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Synthesis of Fluorescent Biochemical Tools Related to the 2-Azetidinone Class of Cholesterol Absorption Inhibitors

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Abstract—Fluorescent analogues of the cholesterol absorption inhibitor (CAI), Sch 58235, have been designed and synthesized as single enantiomers. Biological testing reveals that they are potent CAIs and are suitable tools for the investigation of the azetidinone CAI mechanism of action (MOA). © 2002 Elsevier Science Ltd. All rights reserved.

In the previous paper, we described our efforts to design chemical tools for the investigation of the MOA of the very potent class of cholesterol absorption inhibitors typified by the original lead compound in this series, **1**, Sch 48461.[1,2] The preceding paper demonstrated the importance of metabolic sidechain hydroxylation to activity in a series of iodinated analogues, echoing the enhanced activity typified by our current clinical candidate, **2** (Sch 58235; Fig. 1).[3,4] The importance of understanding the precise biological mechanism by which the 2-azetidinones inhibit cholesterol absorption has warranted significant effort in our research labs.[5] Our ultimate goal is to discern the basic biochemistry related to cholesterol absorption. This report details our efforts to prepare fluorescent analogues of our lead CAIs and the evolution of our efforts towards structures resembling Sch 58235 and its glucuronide **3**.

Our previous work in making biochemical tools in the azetidinone CAI area focused our attention at the *N*-aryl site for substitution changes. It had proven to be the most tolerant of a variety of changes while maintaining biological activity. Our first success at making fluorescent analogues came with the inclusion of a benzothiadiazole at the azetidinone nitrogen position. Biological activity was first shown in a racemic compound related structurally to Sch 48461, then confirmed by the synthesis of enantiomerically pure **7**, the analogue related to Sch 58235. The synthesis of **7** begins with the chiral oxazolidinone **4** (Scheme 1).[6] Titanium tetrachloride mediated condensation with imine **5** gave an intermediate β-amino acyloxazolidinone, the major diastereomer of which could readily be purified to homogeneity by SiO_2 chromatography. Silylation and fluoride catalyzed cyclization gave the desired azetidinone **6** protected at both oxygen positions. Selective deprotection of first the benzylic alcohol, followed by boron trichloride mediated removal of the *p*-methoxybenzyl protecting group gave the fluorescent CAI **7**.[7] Chemical synthesis of the related glucuronide **8** was recalcitrant, though enzymatic approaches to its synthesis proved to be quite effective.[8]

When compound **7** was characterized for its fluorescent properties in biological systems, the fluorescence was deemed to be not bright enough for the binding studies

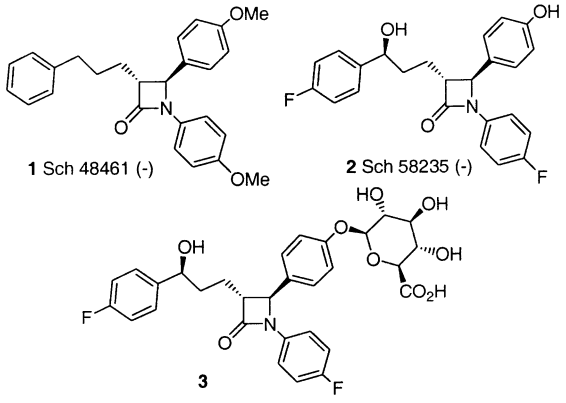

Figure 1. Cholesterol absorption inhibitors.

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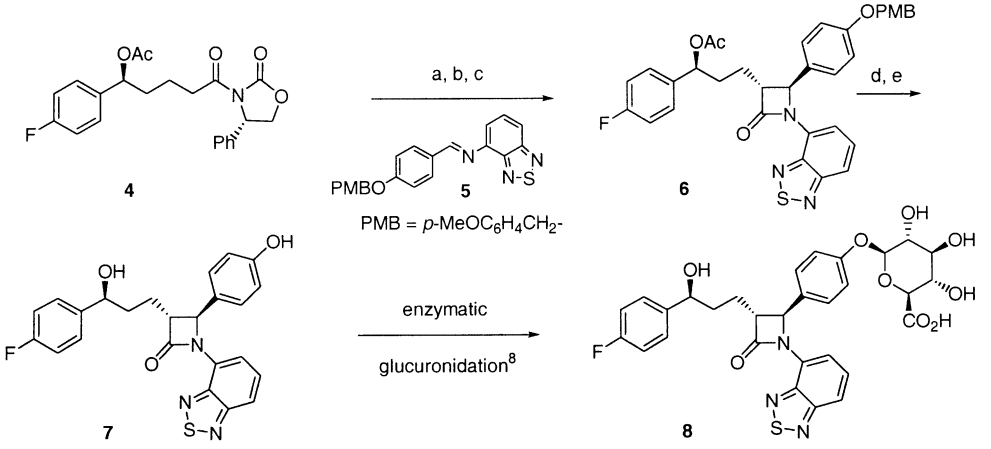
that were planned. Furthermore, the biologically important glucuronide analogue **8** was ~10-fold less bright in similar biological systems. Our charge came to be the preparation of compounds with similar biological properties, but with the fluorescent properties of fluorescein. We decided to take advantage of the *N*-iodophenyl CAI described previously[1] and modify the *N*-aryl group using palladium coupling technology. A suitably fluorescent moiety tethered to an activated carbonyl (5-SFX, Scheme 2) was purchased from Molecular Probes, Inc. We coupled this piece to propargyl amine to generate the fluorescent alkyne **10**. This alkyne was then coupled via a Sonagashira reaction to the *N*-iodophenyl CAI **12** as shown in Scheme 3.[1,9] We found that the methyl ester was best for this purpose, and mild hydrolysis revealed the desired fluorescent CAI, **14**. This analogue turned out to be particularly sensitive to a variety of conditions. For example, under acidic conditions, a lactonization occurred, destroying the fluorescence. Under basic conditions, the lactam would slowly open up, probably assisted by the pendant benzylic hydroxyl. Due to the compound's sensitivity, further work with this analogue was abandoned.

In order to overcome these difficulties, we once again chose to utilize palladium mediated alkynylation of our iodinated CAI. To our list of requirements for our target, we added the need for chemical stability. We turned to the BODIPY® moiety to solve our lactonization problem. It is well known that these dyes by Molecular Probes, Inc. offer significant advantages over fluorescein dyes by greatly extending the pH range in which they fluoresce.[10] As before, propargyl amine was coupled to an activated ester of the BODIPY® dye, followed by palladium mediated coupling of the alkyne to the iodophenyl lactam (Scheme 3). The methyl ester was purified by preparative SiO_2 TLC, then carefully hydrolyzed to give the highly fluorescent CAI **16**. This compound absorbed UV light with a λ_{max} of 505 nm in ethanol (*c* 0.55 mM, $\varepsilon = 33{,}000\ M^{-1}\ cm^{-1}$).[11]

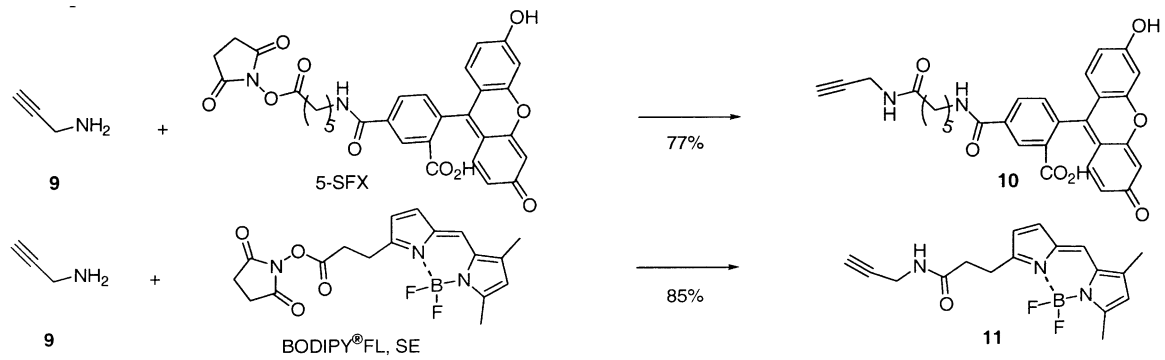
Determination of CAI Activity[4]

The biological activity of all the fluorescent analogues was assessed in a rapid cholesterol absorption assay in rats as described below. The advantage of this rapid assay format is that in addition to determining CAI activity quickly, very small quantities of drug could be used.

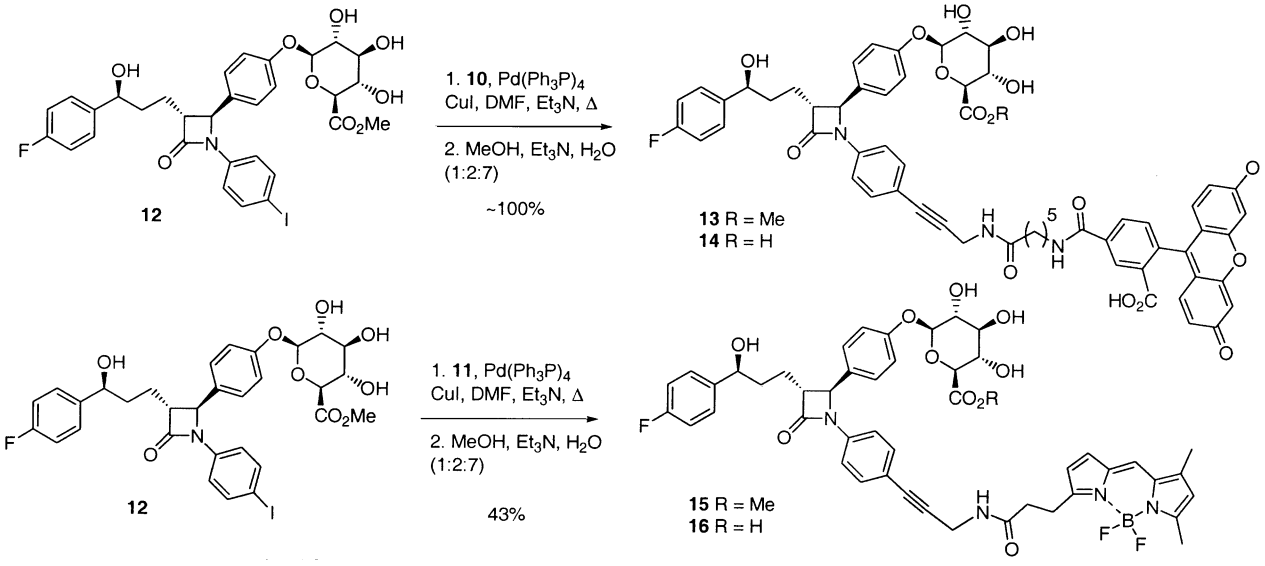
Male Sprague–Dawley rats weighing 250–300 g were used. After an overnight fast, rats were anesthetized (Inactin, 100 mg/kg ip) for the duration of each study and were fitted with a cannula into the small intestine just below the pyloric valve. For the cannulation of the small intestine, a catheter (Surflo® iv catheter (18GX2''), Terumo Medical Corporation; Elkton, MD, USA) was



Scheme 1. Reagents and conditions: (a) imine, $TiCl_4$, *i*-Pr_2NEt, CH_2Cl_2 (54%); (b) BSA, toluene; (c) TBAF (cat) (36%); (d) MeOH, Et_3N, H_2O (1:2:7) (36%); (e) BCl_3, CH_2Cl_2 (89%).



Scheme 2. Synthesis of fluorescent alkynes.



Scheme 3. Synthesis of glucuronidated fluorescent CAIs.

inserted through the fundus of the stomach, advanced 1 cm beyond the pylorus, and ligated in place. Compounds were mixed in rat bile and delivered by bolus injection (1 mL) via the intestinal catheter into the small intestine. One-half hour after the bile doses were delivered, 3 mL of a solution consisting of 2.23 mg/mL L-phosphatidylcholine and 11.8 mg/mL triolein in 19 mM sodium taurocholate (Sigma, St. Louis, MO, USA) buffer (pH 6.4) containing 3 mg cholesterol and 2 μCi ^{14}C-cholesterol (NEN, Boston, MA, USA) was delivered to each rat as a bolus via the intestinal cannula. Ninety minutes after the cholesterol emulsion was delivered, the rats were euthanized. Blood was collected and plasma was separated by centrifugation at 2000 rpm for 15 min at 4 °C. Triplicate aliquots of plasma were analyzed for ^{14}C radioactivity and inhibition of cholesterol absorption relative to bile vehicle control rats was determined.

The results of this assay are shown in Table 1. All fluorescent compounds were potent cholesterol absorption inhibitors in this assay at μg/kg levels of dosing. As a comparison, Sch 58235 has an ID_{50} of 1.6 μg/kg in this test.[4] Thus, all compounds were deemed to be biologically relevant CAIs and were suitable for use in our MOA studies.[12,13]

Table 1. In vivo activity of CAIs in a rapid cholesterol absorption assay in rats

Compd	Dose (μg/kg)	% Inh of ^{14}[C]-cholesterol absorption into plasma
1(−)	2000	95
2(−)	3	63
2(−)	10	74
3	10	91
8	10	66
8	30	83
8	100	85
14	30	85
14	56	79
14	186	88
16	30	58
16	100	80
16	300	92

We have designed and prepared a number of potent azetidinone CAIs with fluorescent absorption and emission properties making them suitable for use as biological tools for the investigation of the mechanism of action of this important class of new pharmacological agents. We have addressed both the photochemical and stability issues in this work and have assessed their biological utility via a rapid cholesterol absorption assay in rats. In particular, compound **16** represents a stable, highly fluorescent tool representative of this class for biological studies.

Acknowledgements

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6. This synthesis parallels a process route for the synthesis of Sch 58235. See ref 1 and references within.
7. Analytical data: Compound **7**: yellow solid, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, 1H, $J=7$ Hz), 7.70 (d, 1H, $J=9$ Hz), 7.57 (dd, 1H, $J=7$, 9 Hz), 7.34–7.25 (m, 4H), 7.03 (t, 2H, $J=7$ Hz), 6.72 (d, 2H, $J=7$ Hz), 5.58 (s, 1H), 4.77 (m, 2H), 3.25 (m, 1H), 2.15–1.98 (m, 4H). UV absorbance λ_{max} 370 nm (62 μM in EtOH), emission λ_{max} 495 nm (1.1 mM in EtOH) or λ_{max} 504 nm (1.1 mM in PBS, note: 30% intensity of EtOH, PBS = 15 mM aq Na_2PO_4, 0.138 M NaCl, pH 7.4). MS (FAB) m/z 450 $(M+H)^+$, HRMS (FAB) exact mass calcd for $C_{24}H_{21}N_3FO_3S$ m/z 450.1288, obsd m/z 450.1286.

Compound **8**:[8] MS (FAB) m/z 648.2 $(M+Na)^+$, HRMS [FAB, $(M+Na)^+$] exact mass calcd for $C_{30}H_{28}N_3O_9SFNa$ m/z 648.1428, obsd m/z 648.1430.

Compound **14**: orange solid isolated as a mixture of amide rotamers of $(Et_3NH^+)_2$ salt, ^{1}H NMR (400 MHz, CD_3OD) δ 8.42 (br s, 1H), 7.99–7.95 (m, 1H), 7.55 (d, 1H, $J=8$ Hz), 7.35–6.90 (m, 16H), 6.56 (m, 2H), 6.40 and 6.29 (2d, 1H, $J=8$ Hz) 4.93–4.78 (m, 2H), 4.58 (m, 1H), 4.13 and 4.08 (2s, 2H), 3.75–3.64 (m, 1H), 3.50–3.10 (m, 6H), 2.91 (q, 6H, $J=7$ Hz), 2.82 (q, 6H, $J=7$ Hz), 2.28–2.23 (m, 2H), 1.91 (s, 1H), 1.89 (s, 1H), 1.75–1.40 (m, 10H), 1.23 (t, 9H, $J=7$ Hz), 1.15 (t, 9H, $J=7$ Hz). MS (ES) m/z 1092.3 $(M+1)^+$, HRMS (FAB) exact mass calcd for $C_{60}H_{55}N_3O_{16}F$ m/z 1092.3566, obsd m/z 1092.3557.

Preparation of compound **11**: To 8 μL (6 mg, 120 μmol) of propargyl amine in 0.5 mL DMF and 1 drop of 0.1 M $NaHCO_3$, was added 10 mg (26 μmol) of the activated ester BODIPY FL, SE (Molecular Probes, Inc.) in 0.1 mL DMF. The reaction was stirred at rt for 12 h. Concentration in vacuo and chromatography over ~5 g SiO_2 eluting with 1% MeOH in CH_2Cl_2 gave the desired alkyne, **11**, as a yellow orange oil. R_f 0.62 in 5% MeOH in CH_2Cl_2 on SiO_2 TLC.

Preparation of compound **15**: To 7.7 mg (10.9 μmol) of *N*-iodophenylazetidinone glucuronide methyl ester **12** in 1 mL DMF was added 7.2 mg (11 μmol) of the BODIPY alkyne **11**. Argon was bubbled through the solution for a few minutes and then 1.2 mg (1 μmol) of palladium tetrakistriphenylphosphine, 0.6 mg (3 μmol) copper(I) iodide and 10 μL (7 μmol) triethylamine were added. The reaction was stirred overnight under argon. The solvent was removed in vacuo and the product purified via prep SiO_2 TLC eluting with a 50:50:17:1 mixture of EtOAc/hexanes/MeOH/HOAc to give the desired fluorescent methyl ester as an orange solid. MS (ES) m/z 909.2 $(M+1)^+$.

Preparation of compound **16**: 4.2 mg of the methyl ester **15** was dissolved in ~1 mL of a 7:2:1 mixture of water/triethylamine/methanol and stirred for 1 h. Evaporation to dryness gave the desired fluorescent azetidinone glucuronide, **16** as an orange solid isolated as (Et_3NH^+) salt, ^{1}H NMR (400 MHz, CD_3OD) δ 7.34–7.24 (m, 6H), 7.20 (d, 2H, $J=8$ Hz), 7.13 (d, 2H, $J=8$ Hz), 7.05–6.95 (m, 3H), 6.92–6.88 (m, 1H), 6.31 (d, 1H, $J=4$ Hz), 6.17 (s, 1H), 4.90 (m, 1H), 4.83 (m, 1H), 4.58 (m, 1H), 4.13 (s, 2H), 3.81 (m, 1H), 3.71 (d, 1H, $J=9$ Hz), 3.63 (m, 1H), 3.55–3.45 (m, 3H), 3.17–2.95 (m, 9H), 2.53 (s, 3H), 2.34 (t, 2H, $J=7$ Hz), 2.25 (br s, 3H), 1.90 (s, 1H), 1.94–1.85 (m, 2H), 1.63–1.55 (m, 2H), 1.28 (t, 9H, $J=7$ Hz), MS (ES): m/z 875.3 $(M+1-HF)^+$.
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11. **16** Absorption λ_{max} 502 nm, emission λ_{max} 510 nm in PBS (c 0.5 μM, $\varepsilon = 20{,}000\ M^{-1}\ cm^{-1}$), Note: under identical conditions, fluorescein λ_{max} 493 nm and the relative fluorescence intensity was 2.5:1 (fluorescein/**16**).
12. Details of our studies with **16** have been accepted for publication: Altmann, S. W.; Davis, H. R., Jr.; Yao, X.; Laverty, M.; Compton, D. S.; Zhu, L.; Crona, J. H.; Caplen, M. A.; Hoos, L. M.; Tetzloff, G.; Priestly, T.; Burnett, D. A.; Strader, C. D.; Graziano, M. P. *Biochim. et Biophys. Acta* In press.
13. The biological activity of compound **7** was measured in our 7 day cholesterol fed hamster assay[1] and found to lower hepatic cholesterol esters 92% versus controls at 3 mg/kg/day.