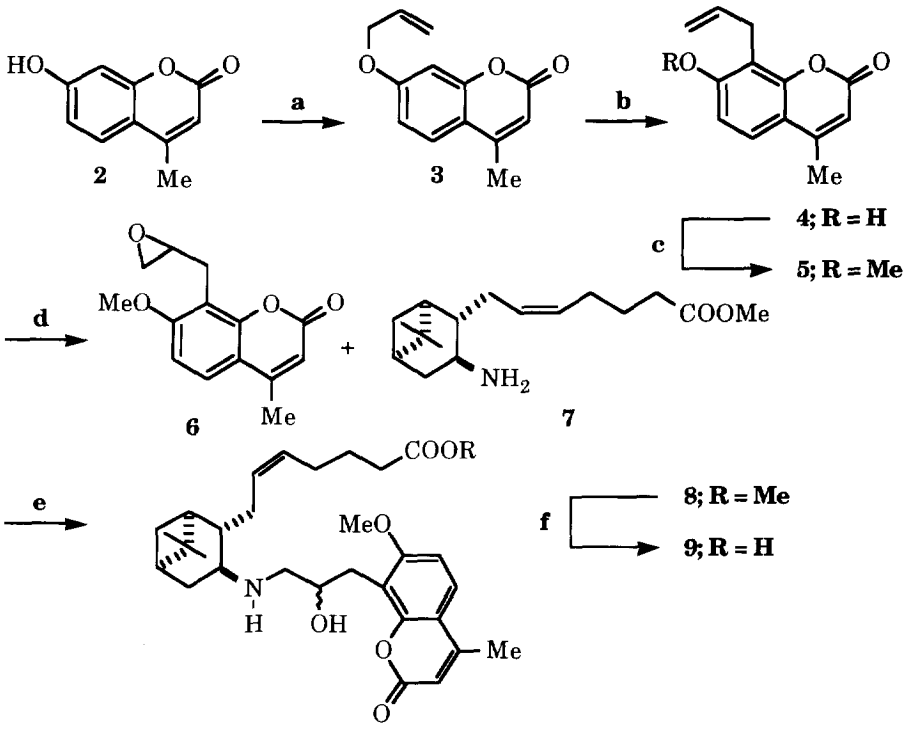


SQ 29,548

Insights into TX receptor localization have been gained with radiolabeled TX-receptor antagonists,[16] and by analysis of receptor protein and mRNAs for receptor isoforms.[17-19] Several radiolabeled TXA_2 agonists and antagonists have been used for characterization of platelet receptors. We were interested in developing a ligand that could be used in conjunction with a flow cytometer for separating specific cells containing TX receptors. Radiolabeled ligands are not suitable for these purposes and so we have developed a fluorescent labeled ligand that is compatible with current cell sorting technology. The key intermediate epoxide was prepared from 7-hydroxy-4-methyl coumarin (Aldrich) **2** as follows. Allylation of **2** (acetone, K_2CO_3, allyl bromide) was followed by Claisen migration of the resulting allyloxy derivative **3** by heating at 160 °Covernight in N,N-dimethylaniline to give the 6-hydroxy-8-allyl-derivative **4**. Protection of the hydroxyl group (acetone, K_2CO_3, dimethyl sulfate) and epoxidation of the resulting methyl ether **5** with *m*-chloroperbenzoic acid in CH_2Cl_2 gave the required epoxide **6**. Condensation of epoxide **6** with methyl 7-[(1S, 2S, 3S, 5R)-3-amino-6,6-dimethyl bicyclo{3,1,1]-hept-2-yl]-hept-5Z-enoate **7**[20] in refluxing methanol for 24 hr gave an epimeric mixture of methyl ester **8**. HPLC analysis of the

crude product revealed a mixture of two pinane coumarin (PCM) analogs in equal amounts. FAB/MS showed intense ions at *m/z* 512 (MH^+) for both the compounds, indicating that they were isomeric.



a. acetone, K_2CO_3, allyl bromide, 92 %; **b**. N,N-dimethylaniline, 160 °C, 65 %; **c**. acetone, K_2CO_3, dimethyl sulfate, 80 %; **d**. MeOH, 80 °C, 75 %; **e**. CH_2Cl_2, *m*CPBA, 72 %; **f**. LiOH, DMA/water, 50 °C, 85%.

Saponification of the methyl ester diastereoisomers with LiOH (3 N, DME/Water, 50 °C, 1 h) gave the free acid **9** as an epimeric mixture at C-15. The C-15 epimers were separated on a semipreparative partition HPLC (ultrasphere silica, 250 x 10 mm i.d.; 5 μm) using methanol/0.01 M ammonium acetate pH 5 (50:50, v/v) at a flow rate of 3 ml/min. The early eluting diastereoisomer 15α-PCM had a retention time of 20.3 min and showed an intense ion at *m/z* 498 (MH^+) on FAB/MS analysis.[21] The 15β-PCM isomer had a retention time of 22.1 min and gave an identical FAB mass spectrum.

The affinity of 15α-PCM and 15β-PCM for the TXA_2 receptor in washed human platelets was studied using a competitive binding assay with the TXA_2 receptor antagonist [^{3}H]SQ 29,548.[22] Blood, anticoagulated with EDTA (5 mM) was drawn from healthy volunteers who had received no medication for at least 10 days prior to study. The blood was centrifuged for 10 min at 160xg, and platelet rich plasma (PRP) was removed. The PRP was centrifuged at 800xg for 15 min, and the

platelet pellet resuspended in 50 mM Tris HCl (pH 7.4), 150 mM NaCl, and 5 mM dextrose to a concentration of 10^6 platelets/μl. The TX receptor antagonist ([^{3}H]SQ 29,548; 0.5 nM) was incubated with 5 x 10^7 platelets in the presence of various concentrations (1 nM to 10 μM) of 15 α-PCM and 15β-PCM. Incubations were carried out in duplicate at 37 °C for 30 min and were terminated by dilution with 4 ml of ice cold 10 mM Tris HCl (pH 7.4). The reaction mixtures were immediately filtered through Whatman GF/C glass fiber filters. The filtrates were then washed with an additional 3 x 4 ml of Tris buffer and total solutions were counted. Binding analyses were performed by computerized non-linear curve fitting using the LIGAND program (Fig. 1). The K_D for 15α-PCM was 138 ± 7nM (n=4) and for 15β-PCM was 424±47nM (n=4).

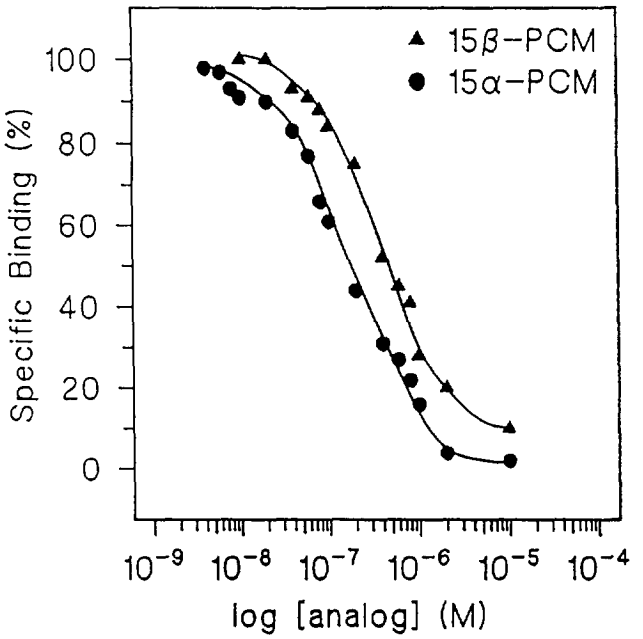


Figure 1:Competitive inhibition of the specific binding of [^{3}H]SQ 29,548 by 15α- and 15β-epimers.

In summary, we have prepared two fluorescent analogs that displace the TX receptor antagonist SQ-29,548 from the platelet TX receptor. The 15α-hydroxy analog (15α-PCM) has a K_D of 138 nM which makes it suitable for fluorescent labeling of the platelet TX receptor.

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References and Notes:

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21. 15α-PCM; UV λ_{max} 319 nm, NMR (CD_3OD) d 7.9 (d, 1H), 7.58 (d, 1H), 7.12 (d, 1H), 6.28 ((d, 1H), 5.30-5.5 (m, 2H), 4.32 (m, 1H), 3.98 (s, 3H), 3.02 (m, 3H), 1.46-2.75 (m, 16H), 1.24 (s, 3H), 1.12 (d, 1H) and 1.02 (s, 3H). 15β-PCM; UV λ_{max} 319 nm, NMR (CD_3OD) d 7.92 (d, 1H), 7.58 (d, 1H), 7.08 (d, 1H), 6.28 ((d, 1H), 5.35-5.50 (m, 2H), 4.25 (m, 1H), 3.97 (s, 3H), 3.02 (m, 3H), 1.46-2.75 (m, 16H), 1.24 (s, 3H), 1.06 (d, 1H) and 1.02 (s, 3H). The fast moving and the slow moving alcohol epimers were assigned the 15α and 15β configuration, respectively, on the basis of their polarities compared to those of the TXA_2 analogs (Nicolaou, K. C.; Magolda, R. L.; Claremon, D. A. *Adv. Prost. Thromb Res.* **1980**, *6*, 481.
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