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ARTICLE

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Discovery of 2-Arylbenzoxazoles as Upregulators of Utrophin Production for the Treatment of Duchenne Muscular Dystrophy

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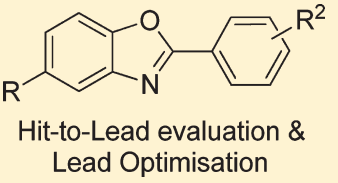
Supporting Information

ABSTRACT: A series of novel 2-arylbenzoxazoles that upregulate the production of utrophin in murine H2K cells, as assessed using a luciferase reporter linked assay, have been identified. This compound class appears to hold considerable promise as a potential treatment for Duchenne muscular dystrophy. Following the delineation of structure−activity relationships in the series, a number of potent upregulators were identified, and preliminary ADME evaluation is described. These studies have resulted in the identification of **1**, a compound that has been progressed to clinical trials.



INTRODUCTION

Duchenne muscular dystrophy (DMD) is one of the most common of the muscular dystrophies, affecting approximately 1 in 3500 males. The disorder is caused by a mutation in the gene *DMD*, located in humans on the X chromosome (Xp21). The DMD gene codes for the protein dystrophin, which plays an essential role in linking the internal cytoskeleton to the extracellular matrix as part of the dystrophin-associated protein complex (DAPC). Loss of functional dystrophin causes destabilization of the DAPC, and this results in a breakdown of muscle fibers and a collapse of membrane integrity.[1] Death from DMD is currently inevitable and typically occurs when the sufferer is in their twenties, usually due to cardiac or respiratory failure. Accordingly, an effective therapy is essential to treat this debilitating and ultimately fatal disease.[2]

Since the discovery of the gene responsible for causing DMD about 20 years ago,[3] a number of pharmacological approaches have been investigated for the treatment of this and other muscular dystrophies, and these have been recently reviewed.[4] In the majority of these studies, treatment is symptomatic only, being intended to improve the phenotype by means such as decreasing inflammation, improving calcium homeostasis, or increasing muscle progenitor proliferation or commitment. A number of small molecule, non-symptomatic approaches to treat DMD have also been described. These are based on either restoring the function of the mutated gene or slowing/preventing degradation of the resulting protein and muscle. The most advanced of these is PTC-124, the mode of action of which is to allow read-through of the nonsense mutations (premature stop codons), which are present in around 5−15% of DMD sufferers, thereby restoring gene function and production of dystrophin protein.[5] Phase 2a clinical trial data have shown that Ataluren treatment is associated with the production of functional dystrophin and statistically significant reductions in the leakage of muscle-derived creatine kinase into the blood. An alternative nonsymptomatic pharmacological approach, which has only begun to be investigated for DMD comparatively recently, is upregulation therapy. This is based on increasing the expression of alternative genes to replace those that are defective. Upregulation of utrophin, an autosomal paralogue of dystrophin, has been proposed as a potential treatment paradigm for DMD.[6,7] It has been estimated that a 2−3-fold increase in levels of systemic utrophin may be sufficient to ablate the dystrophic phenotype and partially or completely restore normal muscle function.[8,9]

Previously, the development of a cell-based assay was reported, which was designed to allow assessment of utrophin upregulation in a physiologically relevant environment and was also suitable for screening thousands of compounds.[10] In this assay, murine H2K cells were engineered to express the UTRN promoter linked to a luciferase reporter gene. Using this construct, measurement of utrophin upregulation could be made using a simple luminescence readout. Following an in vitro screen of a focused set of compounds using the aforementioned assay system, a number of small molecule upregulators of utrophin were identified (Figure 1). Among these were benzoxazole[11] **2** as well as the structurally related benzotriazole **3**.

Although both screening hits showed encouraging levels of potency in vitro, as well as some limited activity after dosing in vivo,[8] the compounds clearly contain several structural features that give cause for concern, most notably the presence of

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potential toxicophores (e.g., a primary aniline function in both **2** and **3**, Figure 1) or of metabolically vulnerable groups (e.g., benzylic methyl and phenolic units present in **2**).[12] This latter assertion was confirmed by the low systemic exposure seen when **2** was dosed orally to mice (Table 6). In this paper, we report the synthesis and biological evaluation of a family of 2-substituted benzoxazoles,[10,13] culminating in the discovery of **1**, a compound that has been progressed to clinical trials. Further work on the benzotriazole series will be reported elsewhere.[14]

CHEMISTRY

There are a number of methodologies that would allow access to the 2-aryl benzoxazole core.[15] The most generally applicable synthetic route employs a cyclocondensation approach between an aminophenol and either a carboxylic acid, using polyphosphoric acid (PPA), or an acid chloride. While thermal activation of the reaction was often the heating method of choice, we discovered that microwave irradiation[16] was also an extremely effective accelerant, particularly for reactions using an acid chloride. Compounds of general structure **4** and **5** were either purchased from commercial vendors or prepared using one of the routes indicated in Scheme 1. 5-Aminobenzoxazoles (**4**, R = 5-NH_2) were converted to amide derivatives, **6**, under standard reaction conditions. Alternatively, reduction of the nitro function present in **5** was effected using either tin(II) chloride or iron powder and subsequent amide formation delivered **6**.

A range of 2-(4-chlorophenyl)benzoxazoles bearing various substituents at the 5-position were prepared using routes shown in Scheme 2. The *N*-methyl derivative **7** was prepared from **8** using sodium hydride and iodomethane. Heating 3-amino-4-hydroxybenzoic acid (**9**) and *p*-chlorobenzoyl chloride in 1,4-dioxane, under microwave activation, afforded carboxylic acid **10**. Subsequent *O*-(7-azabenzotriazol-1yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU)-mediated amide formation gave reversed amide **11**. Sulfonamide **12** was prepared from aniline **13** using standard conditions. Reductive amination of **13**, using sodium triacetoxyborohydride, gave **14** in moderate yield and **13** was also used to prepare isosteric alanine analogue **16**. In the latter case, Fmoc protection was employed (**15**) to simplify isolation of the final product. Keto analogue **17** was synthesized in three steps from commercially available propiophenone **18**, and Lawesson's reagent[17] was used to convert **8** to its corresponding thioamide analogue, **19**.

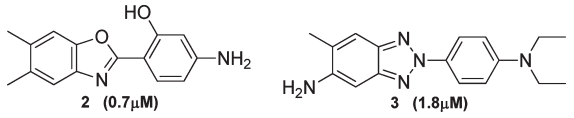


Figure 1. Screening hits (EC_{50}).

Alternative cores were obtained according to the routes shown in Scheme 3. Benzimidazole **20** and benzothiazole **21** were prepared in three and four steps, respectively, from diamine **22** and fluoride **23**. A tandem Sonogashira/cyclization strategy[18] was utilized to furnish benzofuran **24**, a precursor to **25**.

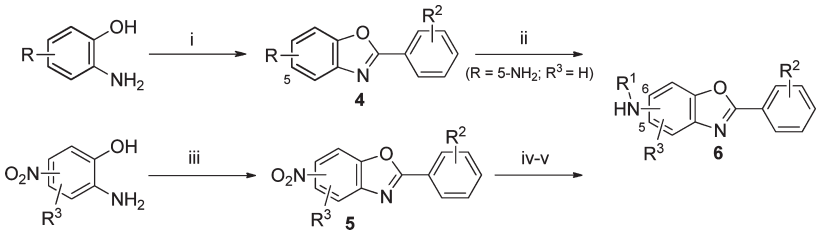
Sulfonyl derivatives were prepared as outlined in Scheme 4. A family of 5-ethylsulfonyl- substituted benzoxazoles were accessed using either a cyclocondensation approach between **26** and the appropriate acyl derivative or via oxidative cyclization of an imine intermediate (methods A, B, or C). Replacement of the ethyl group was accomplished via demethylation of anisoles **27** (R = Me, Pr, ^{i}Pr, and NEt_2), giving **28**, followed by PPA-mediated cyclization with the appropriate aryl carboxylic acid. In some cases, **28** (R = OH, NH_2, and NHMe) was condensed with acyl chlorides to afford either sulfonic acid or additional 5-sulfonylamido-benzoxazole derivatives, respectively. Preparation of 6-ethylsulfonyl-benzoxazoles was achieved using a three step sequence. Aminophenol **29** was formed in two steps via the S_NAr reaction between sodium ethanesulfinate and fluoronitrophenol **30**, followed by hydrogenolysis of the nitro group. Treatment of **29** with the requisite acid chlorides in dioxane, under microwave activation, then furnished the desired compounds.

RESULTS AND DISCUSSION

All analogues described herein were screened using the H2K cell-based assay with benzotriazole **3**[13] as an internal control compound. Initial efforts focused on attempts to replace the substituents present in 2-arylbenzoxazole **2**, while still retaining some level of biological activity (Table 1). Comparable activity was noted with the somewhat larger 5-alkyl substituent (**31**), which indicated that, while more investigation was clearly required, structural changes to the periphery of the molecule were possible. The 6-desmethyl 5-NH_2 variant **32** showed a modest level of activity as compared to **2**. This latter observation suggested that structure−activity crossover existed between the benzoxazole and the benzotriazole classes (as exemplified by **2** and **3**, respectively).

Following these results, we initiated a more extensive hit-to-lead evaluation by investigating structural regions A and C (Figure 2) in parallel. Although only modestly active, **32** bore functionality more amenable to synthetic exploration. Thus, our attention centered on introduction of an amine functionality at the 5-position, for facile

Scheme 1[a]



[a] Conditions: (i) Arylcarboxylic acid, PPA, 120−190 °C or ArCOCl, 1,4-dioxane, microwave, 210 °C (8−97%). (ii) R^1COCl, pyridine, room temperature or R^1COCl, Et_3N or $^iPr_2NEt$, DCM, room temperature (37−60%). (iii) Arylcarboxylic acid, PPA, 180 °C or RCOCl, 1,4-dioxane, microwave, 210 °C (49−73%). (iv) Pd/C, H_2, EtOAc, AcOH or $SnCl_2$, industrial methylated spirit (IMS) (or EtOH), 70 °C, room temperature or Fe, NH_4Cl, IMS (or tetrahydrofuran), H_2O, 70−80 °C (16−80%). (v) Acid chloride, pyridine, or $^iPr_2NEt/Et_3N$, CH_2Cl_2, (4-dimethylaminopyridine), room temperature or carboxylic acid, HATU, pyridine, *N*,*N*-dimethylformamide (DMF), room temperature (8−93%).

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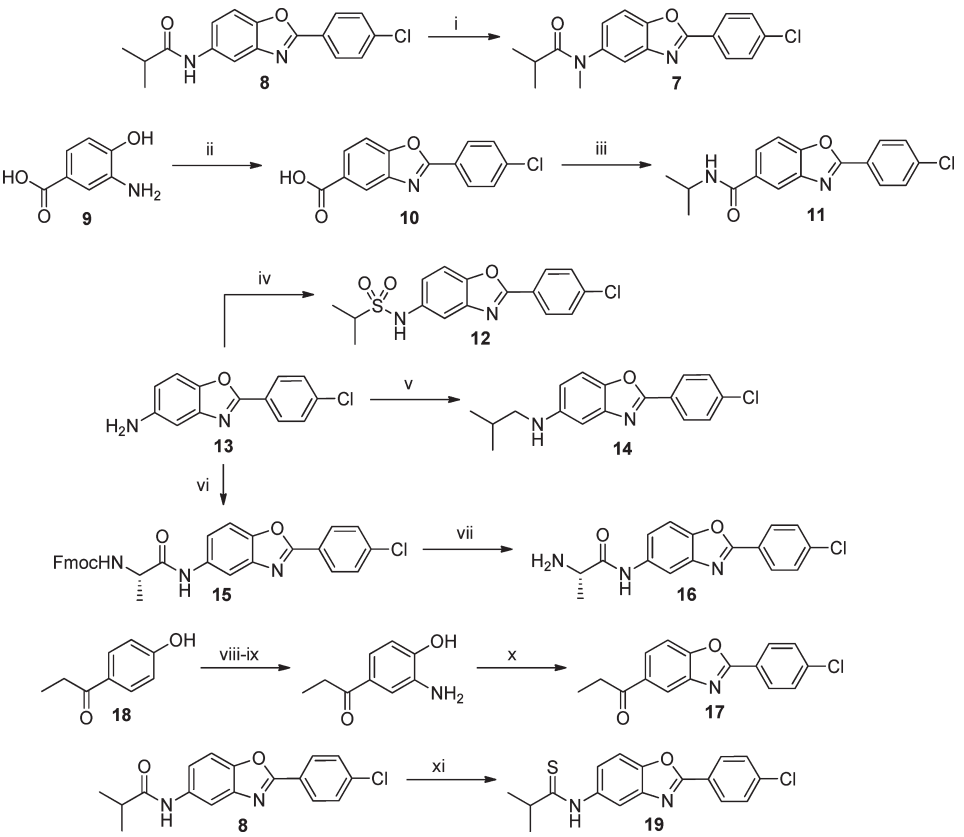
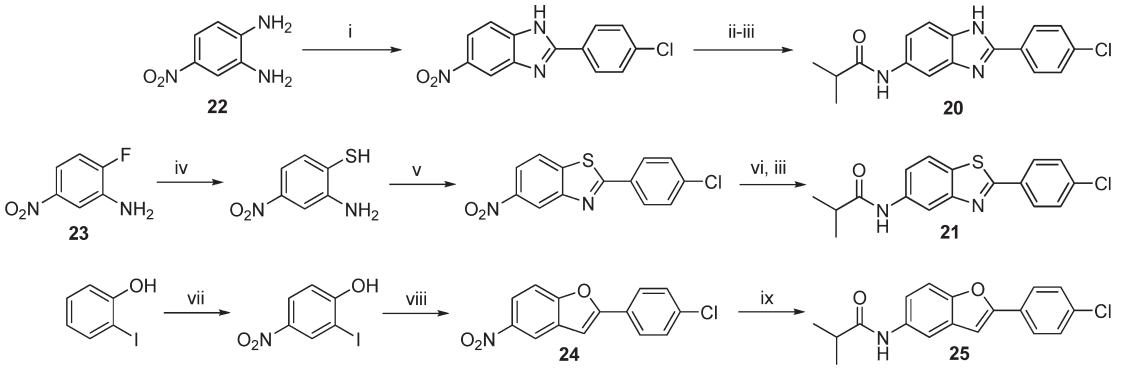
Scheme 2[a]



[a] Conditions: (i) NaH, MeI, DMF (32%). (ii) 4-Chlorobenzoyl chloride, 1,4-dioxane, microwave, 210 °C (79%). (iii) HATU, DIPEA, DMF, $iPrNH_2$, room temperature (18%). (iv) Isopropylsulfonyl chloride, pyridine, CH_2Cl_2 (11%). (v) Isobutyraldehyde, $NaBH(OAc)_3$, DCE, room temperature (48%). (vi) FmocAlaOH, HATU, pyridine, DMF (16%). (vii) Piperidine, DMF (34%). (viii) $Fe(NO_3)_3 \cdot 9H_2O$/IMS, 80 °C (25%). (ix) Fe, NH_4Cl, IMS, 80 °C (60%). (x) *p*-$ClPhCO_2H$, PPA, 180 °C (10%). (xi) Lawesson's reagent, PhMe, reflux (26%).

Scheme 3[a]



[a] Conditions: (i) 4-Chlorobenzoyl chloride, PPA, 150 °C (36%). (ii) $SnCl_2$, IMS, 70 °C (product used crude). (iii) Isobutyryl chloride, pyridine, room temperature (11% over 2 steps for **20**; 44% for **21**). (iv) $Na_2S \cdot 9H_2O$, aqueous sodium bicarbonate, MW (product used crude). (v) 4-Chlorobenzoyl chloride, Eaton's reagent, MW (product used crude). (vi) Fe, NH_4Cl, IMS, reflux (13% over 3 steps from **23**). (vii) HNO_3, CH_2Cl_2 (43%). (viii) 1-Chloro-4-ethynylbenzene, prolinol, Pd(C), CuI, PPh_3, H_2O (7%). (ix) Fe, NH_4Cl, IMS, H_2O, reflux; isobutyryl chloride, pyridine (42% overall).

derivatization and variation of the C2-substituent, thereby allowing for the preparation of a wider range of compounds. Alkyl and aryl amides with four different C2-aryl substituents were initially targeted (Table 1).

Importantly, the activity of 2-(3,4-dichlorophenyl)-substituted aniline **33** confirmed that the phenolic and aniline functions in region C were not essential. Furthermore, the size and nature of the amide substituent appeared to be critical. For analogues bearing a C2-phenyl residue, the smaller acetyl group (**35**) was moderately active, with the ethyl substituent (**36**) conferring increased activity and *n*-propyl derivative **38** exhibiting lower activity. Branching of the alkyl chain was tolerated, with the isopropyl unit (**34** and **39**)

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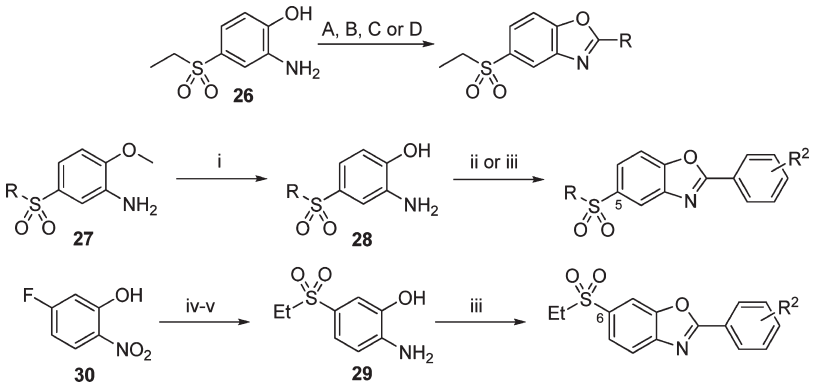
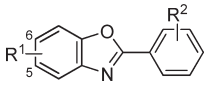
Scheme 4[a]



[a] Conditions: Method A: R-CO_2H, PPA, 125-190 °C (7−63%). Method B: RCOCl, 1,4-dioxane, microwave, 210 °C (7−85%). Method C: OHC-R, EtOH, Δ; $PhI(OAc)_2$, MeOH, room temperature (7%). Method D: $SOCl_2$, $CHCl_3$, reflux (55%) following method A. Other conditions: (i) BBr_3, CH_2Cl_2 or 1,2-DCE, reflux (products used crude). (ii) Aryl-CO_2H, PPA, 120−190 °C (5-76%). (iii) Aryl-COCl, 1,4-dioxane, microwave, 210 °C (36−41%). (iv) $EtSO_2Na$, DMSO, 100 °C. (v) H_2, Pd(C), IMS (46% over 2 steps from **30**).

Table 1. Initial Structure−Activity Relationships (Varying C5 and C2 Substituents)



compound	R^1	R^2	EC_{50} or % vs **3**[a](conc/μM)[b]
2	5,6-di-Me	2-OH, 4-NH_2	0.7
31	5-^{i}Pr	2-OH, 4-NH_2	1
32	5-NH_2	2-OH, 4-NH_2	3.5
33	5-NH_2	3,4-di-Cl	107% (3)
34	5-NHC(O)^{i}Pr	3,4-di-Cl	1.5
35	5-NHC(O)Me	H	3
36	5-NHC(O)Et	H	0.8
37	5-NHC(O)Et	Cl	85% (3)
38	5-NHC(O)Pr	H	3
39	5-NHC(O)^{i}Pr	H	0.8
40	5-NHC(O)Bu	H	69% (10)
41	5-NHC(O)Ph	H	>30
42	5-NHC(O)Pr	4-Cl	3.5
43	5-NHC(O)Ph	4-Cl	>30
44	5-NHC(O)CH_2Ph	4-Cl	5
45	5-NHC(O)$(CH_2)_2$Ph	4-Cl	>30

[a] Versus **3** (max value vs max value); no EC_{50} available. [b] Concentration at max value.

appearing to be particularly favorable. Use of the larger *n*-butyl substituent (**40**) or replacing the alkyl group with a carbocyclic aryl group (**41**) gave a dramatic decrease in activity.

Introduction of the bulkier 4-chlorophenyl group at the 2-position provided a propylamide (**42**) with comparable activity to the unsubstituted example **38**. In this 2-(4-chlorophenyl) series, ethyl amide **37** exhibited activity, and the trend of replacing the amide alkyl group with a phenyl residue leading to reduced activity was again observed (c.f. **42** and **43**). Alkaryl analogues did not offer any advantage, as exemplified by **44** and particularly **45**.

Having established some preliminary structure−activity relationships, a more detailed investigation of region C was undertaken (Figure 2). Screening data for a series of analogues bearing a 5-isopropylamido unit and substituents on the C2-phenyl ring designed to confer a range of electronic and steric properties are summarized

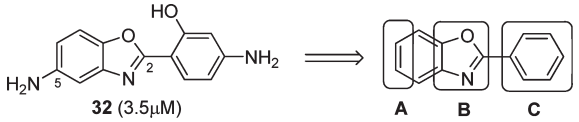
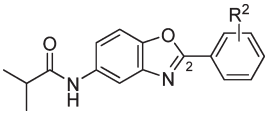


Figure 2. Compound **32** and structural regions for exploration.

Table 2. Structure−Activity Relationships for "C" Region



compound	R^2	EC_{50} or % vs **3**[a] (conc/μM)[b]
8	4-Cl	0.4
34	3,4-di-Cl	1.5
46	4-Me	95% (10)
47	4-OMe	132% (10)
48	4-NEt_2	12% (10)
49	4-CF_3	22% (10)
50	4-F	0.9
51	3-Cl	4.5
52	2-Cl	1.1
53	2,3-di-Cl	10
54	2,4-di-Cl	53% (10)
55	2,5-di-Cl	>30

[a] Versus **3** (max value vs max value); no EC_{50} available. [b] Concentration at max value.

in Table 2. Variation at the C2-position was found to have a significant effect on activity. For example, the para-substituted chloro (**8**), fluoro (**50**), methoxy (**47**), and methyl (**46**) derivatives all gave good levels of activity. Conversely, introduction of a basic diethylamino group (**48**) or a strongly electron-withdrawing trifluoromethyl group (**49**) at the 4-position was less favored. The position of the substituent was also important. *para*-Chloro substitution was

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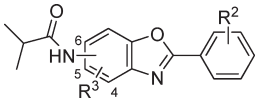
favored over *ortho* and *meta* substitution (compare results for **8**, **51**, and **52**). Disubstitution was also investigated on the C2-phenyl ring.

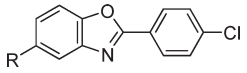
Table 3. Regiochemical and Other SAR



compound	substitution site (NHC(O)^{i}Pr)	R^2	R^3	EC_{50} or % vs **3**[a] (conc/μM)[b]
8	5	4-Cl	H	0.4
46	5	4-Me	H	95% (10)
56	4	4-Me	H	>30
57	6	4-Cl	H	3
58	6	4-Cl	5-Cl	>30
59	6	2,3-di-Cl	H	>30
60	6	3,4-di-Cl	H	4

[a] Versus **3** (max value vs max value); no EC_{50} available. [b] Concentration at max value.

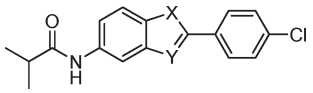
Table 4. Analogues of 8



compound	R	EC_{50} or % vs **3**[a] (conc/μM)[b]
7	NMeC(O)^{i}Pr	>30
8	NHC(O)^{i}Pr	0.4
11	C(O)NH^{i}Pr	>30
12	$NHSO_2{}^i$Pr	>30
14	$NHCH_2{}^i$Pr	>30
16	NHC(O)C^{c}H(Me)NH_2	35% (3)
17	C(O)Et	0.9
19	NHC(S)^{i}Pr	>30

[a] Versus **3** (max value vs max value); no EC_{50} available. [b] Concentration at max value. [c] (*S*)-stereochemistry.

Table 5. Core SAR



compound	X	Y	EC_{50} or % vs **3**[a] (conc/μM)[b]
8	O	N	0.4
20	NH	N	10
21	S	N	1.2
25	O	CH	>30

The 3,4-disubstitution pattern (**34**) proved to be the most favorable combination by comparison to the corresponding 2,3-dichloro, 2,4-dichloro, and 2,5-dichloro analogues (**53**−**55**).

Next, we investigated the position of the amide substitution on the core. Screening results (Table 3) indicated that transposition of the isopropyl amide of **8** to the 6-position resulted in a notable drop in potency (**57**) and the relative activities of **57**, **59**, and **60** indicated that addition of a second halogen atom to the C2-phenyl group was not advantageous. Interestingly, introduction of a second substituent on the core of the compound (**58**) also did not improve activity as compared to **8**. Substitution at the 4-position was also disfavored, as demonstrated by comparing **46** and **56**.

Further replacements to the 5-isopropylamido residue were also examined by screening analogues of **8** (Table 4). *N*-Methylation (**7**) and an isopropylsulfonamide (**12**) were not tolerated. Introduction of either a thioamido (**19**), isobutylamino (**14**) motif, or reversal of the amido function (**11**) similarly resulted in loss of activity. Isosteric alanine analogue **16** also exhibited reduced activity, whereas introduction of a 5-keto group (**17**) resulted in good activity.

In addition, benzimidazole, benzothiazole, and benzofuran ring systems were investigated as replacements[19] for the benzoxazole core (region B, Figure 2). The activity observed for benzothiazole **21** was lower as compared to **8** (Table 5), and the corresponding benzimidazole and benzofuran derivatives were considerably less active (**20** and **25**). Hence, focus was maintained on benzoxazoles.

To establish its in vitro metabolic profile, lead compound **8** was incubated with both mouse and human liver microsomes (MLM and HLM). As can be seen from these data (Table 6), while the compound appeared to be relatively stable in HLM, it exhibited only modest stability in MLM. Turbidometric aqueous solubility assessment of **8** was also undertaken (3 μM) to further profile this family of compounds. Pleasingly, intraperitoneal dosing of **8** in the mouse at 10 mg kg^{-1} indicated its observed plasma exposure was far superior as compared to the original hit compound **2** (Table 6), although still in need of further optimization. Accordingly, our chemistry program then centered on generating new leads with improved microsomal stability and solubility while retaining high activity. Gratifyingly, sulfone **63** (Table 7) showed improved aqueous solubility (turbidometric solubility, 100 μM) and encouraging activity as compared to its isopropylamide analogue, **39** (Table 2). These results prompted the preparation of the 4-chloro-analogue **61**, a compound exhibiting reasonable activity and microsomal stability (Tables 6 and 7). The improved pharmacokinetic characteristics of **61** relative to **8** (Table 6), following dosing at 10 mg kg^{-1} (i.p.) in the mouse, coupled with the lack of any significant in vivo efficacy after dosing **8**

Table 6. In Vitro and in Vivo Profiles of Lead Compounds

compound	MLM[a]	HLM[a]	i.p. dose (mg/kg)	T_{max} (min)	C_{max} (ng/mL)	$T_{1/2}$ (min)	AUC[c]	aqueous solubility (μM)[d]
1	81.5	105	50[b]	15	1486[b]	N/A[b]	420	3−20
2	22	NT	10[b]	5	10.9	40.9	0.6	1
8	19.3	286	10	15	2807	136	229	3
61	30.6	69	10	5	5650	131	579	10
62	101	105		NT	NT	NT	NT	3−10

[a] $T_{1/2}$ (min). [b] Study conducted by Cyprotex (address in Experimental Section). Time points (h): 0.08, 0.25, 0.5, 1, 2, 4, 8, and 24. Male CD1 mice dosed at 50 mg/kg using 95% PBS containing 0.1% Tween 20 and 5% DMSO as vehicle. C_{max} = 1.23−1.75 μg/mL (~4 μM), three animals; data are shown as a mean value in ng/mL. AUC = 7 μg h/mLe. Studies were conducted by Wickham laboratories (address in Experimental Section). Male CD1 mice dosed at 10 mg/kg using 95% PBS containing 0.1% Tween 20 and 5% DMSO as vehicle. Time points for compound **2** (min): 5, 15, 30, 60, 90, 120, 240, and 360; time points for compounds **8** and **61** (min): 5, 15, 30, 60, 90, 120, 240, and 480. The half-life was not calculated because the log concentration vs time plot did not show a linear region of at least three points. [c] μg.min/mL. [d] Turbidometric (final test compound concentration); NT, not tested. Further details can be found here: http://www.cyprotex.com/cloescreen/physicochemical-properties/turbidimetric-solubility/.

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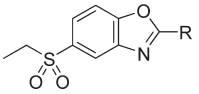
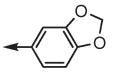
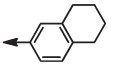
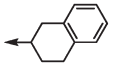
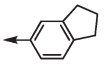
Table 7. Sulfone SAR



Compound	R	EC_{50} / μM or % vs **3**[a] (conc./μM)[b]	Synthetic method
1	Naphthalen-2-yl	0.91	B
61	4-Chlorophenyl	5	A
62	3,4-Dichlorophenyl	3	A
63	Phenyl	69% (10)	A
64	3-Chlorophenyl	>30	A
65	2-Chlorophenyl	62% (30)	A
66	2,3-Dichlorophenyl	40% (10)	A
67	5-Chloro-2-pyridinyl	>30	B
68	2-Fluoro, 3-chlorophenyl	>10	B
69	2-Furyl	>30	B
70	2-Thienyl	29% (30)	C
71		4.2	B
72	2-Quinolinyl	59% (3)	A
73	3-Quinolinyl	>30	A
74	3-Isoquinolinyl	63%(10)	A
75	6-Quinolinyl	1.9	A
76	6-Quinoxalinyl	>30	A
77	Naphthalen-1-yl	>30	B
78	5-Benzo[*b*]-thienyl	86% (10)	A
79		51% (10)	A
80		32% (10)	A
81		2.3	A
82	5-Benzofuryl	73% (10)	B
83	5-1*H*-indolyl	81% (3)	B
84	4-Biphenyl	>30	B
85	4-Phenoxyphenyl	>30	A
86	3-Phenoxyphenyl	>30	A
87	Naphthalen-2-ylmethyl	>30	A
88	Styryl	53% (10)	B
89	Propyl	14% (10)	N/A
90	Cyclohexyl	25% (10)	B

[a] Versus **3** (max value vs max value); no EC_{50} available. [b] Concentration at max value.

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at 10 mg kg^{-1} (i.p.) in the *mdx* mouse[8] provided additional drives to focus our investigations on the sulfone class.

Although amido and keto analogues **17** and **50**, respectively, also exhibited encouraging activity profiles (Tables 2 and 4, vide infra), their murine microsomal stability was significantly less as compared to **61** (MLM $T_{1/2}$ = 13 and 16.1 min, respectively); therefore, these compounds were not progressed any further.

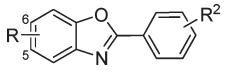
Lead optimization of **61** was achieved by systematic modification of the 2-aryl and sulfonyl substituents. First, an extensive range of C2-substituted analogues of **61** was screened (Table 7). Switching the *para*-chloro substituent to the *ortho* and *meta* positions was disfavored (**64** and **65**), in accordance with earlier observations on amide **8**. In addition, introduction of 2,3-dichlorophenyl, 2-fluoro-3-chlorophenyl, and other monocyclic groups at the 2-position (**66**−**70**) gave rise to low activity. Conversely, 3,4-disubstitution was favored (**62** and **71** to a lesser extent), as discussed previously in the context of amido derivatives. At this juncture, we extended this latter observation by testing compounds bearing 3,4-bicyclic units. However, analogues **72**−**74**, **76**, and **77** exhibited weak to moderate activity, whereas introduction of a 6-quinolinyl motif (**75**) gave improved activity. Benzothiophene **78** and partially saturated analogue **81** provided additional actives, compounds **82** and **83** were moderately active, whereas tetrahydronaphthalenes **79** and **80** were weakly active. A 2-naphthyl substituent (**1**) gave an additional increase in activity, and pleasingly, both compound **1** and **62** showed enhanced microsomal stability as compared to **8** (Table 6). Other results suggested that **78** and **81** (HLM $T_{1/2}$ = 59 and 20 min, respectively) would be less stable than **1** in vivo.

Other types of C2 substituent were also examined. The presence of a biphenyl unit (**84**), phenoxyphenyl groups (**85** and **86**), or a methylene spacer between the core and a naphthyl residue (**87**) gave poorly active compounds. Results in Table 7 also show that stilbene **88** was moderately active, whereas the absence of an aromatic C2 substituent appears to be detrimental (2-propyl derivative **89** and cyclohexane **90**).

Variation of the sulfonyl function present in **61** provided the screening data shown in Table 8. Switching the ethyl group of **61** for methyl, *n*-propyl, or isopropyl groups lead to reduced activity (**91**−**93**). Also, 5-isopropylsulfonyl derivative **94** displayed marginally weaker activity than its 5-ethylsulfonyl counterpart, **62**. Sulfonic acid **96** and sulfonamide analogues (**95** and **97**) exhibited lower activity as compared to **61**. Moving the ethylsulfonyl substituent from the 5-position to the 6-position of the core structure was also disfavored (cf. **61**−**62** and **98**−**99**). 5-Methylsulfone **100** provided another example of an active compound bearing a 2-naphthyl group (Table 8) but was not progressed further due to its lower murine microsomal stability (MLM $T_{1/2}$ = 33.4 min) as compared to **1**.

Auld et al. have published[20,21] examples of complications arising from luciferase-based assays due to stabilization and inhibition of luciferase. It was not believed that this was an issue for the 2-arylbenzoxazoles described herein because clear structure−activity relationships were observed, for example, in the 5-alkylamido series. In any event, we also evaluated selected compounds in a real-time quantitative polymerase chain reaction (RT-PCR) assay, to monitor utrophin expression, as an adjunct to the engineered H2K cell-based assay results. Active compounds were identified by significantly upregulated utrophin levels when compared to background [dimethylsulfoxide (DMSO) control] with **1** and **2** showing the best results. Amides **34** and **60** and sulfone **62** provided additional examples of active compounds in this assay (Table 9 and Figure 3). These observations provide support for the in vitro

Table 8. Variation of Sulfone



compound	R	R^2	EC_{50} or % vs **3**[a] (concn/μM)[b]
1	5-SO_2Et	3,4-benzo fused	0.91
61	5-SO_2Et	4-Cl	5
62	5-SO_2Et	3,4-di-Cl	3
91	5-SO_2Pr	4-Cl	>30
92	5-SO_2Me	4-Cl	53% (10)
93	5-$SO_2{}^i$Pr	4-Cl	10.2
94	5-$SO_2{}^i$Pr	3,4-di-Cl	45% (10)
95	5-SO_2NH_2	4-Cl	60% (30)
96	5-SO_3H	4-Cl	>30
97	5-SO_2NEt_2	4-Cl	28% (10)
98	6-SO_2Et	4-Cl	>30
99	6-SO_2Et	3,4-di-Cl	10.6
100	5-SO_2Me	3,4-benzo fused	95% (3)

[a] Versus **3** (max value vs max value); no EC_{50} available. [b] Concentration at max value.

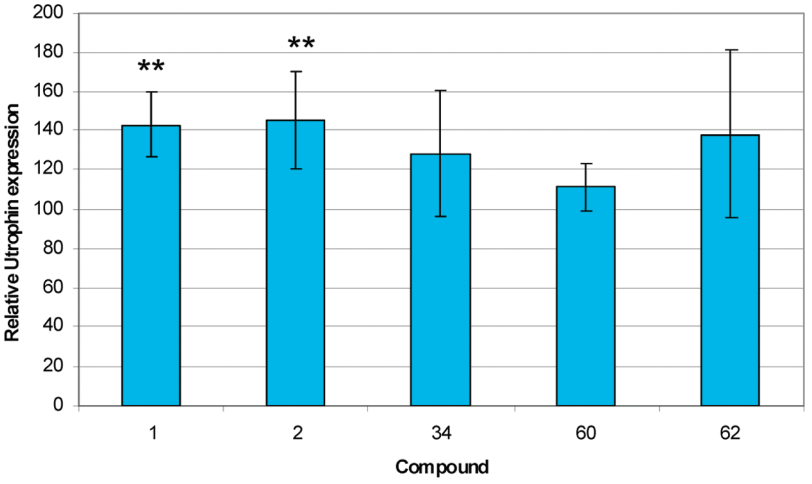


Figure 3. RT-PCR assay results. Compounds **1**, **2**, **60**, and **62** were tested at 10 μM; compound **34** was tested at 3 μM.

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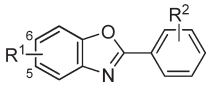
Table 9. RT-PCR Data



compound	R^1	R^2	EC_{50} or % vs 3[a](conc/μM)[b]	% UTRN[c]	*P* value
1	5-SO_2Et	3,4-benzofused	0.91	143.0	0.002
2	5,6-di-Me	2-OH, 4-NH_2	0.7	144.8	0.008
34	5-NHC(O)^{i}Pr	3,4-di-Cl	1.5	128.2	0.058
60	6-NHC(O) ^{i}Pr	3,4-di-Cl	4	111.5	0.077
62	5-SO_2Et	3,4-di-Cl	3	138.3	0.088

[a] Versus **3** (max value vs max value); no EC_{50} available. [b] Concentration at max value. [c] Background (DMSO control) = 100.

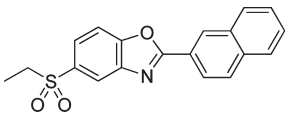


Figure 4. Structure of **1**.

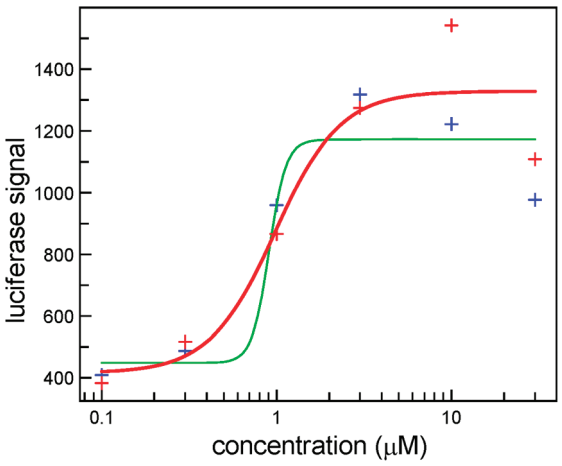


Figure 5. Typical dose−response curve. The green line represents the test compound; the red line represents **3**. Three replicates per data point.

activity of this family being associated with utrophin upregulation rather than any "off target" effects.

Compound **1** (Figure 4) was selected for further investigation based on its overall in vitro and in vivo[22] profile. Further exploration of the 2-arylbenzoxazole class will be reported on in due course.

SUMMARY

Following the appraisal of hits discovered from a focused screening approach, a series of novel 2-arylbenzoxazoles have been identified as potent upregulators of utrophin production as assessed using an H2K cell-based assay with a luciferase reporter readout. The most active examples have in vitro potency below 1 μM and in vitro and in vivo ADME profiles supportive of further investigation. Lead optimization studies have resulted in the identification of **1** as a molecule for further progression. The in vivo properties of compound **1** are discussed in more detailed elsewhere.[22]

EXPERIMENTAL SECTION

Assays. H2K cells[13,23] were plated into 96-well plates 24 h prior to dosing and then cultured with compound for a further 48 h before the luciferase signal was measured. Then, the level of luciferase was determined using the Steady-Glo Luciferase Assay System (Promega), and the plates were read using a FLOUStar plate reader and Stacker unit (BMG Labtech). The primary screen was performed at 10 μM in triplicate. A secondary screen was performed at six concentrations in triplicate to generate EC_{50} values (Figure 5).

The target sequence for the real-time quantitative PCR screen was an amplicon that spans the exon 3−4 boundary, and an amplicon in the β-actin RNA message was used as the endogenous control. Cells were seeded in 6-well plates at 25000 cells per well in 3 mL of appropriate media and incubated for 24 h prior to dosing. Compounds were dosed in a final concentration of 1% DMSO for 72 h. RNA extractions were performed using a QIAGEN RNeasy Plus kit (Qiagen 79654) and QiaShredder (Qiagen 74134), using the manufacturer's instructions. For reverse transcription, Applied Biosystems's (4368814) High-Capacity cDNA Reverse Transcriptase Kit from was used according to manufacturer's instructions. The quantitative PCR (qPCR) method used the ΔΔCT protocol described by Livaks and Schmittgen.[24] The 7300 Real-Time PCR System from Applied Biosystems was used for this assay along with 7300 System SDS software with the SDS Relative Quantification Study Plug in. 7300 System SDS software with the SDS Relative Quantification Study Plug was used for data analysis.

Other in Vitro and in Vivo Studies. Turbidometric aqueous solubility and microsomal stability assessments were conducted by Cyprotex Discovery Limited, 15 Beech Lane, Macclesfield, Cheshire, SK10 2DR, United Kingdom. In vivo pharmacokinetic studies were either performed at Cyprotex Discovery Limited (same address; compounds **2**, **8**, and **61**) or Wickham Laboratories Ltd., Winchester Road, Wickham, Fareham, Hampshire, PO17 5 EU, United Kingdom (compound **1**).

Chemistry/Compound Characterization. HPLC-UV-MS was performed on a Gilson 321 HPLC with detection performed by a Gilson 170 DAD and a Finnigan AQA mass spectrometer operating in electrospray ionization mode. The HPLC column used is a Phenomenex Gemini C18 150 mm × 4.6 mm. Preparative HPLC was performed on a Gilson 321 with detection performed by a Gilson 170 DAD. Fractions were collected using a Gilson 215 fraction collector. The preparative HPLC column used was a Phenomenex Gemini C18 150 mm × 10 mm, and the mobile phase was acetonitrile/water.

^{1}H NMR spectra were recorded on a Bruker instrument operating at 300 MHz. NMR spectra were obtained as $CDCl_3$ solutions (reported in ppm), using chloroform as the reference standard (7.25 ppm) or DMSO-d_6 (2.50 ppm). When peak multiplicities are reported, the

following abbreviations are used s (singlet), d (doublet), t (triplet), m (multiplet), br (broadened), dd (doublet of doublets), dt (doublet of triplets), and td (triplet of doublets). Coupling constants, when given, are reported in Hertz (Hz).

Compounds were prepared, as assessed using the aforementioned standard spectroscopic techniques, in ≥95% purity unless otherwise stated. It is also stated herein if LC data were not available. Starting materials were either procured from commercial sources or prepared as described herein.

Column chromatography was performed either by flash chromatography (40−65 μm silica gel) or using an automated purification system (SP1 Purification System from Biotage). Microwave reactions were performed in an Initiator 8 (Biotage) or an Explorer 48 system (CEM).

Method B. *5-(Ethylsulfonyl)-2-(naphthalen-2-yl)benzo[d]oxazole (**1**).* To 2-amino-4-(ethylsulfonyl)phenol (604 mg, 3.0 mmol) in dry 1, 4-dioxane (2.5 mL) was added 2-naphthoyl chloride (572 mg, 3.0 mmol) at room temperature. The reaction vessel was heated, under microwave activation, at 210 °C for 15 min. After it was cooled, the mixture was slowly poured into water, and the resulting precipitate was filtered and washed with aqueous sodium hydroxide (1 M NaOH) and then water to give the crude product. Purification by flash chromatography, eluting with 4:1 petrol ether:ethyl acetate (EtOAc) gave 750 mg (75%) of the title compound. LCMS RT = 6.94 min, MH^+ 338.1. ^{1}H NMR (DMSO-d_6): 8.90 (1H, br), 8.34 (1H, d, J = 1.4 Hz), 8.30 (1H, dd, J = 8.6 and 1.7 Hz), 8.24−8.05 (4H, m), 7.99 (1H, dd, J = 8.5 and 1.8 Hz), 7.73−7.64 (2H, m), 3.41 (2H, q, J = 7.3 Hz), 1.15 (3H, t, J = 7.3 Hz).

Method A. *2-Phenylbenzo[d]oxazol-5-amine (Intermediate for **35**).* To PPA at 110 °C were added simultaneously 2,4-diaminophenol dihydrochloride (7.88 g, 40.0 mmol) and benzoic acid (4.88 g, 40.0 mmol). The resulting mixture was then heated to 180 °C for 3−5 h. The solution was then poured into water. The resulting precipitate was collected by filtration and washed with saturated sodium bicarbonate solution. The crude product was recrystallized from ethanol/water to afford 8.15 g (97%) of the title compound. LCMS RT = 5.17 min, MH^+ 211.1. ^{1}H NMR (DMSO-d_6): 8.15−8.12 (2H, m), 7.60−7.56 (3H, m), 7.42 (1H, d, J = 8.7 Hz), 6.89 (1H, d, J = 2.1 Hz), 6.68 (1H, dd, J = 8.6 and 2.2 Hz), 5.12 (2H, s).

All compounds below were prepared following the same general method and purified either by trituration, recrystallization, or column chromatography. In some cases, the crude precipitate was basified with aqueous NaOH solution and then collected by filtration (prior to purification). Reaction temperatures ranged from 125 to 190 °C, and some reactions were left stirring overnight.

*2-(4-Chlorophenyl)benzo[d]oxazol-5-amine (**13**).* Yield, 95%. LCMS RT = 6.01 min, MH^+ 245.1 (93% purity). ^{1}H NMR ($CDCl_3$): 8.08 (2H, d, J = 9.0 Hz), 7.43 (2H, d, J = 9.0 Hz), 7.28 (1H, d, J = 9.0 Hz), 6.97 (1H, d, J = 2.2 Hz), 6.66 (1H, dd, J = 8.6 and 2.3 Hz), 3.67 (2H, br s). Compound **13** was used to prepare **8** (see Method K).

*2-(4-Chlorophenyl)-5-(ethylsulfonyl)benzo[d]oxazole (**61**).* Yield, 42%. LCMS RT = 6.63 min, MH^+ 322.1. ^{1}H NMR ($CDCl_3$): 8.39 (1H, d, J = 1.7 Hz), 8.27 (2H, d, J = 8.7 Hz), 8.00 (1H, dd, J = 8.5 and 1.8 Hz), 7.80 (1H, d, J = 8.5 Hz), 7.60 (2H, d, J = 8.6 Hz), 3.23 (2H, q, J = 7.6 Hz), 1.36 (3H, t, J = 7.4 Hz).

*2-(3,4-Dichlorophenyl)-5-(ethylsulfonyl)benzo[d]oxazole (**62**).* Yield, 32%. LCMS RT = 7.25 min. ^{1}H NMR (DMSO-d_6): 8.39 (1H, d, J = 2.0 Hz), 8.35 (1H, d, J = 1.4 Hz), 8.19 (1H, dd, J = 8.4 and 2.0 Hz), 8.10 (1H, d, J = 8.6 Hz), 7.99 (1H, dd, J = 8.6 and 1.8 Hz), 7.94 (1H, d, J = 8.4 Hz), 3.41 (2H, q, J = 7.3 Hz), 1.13 (3H, t, J = 7.3 Hz).

Method J. *N-(2-Phenylbenzo[d]oxazol-5-yl)acetamide (**35**).* To a solution of 2-phenylbenzo[d]oxazol-5-amine (50 mg, 0.24 mmol) in dichloromethane (2 mL) at room temperature was added acetyl chloride (20.5 mg, 0.26 mmol) followed immediately by diisopropylethylamine (82.1 mg, 0.64 mmol). The resulting mixture was stirred at room temperature for 18 h. Dichloromethane was added, and the organic layer was washed with saturated aqueous sodium carbonate (Na_2CO_3). The combined organic layers were dried over anhydrous $MgSO_4$ and evaporated. The resulting solid was dissolved in methanol, passed through an acidic scavenger column (silica-based quaternary amine SPE-AX, supplied by Biotage), and then evaporated to afford 25 mg (41%) of the title compound. LCMS RT = 5.16 min, MH^+ 253.1. ^{1}H NMR (DMSO-d_6): 10.14 (1H, s), 8.21−8.14 (3H, m), 7.71 (1H, d, J = 8.8 Hz), 7.65−7.60 (3H, m), 7.51 (1H, dd, J = 9.0 and 2.1 Hz), 2.09 (3H, s)

Method K. Method K was the same as Method J, except triethylamine was used as a base instead of diisopropylamine.

*N-(2-(4-Chlorophenyl)benzo[d]oxazol-5-yl)isobutyramide (**8**).* Yield, 46%. LCMS RT = 7.04 min, MH^+ 315.1. ^{1}H NMR (DMSO-d_6): 10.03 (1H, s), 8.22−8.18 (3H, m), 7.74−7.67 (3H, m), 7.56 (1H, dd, J = 8.9 and 2.1 Hz), 2.67−2.59 (1H, m), 1.14 (6H, d, J = 6.8 Hz).

■ ASSOCIATED CONTENT

Supporting Information. Synthesis details and analytical data for all other methods and compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

DAPC, dystrophin-associated protein complex; DMD, Duchenne muscular dystrophy; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; EtOAc, ethyl acetate; HATU, *O*-(7-azabenzotriazol-1yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HLM, human liver microsomes; IMS, industrial methylated spirit; MLM, mouse liver microsomes; NaOH, sodium hydroxide; Na_2CO_3, sodium carbonate; PPA, polyphosphoric acid

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