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Long-chain formoterol analogues: an investigation into the effect of increasing amino-substituent chain length on the β_2-adrenoceptor activity

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Abstract—The synthesis of a series of long-chain formoterol analogues in which the terminal ether residue of the β-phenethylamino-substituent has been extended beyond the methyl ether residue present in the parent compound are described. Evaluation of these analogues as β_2-adrenoceptor agonists was used to provide an insight into the factors controlling the magnitude and duration of receptor activation.

The two currently prescribed inhaled long-acting β_2-adrenoceptor agonists formoterol **1** and salmeterol **2** play a central role as bronchodilators in the treatment of the respiratory diseases asthma,[1] and COPD.[2] Formoterol is characterised as a high-efficacy agonist with a rapid onset of action.[3] In contrast, salmeterol is a low-efficacy agonist with a relatively slow onset of action.[4] Despite these differences both compounds provide sustained relief of bronchoconstriction following a twice-daily dosing regimen. In common with others, we are interested in identifying inhaled β_2-agonists with extended durations of action, which are suitable for once-daily dosing as the next generation of compounds from this therapeutic class.[5]

To rationalise the duration of action of formoterol and salmeterol two hypothese have been put forward involving a key interaction with either the receptor, termed the exosite theory,[6] or the surrounding membrane, termed the diffusion microkinetic theory.[7] In the former, a secondary interaction distant from the primary agonist binding region is implicated in which the long-chain of salmeterol anchors to an additional portion of the β_2-adrenoceptor, termed the exosite. This interaction is believed to enable repeated stimulation to occur without complete dissociation of the ligand, leading to the sustained effect. Key to this interaction is the location of the ether residue within the 6-(4-phenylbutoxy)hexyl amino chain of salmeterol, which is thought to provide the conformational freedom for the dual binding modes to occur.[4,8] In the diffusion microkinetic theory, the basic centre and lipophilic nature of both formoterol and salmeterol allows them to partition effectively into the lipid bilayers of smooth muscle following local delivery to the lung. This accumulation of material within the lipid bilayer functions as a depot to maintain an effective concentration of agonist over time, resulting in the observed duration of effect. Additionally for this theory, the greater lipophilicity and nature of the membrane interaction for salmeterol have been proposed to account for the slow onset of action due to entry into the β_2-adrenoceptor directly from the lipid bilayer as the primary route.[9] Some controversy surrounds these two hypothese centering around the role and extent to which the exosite interaction with salmeterol defines the observed pharmacology.[10,11] However, an interaction with this region of the receptor for salmeterol appears to be well established, through natural,[12] and engineered mutations,[13] even if the nature and degree to which the exosite interaction contributes to the molecules biological activity remains to be fully defined.

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Although the six-atom nitrogen-to-oxygen separation is believed to be an essential structural element for the activity observed with salmeterol,[4] the hexyl spacer does allow for significant conformational freedom. A consequence of this is to make any accurate prediction of the active spatial orientation in this region of the molecule difficult. Our interest centred on the observation that this proposed key six-atom separation between the basic nitrogen and ether oxygen is also present in a number of other well described β_2-adrenoceptor agonists, which include picumeterol **3**, sibenadet **4**, formoterol **1**, fenoterol **5** and TA-2005 **6**.[11,14] The last three of which all incorporate a *para*-oxygen substituted β-phenethyl-amino-substituent as the common six-atom spacer. Therefore, potentially enabling an equivalent interaction for the common oxygen atom of the ether side chains, as highlighted in Figure 1. The reduced conformational mobility of the β-phenethyl moiety possibility offering the opportunity of a more energetically favourable interaction.

Based upon the above correlation, we hypothesised that the potential exists with formoterol analogues for enabling the same secondary receptor binding, put forward for salmeterol. This we anticipated could be achieved by extension of the *para*-β-phenethyl terminal ether residue, to facilitate an interaction with the exosite region of the β_2-adrenoceptor. Further support for such a commonality of binding modes with the above catecholamine-based agonists can be derived from receptor mutagenesis in combination with molecular modelling studies. The key interactions are highlighted for salmeterol and formoterol in Figure 2. In summary, the important interactions between agonists and the β_2-adrenoceptor have been established as involving; the catechol mimic and serine residues 204 and 207 on the fifth transmembrane spanning domain (TM5),[15] the protonated amine and Asp-113 on TM3,[16] and the benzylic alcohol with the chirally discriminating Asn-293 on TM6.[17] In addition to these primary interactions, Tyr-308 on TM7 has been identified from molecular modelling studies as defining a limiting-surface of the binding pocket for the amino-substituents of formoterol, salmeterol and TA-2005.[18] This interaction in the case of salmeterol enables the much larger 4-phenylbutyl ether substituent to orientate itself, facilitated by the presence of the ether linkage, back into the β_2-adrenoceptor.[8] A consequence of this alignment is to bring the 4-phenylbutyl ether moiety into an interaction with the amino acids localised around positions 149–158 on TM4, which define the key residues of the proposed exosite.[13]

In this letter we describe our initial results from sequentially increasing the size of the β-phenethyl oxygen substituent for a series of formoterol analogues to match the equivalent terminal ether residue of salmeterol. We anticipated that determination of the β_2-adrenoceptor activity for this series of homologues would provide an insight into the possibility of an analogous interaction to that proposed for salmeterol with the exosite region.

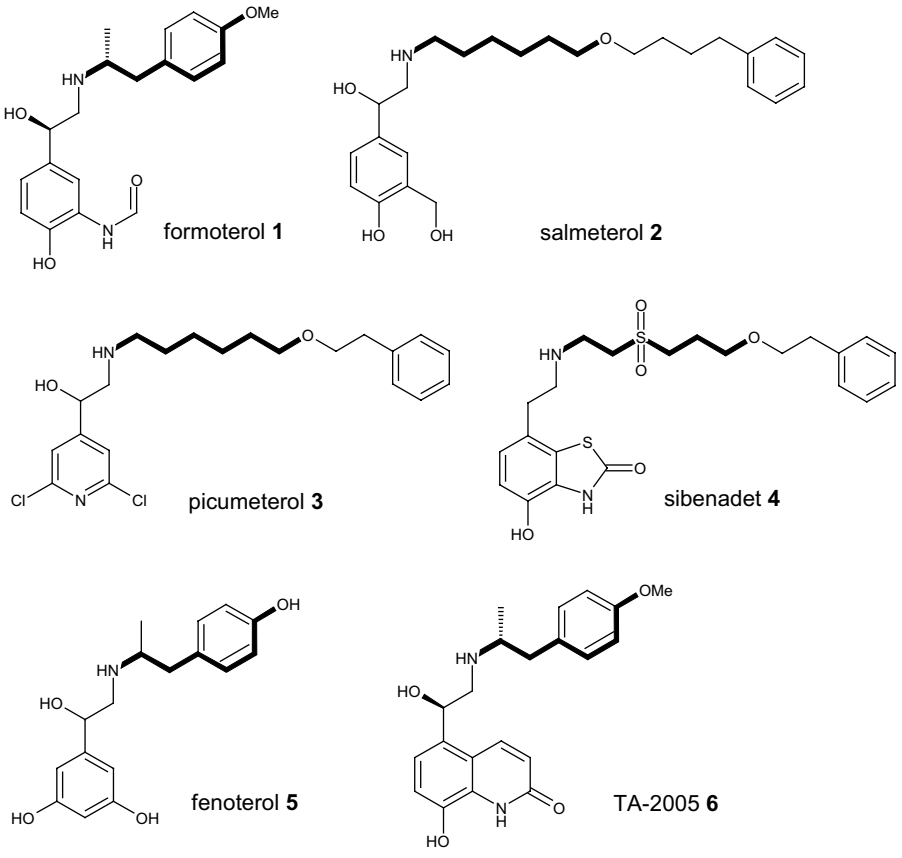


Figure 1. Comparison of β_2-adrenoceptor agonist structures of formoterol, salmeterol, picumeterol, sibenadet, fenoterol and TA-2005.

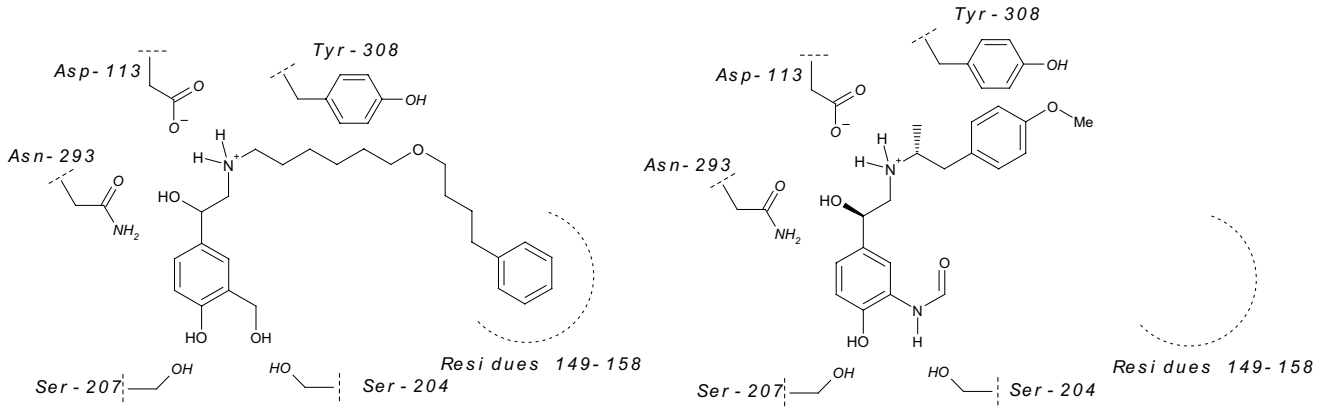
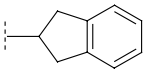


Figure 2. Key β_2-adrenoceptor/agonist interactions for salmeterol and formoterol.

Table 1. Structures and position of the β-phenethyl ether substituents R

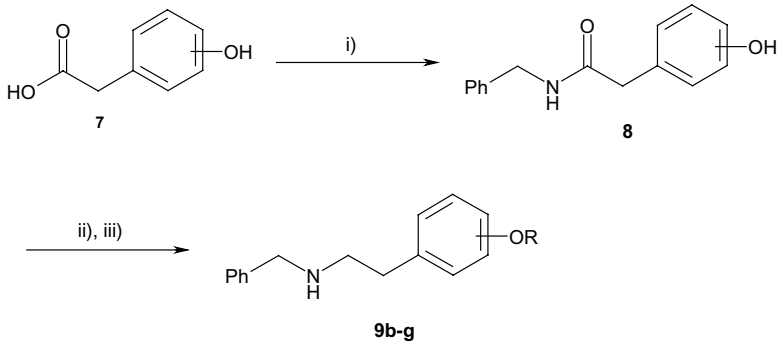
Compound	Substituent: R	Regiochemistry
a	–Ph	*para*
b	$-CH_2CH_2Ph$	*para*
c	$-CH_2CH_2CH_2Ph$	*para*
d	$-CH_2CH_2CH_2CH_2Ph$	*para*
e		*para*
f	$-CH_2CH_2CH_2CH_2Ph$	*meta*
g	$-CH_2CH_2CH_2OPh$	*meta*

In addition a selection of closely related derivatives were prepared and studied to further investigate the factors controlling the magnitude and duration of the effect at the β_2-adrenoceptor.

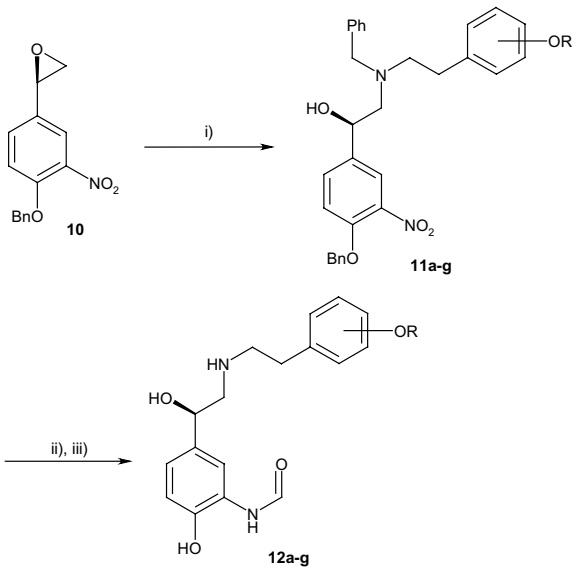
To explore the above possibility a series of *N*-benzylated β-phenylethylamines bearing *para*-substituted ether residues of increasing chain length and terminated with a phenyl moiety were prepared as key intermediates, entries **9a**–**d** in Table 1. Additionally, as a way to further explore the structure–activity relationship, related examples were prepared by varying the nature and position of the ether residue, entries **9e**–**g** in Table 1. The synthetic sequence used to prepare the intermediate amines **9b**–**g** is shown in Scheme 1. Starting from the appropriately substituted hydroxyphenylacetic acids **7**, carbodiimide couplings yielded the corresponding *N*-benzylated carboxamides **8**. The phenolic residues were then alkylated with in situ generated alkyl iodides or, in the case of the 2-indanyl example, via a Mitsunobu reaction. Subsequent reduction of the amide functionality, with either diborane or lithium aluminium hydride, gave the desired secondary amines **9b**–**g**. To prepare the bis-arylether intermediate **9a** an alternative sequence was employed. Starting from commercial 4-phenoxy-β-phenylethylamine, *N*-benzoylation followed by amide reduction with lithium aluminium hydride produced the desired benzylated amine **9a** in 48% overall yield.

The above secondary benzylated amines **9a**–**g** were then reacted under conditions of either conventional or microwave heating to open the chiral epoxide **10**. Reaction occurring selectively at the least hindered position, yielding the ethanolamine derivatives **11a**–**g**.[19] Subsequent functional group manipulation, via hydrogenation of the nitro residues over platinum oxide, followed by formylation of the liberated aniline functionality gave the corresponding formamides. Finally, hydrogenolysis of the benzyl protecting groups yielded the target molecules **12a**–**g**, as single enantiomers of the more active (*R*)-configuration (Scheme 2).[20]

Compounds **12a**–**g** were screened in a filtration binding assay to determine their affinities for the human



Scheme 1. Synthesis of the amines **9b**–**g**: (i) $BnNH_2$, EDCI, HOAt, CH_2Cl_2, rt (88–93%); (ii) R–Cl, $CsCO_3$, KI, CH_3CN, 90 °C (86–90%); or, R–OH, PPh_3, DEAD, THF, rt (26%); (iii) B_2H_6, THF, reflux, then MeOH, reflux (44–95%); or, $LiAlH_4$, THF, reflux (29–33%).



Scheme 2. Synthesis of the β_2-adrenoceptor agonists **12a**–**g**: (i) **9a**–**g** toluene, reflux, 18 h (53–82%); or, neat, Prolabo Synthewave™ 402 microwave, 25 min at 110 °C (63–92%); (ii) 1 atm H_2, PtO_2, 1:1 toluene: THF, rt (88–98%); (iii) aged mixture HCO_2H/Ac_2O, 1:1 toluene–THF, rt (62–86%); or, HCO_2H, oxalyl chloride, imidazole, Et_3N, CH_2Cl_2, rt (25%); (iv) 1 atm H_2, Pd/C, MeOH, rt (58–89%).

β_2-adrenoceptor.[21] Additionally, IC_{50} values for the compounds ability to inhibit the electrically induced contraction of superfused guinea-pig trachea were determined as a measure of the functional response at the β_2-adrenoceptor.[22] Biological data and calculated log $D_{7.4}$ values,[23] as an assessment of the membrane partitioning capability, are shown in Table 2. Membrane partitioning being a key determinant of the compounds duration of action potential based upon the diffusion microkinetic theory.[7]

Comparison of the affinities of the compounds **12a**–**g** for the β_2-adrenoceptor showed little variability in the K_i values with the exception of the biphenyl ether **12a**, which was slightly reduced. Interestingly, all of the compounds as the more active (*R*)-enantiomer exhibited decreased affinities for the β_2-adrenoceptor relative to racemic formoterol. A major proportion of this small drop in affinity could potentially be accounted for by the absence of the (*R*)-α-methyl substituent of the β-phenethyl moiety in compounds **12a**–**g**, when compared with formoterol.[20] These binding data indicate that no marked change in affinity for the β_2-adrenoceptor is apparent for **12d** where, based upon the proposed hypothesis the terminal ether residue is in an equivalent position to that present in salmeterol.

Table 2. Biological data and calculated log $D_{7.4}$ values for formoterol, salmeterol and compounds **12a**–**g**

Compound	β_2-Adrenoceptor K_i (nM)	clog $D_{7.4}$	Guinea-pig trachea IC_{50} (nM)[a]
Formoterol **1**	2.6	0.06	1.4
Salmeterol **2**	1.0	1.24	15
12a	35.4	1.95	120
12b	7.8	1.67	1.1
12c	5.9	2.35	38
12d	9.2	2.83	71
12e	11.6	2.95	251
12f	8.5	2.83	114
12g	3.3	2.00	164

[a] Determined from maximal response after compound present in superfusate for initial 30 min ($n \geqslant 3$).

The functional consequences of the above interaction with the β_2-adrenoceptor were measured in a guinea-pig tracheal strip assay.[22] We noted a greater variability in the ability of the compounds **12a**–**g** to agonise the receptor, measured by inhibition of contraction, when compared with their binding affinities. IC_{50} values ranged from being comparable with formoterol, to greater than 200-fold less potent. In all instances greater than an 80% inhibition of the electrically-stimulated contraction was achieved at the higher concentrations investigated in the dose response studies (1 μM). Thus, indicating that all the compounds retain a significant degree of the high-efficacy agonist properties associated with formoterol. Comparing the *para*-substituted isomers: the biphenyl ether **12a** in accord with the above binding data resulted in a marked 86-fold fall in functional potency relative to formoterol. Increasing the separation between the phenyl residues by incorporation of an alkyl chain resulted in an improvement in functional activity to the level of formoterol for the *para*-substituted 2-phenethyl ether derivative **12b**, with the functional potency decreasing upon increasing alkyl chain length in going from two to four methylene groups, entries **12b**–**d**. However, a satisfactory level was still retained in the *para*-4-phenylbutyl ether derivative **12d**, with the terminal ether residue equivalent to salmeterol. This decrease in potency of up to 51-fold relative to formoterol may provide a better opportunity for formulation as an inhalation product with activity in this range.[24] The 2-indanyl derivative **12e**, a cyclic analogue of the potent **12b**, resulted in a marked 228-fold fall in functional activity although this was again not apparent from the binding data. Therefore, this result potentially indicates that steric bulk and conformational restriction about the aryl ether linkage are relatively poorly tolerated for receptor activation in this series of compounds, a rationale, which is further supported by the reduced activity seen for the biphenyl ether **12a**. Switching the point of attachment of the ether linkage in the β-phenethyl moiety to a *meta*-relationship and hence reducing the six-atom separation between basic nitrogen and ether oxygen by one atom resulted in essentially no change in functional activity, entry **12f**, compared with the *para*-isomer **12d**. Thus, these data suggest that the role of the side-chain ether oxygen in this β-phenethyl series is less critical for receptor activation compared with salmeterol. Incorporation of a second ether residue into the four-atom *meta*-linker, entry **12g**, was tolerated with little change in activity compared to the carbon analogue **12f**.

Comparison of the above functional data with the binding affinities highlights the need for a functional assessment to understand the impact of the above structural changes on receptor activation. The nature and position of the ether residue in the series of compounds **12** having

been shown to have a significant effect on functional β_2-adrenoceptor activation. Analysis of the above structure–activity relationship does not highlight a trend for an optimal interaction to indicate that the most favourable profile is attained with **12d**, which based upon the hypothesised structural equivalence is proposed as the closest analogue in this series to salmeterol. However, this approach has enabled functionally-active and high-efficacy β_2-adrenoceptor agonists to be identified of increased lipophilicity of up to greater than 2.8 calculated $\log D_{7.4}$ units higher than formoterol. Based upon the diffusion microkinetic theory, these compounds have the potential to produce the desired increase in duration of action. Representative duration of action data from a superfused guinea-pig tracheal strip assay, in which a drug-free washout phase of up to 9.5 h was included, for formoterol, **12d**, and **12g**, are shown in Figure 3.[22] The concentrations shown for each compound are selected to most closely approximate to an IC_{80} at the end of the 30 min compound administration phase. Durations of action were taken as the time to return to 50% of the maximal effect for each compound from the end of the 30 min compound administration phase and are summarised in Table 3 with measured $\log D_{7.4}$,[25] and K_{IAM} values,[26] as an assessment of the membrane partitioning capability.[27]

The measured data to assess the membrane partitioning capabilities for formoterol, **12d**, and **12g** were found to correlate with the calculated $\log D_{7.4}$ values presented in Table 2. Taking the differences in the K_{IAM} values for the long-chain analogues **12d** and **12g**: a greater than 107-fold and 30-fold increased preference for a phospholipids, over an aqueous phase, respectively, was determined when compared with formoterol. This increased capacity for retention in lipid bilayers was reflected in an increase in the duration of the relaxant effect over formoterol of, 3.4-fold and 2.8-fold, respectively, for **12d** and **12g** when compared at equi-effective concentrations. More than a doubling of the duration of effect over formoterol being obtained not only for **12d** where the hypothesised key six-atom nitrogen-to-oxygen separation is present, but also **12g** where it has been reduced by one atom. A one-atom shift in the position of the ether oxygen having previously been shown to have a marked impact on the duration of action for a series of salmeterol analogues.[4] Thus, when we assessed the differences seen between **12d** and **12g** the extent of the increase in lipophilicity appeared to best explain the degree of enhancement in duration of action observed over formoterol rather than as a consequence of a specific interaction with the exosite region of the β_2-adrenoceptor. Additionally, from the data shown in Figure 3, the maximal effect for **12d**, like formoterol, is achieved within 5 min of the end of the initial 30 min drug administration phase, which is consistent with the potential for a rapid onset of action to be conserved.[22]

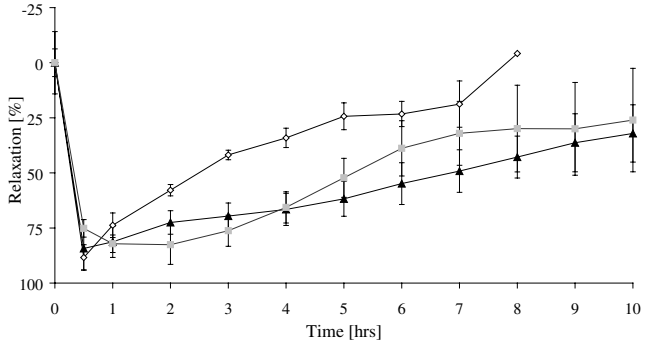


Figure 3. Electrically-stimulated superfused guinea-pig tracheal strip assay (Electrical stimulation was delivered every 2 min, data shown are for each hour following starting the superfusion of the test compound, and at the end of the 30 min drug administration phase. Data are expressed as mean ± standard error of the mean) data for formoterol, **12d** and **12g** ◇ formoterol 30 nM ($n=3$), ▲ **12d** 100 nM ($n=5$), ■ **12g** 1000 nM ($n=4$).

Table 3. Durations of action and measured $\log D_{7.4}$ values for formoterol, **12d** and **12g**

Compound	Duration-of-action (h)	$\log D_{7.4}$	K_{IAM}
Formoterol **1**	1.92	0.79	0.70
12d	6.62	2.76	>2.73[a]
12g	5.43	2.49	2.18

[a] $\log K_{IAM}$ value above upper limit of measurable range for assay system.

In summary, a series of long-chain formoterol analogues in which the ether residue of the β-phenethyl-amino-substituent was varied have been evaluated as β_2-adrenoceptor agonists to explore if an analogous exosite interaction to that proposed for salmeterol was evident. In vitro studies for this series of compounds highlighted the need for a functional assessment to fully understand the structure–activity relationship for receptor activation. Although evaluation of the data does not rule out an interaction with the exosite region of the receptor for the compounds **12**, no variation in the degree and duration of agonist activity to support a dual binding mode was apparent. However, this approach has enabled high-efficacy β_2-adrenoceptor agonists of increased lipophilicity to be identified, which from the diffusion microkinetic theory would be anticipated to have the potential for an extended duration of action as a result of greater membrane partitioning. This has been demonstrated for the compounds **12d** and **12g**, which produced a greater than 2.5-fold longer duration of action compared to the currently prescribed twice-daily β_2-adrenoceptor agonist formoterol. Additionally in the case of **12d**, this increased duration of action was also associated with the desired rapid onset of action profile. Further studies to explore the potential of the long-chain analogue **12d** (NVP-QAC455) as a once-daily long-acting β_2-adrenoceptor agonist will be described in due course.

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