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Elaboration of a Fragment Library Hit Produces Potent and Selective Aspartate semialdehyde Dehydrogenase Inhibitors

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Abstract

Aspartate- β -semialdehyde dehydrogenase (ASADH) lies at the first branch point in the aspartate metabolic pathway which leads to the biosynthesis of several essential amino acids and some important metabolites. This pathway is crucial for many metabolic processes in plants and microbes like bacteria and fungi, but is absent in mammals. Therefore, the key microbial enzymes involved in this pathway are attractive potential targets for development of new antibiotics with novel modes of action. The ASADH enzyme family shares the same substrate binding and active site catalytic groups; however, the enzymes from representative bacterial and fungal species show different inhibition patterns when previously screened against low molecular weight inhibitors identified from fragment library screening. In the present study several approaches, including fragment based drug discovery (FBDD), inhibitor docking, kinetic, and structure activity relationship (SAR) studies have been used to guide ASADH inhibitor development. Elaboration of a core structure identified by FBDD has led to the synthesis of low micromolar inhibitors of the target enzyme, with high selectivity introduced between the Gram-negative and Gram-positive orthologs of ASADH. This new set of structures open a novel direction for the development of inhibitors against this validated drug-target enzyme.

Graphical Abstract

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Keywords

Aspartate semialdehyde dehydrogenase; enzyme inhibitors; fragment based drug discovery; structure-activity relationships

1. Introduction

The growing threat of microbial infections caused by increased microbial resistance to existing antibiotics has led to a renewed and urgent need for new antibiotics.¹ The expanding population of microbes that have developed resistance to many of the available front-line antibiotics require new drugs which can act through novel mechanisms to thwart this increasing resistance.

The aspartate pathway² in microorganisms and plants controls one-quarter of *de novo* amino acid biosynthesis.³ ASADH, coded by the *asd* gene, catalyzes the production of aspartate semialdehyde (ASA) that is located at a critical junction in this pathway. Numerous studies have showed that the deletion of this gene is fatal to microbes, with genetically-modified bacterial strains lacking the *asd* gene no longer viable.^{4,5} In addition to the synthesis of these essential amino acids, a variety of important metabolites that are required for microbial growth and survival are also produced by the aspartate pathway. Methylation reactions which are crucial for cell growth and viability are mediated by S-adenosyl methionine (AdoMet),^{6,7} one of the key end products of this pathway. Additionally, 4,5-dihydroxy-2,3-pentanedione and acyl homoserine lactones produced from this pathway are two classes of signaling molecules used in bacterial quorum sensing.⁸ These quorum sensing molecules control the expression of a large number of bacterial genes, including those that produce virulence factors such as secreted toxins, proteases and hemolysins that cause disease pathology.⁹ Furthermore, this pathway furnishes components required for the assembly of the polysaccharide matrix of biofilms that protect microbes against phagocytes and antibiotics. Because of these many important microbial events that are controlled by the aspartate pathway, it is clear why blockage of this pathway would be fatal to microorganisms. The identification of effective inhibitors against this target enzyme (ASADH) will provide lead compounds for the development of new biocides with unique mechanisms of action. In addition, achieving selective inhibition of ASADHs from different microorganisms can lead to species-specific biocides that should further delay the development of drug resistance.

Previous work against this target enzyme has utilized an extensive background of structural information^{10–14} to employ various approaches, including structure-guided design,¹⁵ library screening, and fragment based drug discovery (FBDD)^{16,17} to identify and develop lead compounds. Several substrate analog inhibitors have been found to exhibit good selectivity between the Gram-negative and Gram-positive bacterial orthologs and the fungal forms of ASADH.¹⁸ In addition, phthalate and benzene tricarboxylate derivatives identified from fragment library screening were shown to occupy unique binding orientations at the active site of ASADH.¹⁹ However, extension of the phthalate core structure into a fortuitous acetate binding site did not yield the enhanced affinity that was expected to be achieved

through the combined affinities at these multiple binding sites.²⁰ All of the inhibitors that have been developed so far show only modest affinity against these target enzymes.

In the present work, we have reexamined the structure-activity properties of these earlier inhibitors, and have now applied a systematic approach to elaborate this phthalate core structure. This approach has resulted in the synthesis of a series of enzyme inhibitors, with the most effective compounds inhibiting the ASADH from *S. pneumoniae* with significantly enhanced selectivity and with greater affinity than previously observed.

2. Results

2.1. General chemistry

Based on the numerous productive interactions that have been observed with the phthalate derivatives bound at the active site of ASADH,¹⁹ analogs were designed in which a heteroatom was introduced into the side chain of this core structure to allow additional structural elaborations. To produce the parent compounds the carboxylates of commercially available 4-methylphthalic acid (**1**) were protected by esterification (**1Me**), followed by free radical bromination (**2Me**). Coupling to either 2-aminoacetate methyl ester or 3-aminopropionate methyl ester by nucleophilic displacement of the introduced bromine, followed by base-catalyzed hydrolysis yielded the corresponding amine-containing 4-(carboxymethylaminomethyl) and 4-(2-carboxyethylaminomethyl)phthalate analogs **3** and **4**, respectively (Scheme 1). Coupling to alanine methyl ester or serine methyl ester followed by hydrolysis produced analogs **5** and **6**, respectively (Scheme 1). The affinities of these parent compounds for representative Gram-negative (*Vibrio cholerae*) and Gram-positive (*Streptococcus pneumoniae*) forms of ASADH were examined, and then systematically improved through the production of an extensive series of derivatives at the introduced secondary amine.

2.2. Preliminary evaluation of enzymatic activity

Unfortunately compound **4**, with the longer *N*-carboxyethyl side chain, did not show appreciable inhibition of *vc*ASADH, but does inhibit *sp*ASADH with a relatively weak K_i of 2.4 mM. These values are comparable to those that were observed for the inhibition by 3-(3-carboxypropyl)phthalate,¹⁹ despite this longer side chain, the introduction of a nitrogen heteroatom in the side chain, and the shift of the side chain from the 3- to the 4-position. However, the one-carbon shorter homolog (**3**) shows some improvement, functioning as a 0.5 mM inhibitor of *vc*ASADH and a two-fold stronger inhibitor of *sp*ASADH (Table 1).

Attempts to synthesize the corresponding 3-position analogs to compounds **3** and **4** were thwarted by cyclization between the side chain amine nitrogen and the 2-carboxyl group. Blocking this nitrogen by methylation did allow completion of this synthesis (Scheme 2), but this compound (**9**) showed only weak inhibition ($K_i = 1.8$ mM) of *sp*ASADH and no inhibition of *vc*ASADH, similar to the affinities that were observed with compound **4**.

2.3. Introduction of substituted benzyl groups

Varying the nature of the *N*-carboxymethyl side chain through the introduction of an α -L-methyl group (**5**) or an α -L-hydroxymethyl group (**6**) had no appreciable effect on the binding affinity to either ASADH ortholog (Table 1). To explore the improved affinity of the parent compound **3** to the ASADHs several additional derivatives were synthesized through coupling with various halides at the secondary amine position. However, the introduction of simple alkyl, allyl, nitrile, aryl or aldehyde groups (**10–13**, **37**) did not alter the inhibitory properties of these compounds (Table 1). Next, in an attempt to improve the binding affinity, a series of substituted benzyl substituents were introduced at this amine position. Now significant changes were observed in both binding affinity and in binding selectivity between the two forms of ASADH, with the improvement in activity highly dependent both on the nature and on the position of the aromatic substitutions. Coupling with benzyl halides substituted with either bromo (**18** and **19**) or methyl groups (**21** and **22**) at the *ortho*- or *meta*-positions did not yield any improvement in affinity, but similar substitutions at the *para*-position of the benzyl ring (**20** and **23**) resulted in a 10-fold improvement in the K_i values with *sp*ASADH (Table 2). Surprisingly, these *para*-substituted derivatives did not show any improved affinity for *vc*ASADH.

2.4. Incorporation of hydrophobic benzyl substituents

Next, the effect of changes in the methyl substituent was examined. Replacement of the *o*-methyl or *m*-methyl groups with either trifluoromethyl (**24** and **25**) or trifluoromethoxy groups (**27** and **28**) did not alter the affinity of these derivatives for either *sp*ASADH or *vc*ASADH. However, replacing the *p*-methyl group with these same substituents (**26** and **29**) led to a twofold additional improvement in binding affinity for *sp*ASADH (Table 2). Changing *p*-trifluoromethoxy (**29**) to *p*-difluoromethoxy (**30**) reversed this improvement, while an additional two-fold improvement ($K_i = 16 \mu\text{M}$) was observed for the *p*-*tert*-butylbenzyl derivative (**33**) as an inhibitor of *sp*ASADH (Table 2). To further enhance the hydrophobic nature of the *p*-benzyl substituent a perfluorinated 2-propyl derivative was prepared (**34**). This compound shows the most potent inhibition among this family of structures, with a K_i of $9 \mu\text{M}$ for *sp*ASADH and over 70-fold selectivity for this enzyme form relative to the Gram-negative ASADH ortholog from *V. cholerae*. As was seen for the parent compounds, for each of these synthesized derivatives no significant changes were observed in their affinity for the Gram-negative *vc*ASADH.

2.5. Examination of additional hydrophobic derivatives

Now that the importance of *para*- hydrophobic substituents has been established some additional groups were incorporated in place of the benzyl group to examine the nature of this hydrophobic interaction. Substitution with a naphthyl group coupled at either the 1- or 2-position (**14** and **15**) resulted in slightly worse inhibition, while the replacement of the benzyl group with a morpholine (**16**) gave only a slight improvement. The greatest change was observed with the incorporation of a biphenyl group (**17**), resulting in 25-fold improved inhibition of *sp*ASADH relative to the parent benzyl derivative (Table 1).

2.6. Exploration of a structure-activity outlier

The only outlier from this correlation between higher affinity and increased *para*-hydrophobicity was the observation that the *p*-carboxylbenzyl derivative (**31**) has comparable affinity to that observed for the *p*-methylbenzyl derivative (**23**). It seems reasonable to suggest that the improved affinity for this charged benzyl derivative is likely due to the orientation of this side chain in a different binding position, perhaps making an electrostatic interaction with a positively-charged amino acid side chain near the active site. To test this idea the *p*-carboxyl group was replaced with a polar but uncharged substituent, a carboxamide group (**32**). As predicted, this derivative shows a 3-fold loss of affinity to a value that is now comparable to that of the underivatized parent compound (**3**) (Table 2).

Now that the significance of these *p*-substituted benzyl derivatives as potent and selective ASADH inhibitors has been demonstrated, several longer homologs of these derivatives were synthesized starting from parent compound **4** (Scheme 1) to determine if the improved affinities of these derivatives would be able to overcome the lower affinity of this parent compound. However, in these cases neither the *p*-bromobenzyl nor the *p*-trifluoromethylbenzyl derivatives of compound **4** gave better than low millimolar inhibition of *sp*ASADH, and no inhibition of *vc*ASADH was observed with these derivatives (data not shown).

3. Discussion

3.1. Target validation and preliminary inhibitor identification

The past few decades have seen increased attention of the drug development community focusing on diseases such as cancer and cardiovascular diseases, the leading causes of human mortality. However, the development of an increasingly diverse set of defensive mechanisms by microbes against most of the current antibiotics demands a renewed focus from the scientific community to identify unique and selective pathways that can be targeted for development of new classes of antibiotics.

The aspartate pathway, absent in mammals but present in microbes and plants, is a critical and essential pathway that meets these criteria. We have targeted aspartate semialdehyde dehydrogenase (ASADH), the enzyme which controls the first branch point in this pathway, for the design of new, potent, and selective inhibitors. Inhibition of ASADH will provide the basis for development of selective drugs that can function through a novel mechanism of action against this new target.

Fragment library screening against this enzyme target had previously led to the identification of several non-substrate-like structures that function as modest inhibitors of the ASADHs.¹⁶ Among these compounds were some aromatic inhibitors that offered the possibility of additional structural elaborations. Subsequent molecular modeling and docking studies starting with these core structures led to the identification of aryl di- and trianionic compounds that were determined to be low millimolar inhibitors of both Gram-negative and Gram-positive forms of ASADH.¹⁷ Extending one of the carboxylate side chains into an adjacent anionic binding pocket was then explored to improve inhibitor affinity. Unfortunately, some steric clashes coupled with multiple possible binding

orientations¹⁹ blocked the further development of this core structure into more potent inhibitors. What was needed to realize the objective of potent inhibition by this class of structures was the introduction of additional functionality that would favor the adoption of a single orientation when bound to the enzyme.

3.2. Identification and optimization of aryl dicarboxylate inhibitors

Some parent aryl dicarboxylate structures have now been designed with the inclusion of a secondary amine side chain that can be further elaborated to test this approach (Scheme 1). Initial attempts were made to directly mimic the 3-(3-carboxypropyl)phthalate structure that showed good binding orientation at the active site of *sp*ASADH¹⁹ through replacement of the carboxypropyl group with an aminoethylcarboxylate side chain. This synthetic approach failed because of rapid cyclization between the introduced secondary amine and the carboxyl group at the 2-position. Protection of this amine through the presence of a methyl group did allow synthesis of the desired structure (Scheme 2) which, unfortunately, was no more potent than the original compound.

Moving the aminoalkylcarboxyl side chain from the 3- to the 4-position on the benzyl ring avoided this cyclization reaction, and has led to a much more productive series of ASADH inhibitors. The parent compound **3** is already a high micromolar inhibitor of the ASADHs with a 2-fold preference for the Gram-positive *sp*ASADH. Exploring the reactivity of the introduced secondary amine through the incorporation of a set of simple substituents did not alter either the affinity or the selectivity of these derivatives relative to the parent compound (Table 1). However, changes in affinities were observed when substituted aryl substituents were introduced at this amine position. Simple *o*- or *m*-substituted benzyl groups were slightly poorer inhibitors of *sp*ASADH when compared to the parent benzyl substituted analog (**13**), but the corresponding *p*-substituted benzyl derivatives were substantially more potent *sp*ASADH inhibitors and also showed 10-fold selectivity for this enzyme form relative to *vc*ASADH (Table 2). The more hydrophobic fluorinated *p*-methylbenzyl derivatives gave a 2-fold additional improvement in affinity, with slight differences observed between di- and trifluoromethyl and between methyl and methoxy.

3.3. Role of hydrophobic functional groups

Placing a hydrophobic group at the *para*-position is likely accessing a hydrophobic pocket adjacent to the active site of *sp*ASADH. Consistent with this hypothesis is the observation that the *p*-*tert*-butylbenzyl derivative (**33**) is a much more potent inhibitor in this series and also has over 40-fold selectivity for *sp*ASADH (Table 2). The most hydrophobic *para*-substituent tested, perfluoroisopropyl, is, as expected, the most potent inhibitor of *sp*ASADH, with a low micromolar K_i value and 70-fold selectivity over the Gram-negative *vc*ASADH. Further elaboration at this position through the introduction of either a *p*-styrene or a naphthyl group in place of the benzyl group leads to a loss of activity, presumably due to unfavorable interactions with the larger steric bulk. The only additional aromatic hydrophobic group that allowed improved binding affinity was a *p*-biphenyl group (**17**), suggesting the presence of a deep but narrow hydrophobic pocket near the active site that is being accessed by this side chain. Modifications in the *N*-carboxymethyl side chain through the introduction of α -substituents (**5** and **6**) does not lead to enhanced affinity, and carbon

chain homology (4) causes a substantial affinity loss, even in the presence of the more optimal benzyl derivatives (35 and 36).

3.4. Inhibitor selectivity

A major cause of “off-target” effects with drug candidates is not the lack of affinity for the target of interest, but a lack of specificity that allows additional interactions with unintended targets. There are no close mammalian orthologs of ASADH that would provide obvious competing target proteins. While it is always difficult to predict possible interactions between new chemical entities and the wide range of possible *in vivo* binding interactions, the most potent of these inhibitors now displays enhanced selectivity against the identical enzyme from another bacterial species. This increases the confidence in the exquisite selectivity of this developed class of ASADH inhibitors.

3.5. Potential active site interactions

There have been numerous structures of substrate analog inhibitors¹⁵ and reaction intermediates^{11,13} bound at the active site of different ASADHs. Modeling studies of substrate binding to a wide range of ASADHs from different species concluded that the substrate utilizes the same set of active site binding interactions and binds in the same orientation despite the sequence diversity in this enzyme family.²¹ This nearly identical substrate binding presents a significant challenge in designing substrate analog inhibitors that can demonstrate species-selectivity against this target enzyme. Fortunately, the original compound in this phthalate series, 3-(3-carboxypropyl)phthalate, binds in a unique position in the active site of ASADH, base-stacking with the nicotinamide ring of the NADP cofactor while also making electrostatic interactions between the inhibitor carboxylate groups and the side chains of Ser96, Arg99 and Arg245.¹⁹ However, minor structural alterations leads to substantial changes in the bound orientation of these structurally-related inhibitors, with the one carbon shorter homolog, 3-(3-carboxyethyl)phthalate, using the same electrostatic binding partners but flipped by 180° in this binding pocket. This orientational and conformational flexibility is the apparent cause for the much lower measured affinity than would be predicted based on the number of favorable binding interactions observed in the optimally-oriented enzyme-inhibitor structure. In an attempt to constrain this orientational flexibility an amine was introduced into the carboxypropyl side chain (compound 3) that can serve as a site for further structural elaborations. Derivatization of this introduced secondary amine through the introduction of *para*-hydrophobic benzyl substituents appears to make additional productive binding interactions that constrain these multiple orientations, leading to the realization of potent and selective inhibition of ASADH.

Docking of the most potent inhibitors into the active site of *sp*ASADH offers a possible explanation both for the improved affinity of these derivatives and for the observed ortholog selectivity. Using the structure of 3-(3-carboxypropyl)phthalate in complex with *sp*ASADH (PDB ID 4r4j) as a guide, the various *para*-substituted benzylamine derivatives were modeled into the active site. This enzyme structure contains an adjacent pocket near the active site, surrounded by several hydrophobic amino acids (Leu326, Ala330, Ala331 and Val14) that could make favorable interactions with the *para*-hydrophobic substituents of the most potent inhibitors. The shape of this hydrophobic pocket could also potentially

accommodate a *para*-substituent on the benzyl ring as large as phenyl (**17**), but interactions with hydrophobic substituents at either the *ortho*- or *meta*-position would be less favorable. Further development of this series of inhibitors would benefit from structures bound to *sp*ASADH to identify the actual binding interactions that have been incorporated and to gain a better sense of the directions that these *p*-substituted benzyl derivatives can be elaborated.

The Val14 in this putative binding pocket is fully conserved throughout the bacterial ASADH family. However, in the corresponding region in the structure of *vc*ASADH (PDB ID 2qz9) several substitutions with polar amino acids in the 325 to 331 loop, including Leu326 replaced by Asn and Asn325 replaced by a Gln that would now extend into this site, would make this pocket much less hydrophobic. These substitutions could explain the failure of these inhibitors to show improved affinity with this Gram-negative ortholog of ASADH. These same replacements are found at these positions in other Gram-negative orthologs of ASADH, supporting the planned development of this series of inhibitors as Gram-positive selective antimicrobials.

4. Conclusions

A secondary amine was introduced into the carboxypropyl side chain of a previously characterized modest and non-selective inhibitor of ASADH to allow further structural elaborations. Significant improvements in binding affinities, approaching three-orders of magnitude, and dramatically enhanced ortholog selectivity have now been achieved for certain substituted *N*-benzyl derivatives, with the highest affinities driven by the incorporation of hydrophobic *para*-substituents on the introduced benzyl ring.

5. Experimental

5.1. General Chemistry

All compounds and reagents purchased from commercial sources were used without further purification. Reaction progress was monitored by TLC carried out using silica coated glass plates (Analtech) and was visualized by 254 nm UV light. Flash column chromatography was conducted in a 20 mm x 250 mm column with 40–63 μ SiliaFlash P60 silica gel (Silicycle) using the specified ethyl acetate:hexanes gradient for elution. ^1H NMR spectra were recorded on either a Varian VXR 400 MHz or a Varian INOVA 600 MHz spectrometer, and were calibrated using residual non-deuterated solvent as internal reference (CDCl_3 , $\delta = 7.26$, for the esters; D_2O , $\delta = 4.80$, for the sodium salts).

5.2. Parent compound synthesis

5.2.1. 4-methylphthalate dimethyl ester (1Me)—To a solution of compound **1**, 4-methyl-1,2-dicarboxylic acid (5 g, 27.7 mmol) in 12 ml of methanol, 5 ml of sulfuric acid was added at r. t., the reaction mixture was allowed to stir in a microwave reactor at 80 °C for 8 h. Saturated NaHCO_3 solution was added to the reaction mixture until slightly basic, with the product precipitating as an oily liquid. The product was extracted with several portions of dichloromethane (DCM), dried over anhydrous sodium sulphate and concentrated in vacuum. The compound was purified by column chromatography using 0–10% ethyl acetate:hexanes to yield ester **1Me**. ^1H NMR (600 MHz, CDCl_3): $\delta = 2.39$ (s,

3H), 3.87 (s, 3H), 3.88 (s, 3H), 7.30 (d, $J=7.8$ Hz, 1H), 7.45 (s, 1H), 7.65 (d, $J=7.8$ Hz, 1H). MS (ESI) m/z : (obs) $[M+Na]^+$ 231.1, (calc) 231.2.

5.2.2. 4-bromomethylphthalate dimethyl ester (2Me)—To a solution of compound **2** (5 g, 24.8 mmol) in 25 ml dichloromethane was added NBS (4.9 g, 27.3 mmol). The reaction mixture was stirred in the presence of light for 3 h, concentrated under vacuum and purified by flash column chromatography using 0–20% ethyl acetate:hexanes to obtain compound **2Me**. 1H NMR (600 MHz, $CDCl_3$): $\delta=3.89$ (s, 6H), 4.46 (s, 2H), 7.53 (d, $J=6.6$ Hz, 1H), 7.68–7.71 (m, 2H). MS (ESI) m/z : (obs) $[M+Na]^+$ (isotopic) 309.3, 311.1 (calc) (isotopic) 309.0, 311.0.

5.2.3. N-carboxymethyl-3,4-dicarboxybenzylamine (3)—To a solution of compound **2Me** (3.1 g, 10.7 mmol) in DMF was added $NaHCO_3$ (4.6 g, 54 mmol) and glycine methyl ester.HCl (6.8 g, 54 mmol). The reaction mixture was stirred at r. t. for 8 h. DMF was air evaporated and the reaction mixture was purified by flash column chromatography using 10–50% ethyl acetate:hexanes to obtain compound **3Me**. 1H NMR (600 MHz, $CDCl_3$): $\delta=3.38$ (s, 2H), 3.70 (s, 3H), 3.84 (s, 2H), 3.86–3.87 (s, 6H), 7.48 (d, $J=7.8$ Hz, 1H), 7.64 (s, 1H), 7.68 (d, $J=7.8$ Hz, 2H). MS (ESI) m/z : (obs) $[M+Na]^+$ 318.4, (calc) 318.3. This ester was hydrolyzed by adding H_2O (0.1 ml) to a solution of compound **3Me** (0.04 g, 0.14 mmol) in THF (0.4 ml). Then 1 N NaOH (0.4 ml, 0.42 mmol) was added to the reaction mixture and stirred at r. t. for 3 h. The reaction mixture was concentrated and dried for several hours under high vacuum to yield pure compound **3**. 1H NMR (400 MHz, D_2O): $\delta=3.12$ (s, 2H), 3.66 (s, 2H), 7.48 (d, $J=7.8$ Hz, 1H), 7.27–7.32 (m, 2H), 7.40 (s, 1H).

5.2.4. N-carboxyethyl-3,4-dicarboxybenzylamine (4)—To a solution of compound **2Me** (0.52 g, 1.8 mmol) in DMF was added $NaHCO_3$ (0.8 g, 9 mmol) and β -alanine methyl ester.HCl (1.3 g, 9 mmol). The reaction mixture was stirred at r. t. for 8 h. DMF was air evaporated and the reaction mixture was purified by flash column chromatography using 10–60% ethyl acetate:hexanes to obtain compound **4Me**. 1H NMR (600 MHz, $CDCl_3$): $\delta=3.38$ (s, 2H), 3.70 (s, 3H), 3.84 (s, 2H), 3.86 (s, 3H), 3.87 (s, 3H), 7.48 (d, $J=7.8$ Hz, 1H), 7.64 (s, 1H), 7.68 (d, $J=7.8$ Hz, 2H). MS (ESI) m/z : (obs) $[M+Na]^+$ 332.4, (calc) 332.3. A solution of compound **4Me** (0.071 g, 0.229 mmol) was base-hydrolyzed as described above to yield pure compound **4**. 1H NMR (600 MHz, D_2O): $\delta=2.47$ (t, $J=7.2$ Hz, 2H), 2.66 (t, $J=7.2$ Hz, 2H), 3.59 (s, 2H), 7.18 (d, $J=7.8$ Hz, 1H), 7.21 (s, 1H), 7.30 (d, $J=7.8$ Hz, 1H).

5.2.5. N-((2-methyl)carboxymethyl)-3,4-dicarboxybenzylamine (5)—To a solution of compound **2Me** (1.36 g, 4.7 mmol) in DMF was added $NaHCO_3$ (2.0 g, 23.7 mmol) and L-alanine methyl ester.HCl (3.3 g, 23.7 mmol). The reaction mixture was stirred at r. t. for 8 h. DMF was air evaporated and the reaction mixture was purified by flash column chromatography using 10–60% ethyl acetate:hexanes to obtain compound **5Me**. 1H NMR (600 MHz, $CDCl_3$): $\delta=1.32$ (d, $J=6.6$ Hz 3H), 3.49 (m, $J=6.6$ Hz 1H), 3.71 (s, 4H), 3.84 (s, 2H), 3.87–3.90 (m, 8H), 7.51 (d, $J=7.8$ Hz 1H), 7.66 (s, 1H), 7.69 (d, $J=7.8$ Hz, 1H). MS (ESI) m/z : (obs) $[M+Na]^+$ 332.4, (calc) 332.3. A solution of compound **5Me** (0.071 g, 0.229 mmol) was base-hydrolyzed as described above to yield pure compound **5**. 1H NMR (600

MHz, D₂O): δ =1.16 (d, J =6.6 Hz 3H), 3.11 (m, J =7.2 Hz 1H), 3.52 (d, J =12.6 Hz 1H), 3.68 (d, J =12.6 Hz 1H), 7.27 (d, J =8.4 Hz 1H), 7.31 (s, 1H), 7.40 (d, J =7.8 Hz, 1H).

5.2.6. *N*-((2-hydroxymethyl)carboxymethyl)-3,4-dicarboxybenzylamine (6)—To a solution of compound **2Me** (1.15 g, 4.0 mmol) in DMF was added NaHCO₃ (1.7 g, 20.1 mmol) and L-serine methyl ester.HCl (3.1 g, 20.1 mmol). The reaction mixture was stirred at r. t. for 8 h. DMF was air evaporated and the reaction mixture was purified by flash column chromatography using 10–60% ethyl acetate:hexanes to obtain compound **6Me**. ¹H NMR (600 MHz, CDCl₃): δ =2.43 (s, 2H), 3.39 (m, 1H), 3.64 (m, 1H), 3.74 (s, 3H), 3.80 (m, 2H), 3.81–3.89 (m, 6H), 3.95 (d, J =14.4 Hz 1H), 7.50 (d, J =7.8 Hz, 1H), 7.66 (s, 1H), 7.70 (d, J =7.8 Hz, 1H). MS (ESI) m/z : (obs) [M+Na]⁺ 348.4, (calc) 348.3. A solution of compound **6Me** (0.071 g, 0.229 mmol) was base- hydrolyzed as described above to yield pure compound **6**. ¹H NMR (600 MHz, D₂O): δ =3.17 (t, J =5.2 Hz 1H), 3.61 (d, 13.2 Hz 1H), 3.68–3.70 (m, 2H), 3.78 (d, J =12.8 Hz 1H), 7.31 (d, J =8 Hz, 1H), 7.35 (s, 1H), 7.42 (d, J =7.8 Hz, 1H).

5.2.7. 3-methylphthalate dimethyl ester (7Me)—To a solution of compound **7**, 3-methylphthalic acid (5 g, 24 mmol) in 12 ml of methanol at r. t. was added 5 ml of conc. sulfuric acid, with the reaction mixture stirred in a microwave reactor at 80 °C for 8 h. Saturated NaHCO₃ solution was added to the reaction mixture until slightly basic, and the product was extracted with DCM and concentrated in vacuum. The compound was purified by column chromatography using 0–10% ethyl acetate:hexanes to furnish ester **7Me**. ¹H NMR (600 MHz, CDCl₃): δ =2.33(s, 3H), 3.87 (s, 3H), 3.93 (s, 3H), 7.35 (t, J =7.8 Hz, 1H), 7.39 (d, J =7.8 Hz, 1H), 7.80 (d, J =7.2 Hz, 1H). MS (ESI) m/z : (obs) [M+Na]⁺ 231.1, (calc) 231.2.

5.2.8. 3-bromomethylphthalate dimethyl ester (8Me)—To a solution of compound **7Me** (1.8 g, 8.8 mmol) in dichloromethane was added NBS (1.72 g, 9.7 mmol). The reaction mixture was stirred in presence of light for 3 h, concentrated in vacuum and purified by flash column chromatography using 0–15% ethyl acetate:hexanes to obtain compound **8Me**. ¹H NMR (600 MHz, CDCl₃): δ =3.88 (s, 3H), 3.95 (s, 3H), 4.52 (s, 2H), 7.46 (t, J =7.8 Hz, 1H), 7.61 (d, J =7.8 Hz, 1H), 7.90 (d, J =7.8 Hz, 1H). MS (ESI) m/z : (obs) [M+Na]⁺ (isotopic) 309.2, 311.1(calc) (isotopic) 309.0, 311.0.

5.2.9. *N*-methyl, *N*-carboxymethyl-2,3-dicarboxybenzylamine (9)—To a solution of compound **8Me** (0.42 g, 1.46 mmol) in DMF was added NaHCO₃ (0.61 g, 7.32 mmol) and *N*-methyl glycine methyl ester.HCl (1.1 g, 7.35 mmol). The reaction mixture was stirred at r. t. for 8 h. DMF was air evaporated and the reaction mixture was purified by flash column chromatography on silica gel using 10–40% ethyl acetate:hexanes to obtain compound **9Me**. ¹H NMR (600 MHz, CDCl₃): δ =2.23(s, 3H), 3.15 (s, 2H), 3.57 (s, 3H), 3.65 (s, 2H), 3.76 (s, 3H), 3.78 (s, 3H), 7.31 (t, J =7.8 Hz, 1H), 7.54(d, J =7.8 Hz, 1H), 7.76 (d, J =7.8 Hz, 1H). MS (ESI) m/z : (obs) [M+Na]⁺ 332.4, (calc) 332.3. This ester was hydrolyzed as described above to yield pure compound **9**. ¹H NMR (600 MHz, D₂O): δ =2.10(s, 3H), 2.98 (s, 2H), 3.55 (s, 2H), 7.29 (t, J =7.2 Hz, 1H), 7.50(d, J =7.2 Hz, 1H), 7.53(d, J =7.2 Hz, 1H).

5.3. General protocol for *N*-derivatized dicarboxybenzylamines (10–34)

To a solution of compound **3Me** (1 eq.) in acetonitrile was added 1.1 eq of various alkyl halides (Table 1) or various benzyl halides (Table 2) along with 1.1 eq of NaHCO₃. The reaction mixture was stirred at r. t. for 3 h. CH₃CN was evaporated in vacuum and purified by flash column chromatography on silica gel using 0–25% ethyl acetate:hexanes to obtain corresponding *N*-derivatized benzylamine esters. Ester hydrolysis was carried out by adding 0.1 ml of H₂O to a solution of *N*-derivatized benzylamine esters (1 eq) in THF (0.4 ml) with stirring. Then, 1 N NaOH (3.3 eq) was added to the reaction mixture and stirred at r. t. for 3 h. The reaction mixture was concentrated and dried for several hours under high vacuum to yield the corresponding sodium salts of the *N*-derivatized benzylamines, compounds **10–34**.

5.3.1. *N*-methyl, *N*-carboxymethyl-3,4-dicarboxybenzylamine (10)

triester: ¹H NMR (600 MHz, CDCl₃): δ=2.36 (s, 3H), 3.28 (s, 2H), 3.69 (s, 3H) 3.73 (s, 2H), 3.88 (s, 6H), 7.52(d, *J*= 7.8 Hz, 1H), 7.66 (s, 1H), 7.68(d, *J*=7.8 Hz, 2H). MS (ESI) *m/z*: (obs) [M+Na]⁺ 332.4, (calc) 332.3.

sodium salt: ¹H NMR (600 MHz, D₂O): δ=2.04 (s, 3H), 2.85(s, 2H), 3.46 (s, 2H), 7.15–7.17(m, *J*=7.2 Hz, 2H), 7.25 (d, *J*=7.2 Hz, 1H).

5.3.2. *N*-allyl, *N*-carboxymethyl-3,4-dicarboxybenzylamine (11)

triester: ¹H NMR (600 MHz, CDCl₃): δ=3.24 (s, 2H), 3.31 (s, 2H), 3.66 (s, 3H) 3.82 (s, 2H), 3.88–3.89 (s, 6H), 5.13–5.21 (m, 2H), 5.78–5.84 (m, 1H) 7.54 (d, *J*= 7.8 Hz, 1H), 7.68(m, *J*=7.8 Hz, 2H). MS (ESI) *m/z*: (obs) [M+Na]⁺ 358.4, (calc) 358.3.

sodium salt: ¹H NMR (600 MHz, D₂O): δ= 2.9 (s, 2H), 3.04 (s, 2H), 3.58(s, 2H), 5.02–5.07 (m, 2H), 5.69–5.73 (m, 1H), 7.15–7.17(m, 2H), 7.25 (d, 1H).

5.3.3. *N*-acetonitrile, *N*-carboxymethyl-3,4-dicarboxybenzylamine (12)

triester: ¹H NMR (400 MHz, CDCl₃): δ=3.42 (s, 2H), 3.68 (s, 2H), 3.72 (s, 3H) 3.84 (s, 2H), 3.89 (s, 6H), 7.55 (d, *J*= 7.6 Hz, 1H), 7.7 (m, *J*=7.6 Hz, 2H). MS (ESI) *m/z*: (obs) [M+Na]⁺ 357.4, (calc) 357.3.

sodium salt: ¹H NMR (400 MHz, D₂O): δ= 2.97 (s, 2H), 2.99 (s, 2H), 3.57(s, 2H), 7.17–7.20(m, 2H), 7.27(d, 1H).

5.3.4. *N*-benzyl, *N*-carboxymethyl-3,4-dicarboxybenzylamine (13)

triester: ¹H NMR (400 MHz, CDCl₃): δ=3.30 (s, 2H), 3.68 (s, 3H), 3.79 (s, 2H), 3.87 (s, 2H), 3.89(s, 3H), 3.91 (s, 3H), 7.23–7.34 (m, 5H), 7.59 (d, *J*=8 Hz, 1H), 7.72 (m, *J*=8 Hz, 2H). MS (ESI) *m/z*: (obs) [M+Na]⁺ 408.3, (calc) 408.4.

sodium salt: ¹H NMR (600 MHz, D₂O): δ= 2.88 (s, 2H), 3.62 (s, 4H), 7.20(m, 7H), 7.29(m, 1H).

5.3.5. *N*-(1-naphthyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (14)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.28 (s, 2H), 3.67 (s, 3H), 3.87 (s, 2H), 3.89(s, 3H), 3.91 (s, 3H), 4.26 (s, 2H), 7.38(s, J =7.8 Hz, 1H), 7.44–7.53 (m, 4H), 7.65 (m, J =7.8 Hz, 2H), 7.75(d, J =8.4 Hz, 1H), 7.81(d, J =8 Hz, 1H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 458.2, (calc) 458.5.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 3.05 (s, 2H), 3.88 (s, 2H), 4.18 (s, 2H), 7.32(d, 1H), 7.38(s, 1H), 7.41–7.49 (m, 5H), 7.83 (d, J =7.8 Hz, 1H), 7.88–7.90 (m, J =7.8 Hz, 1H), 7.93 (d, J =8.4 Hz, 1H)

5.3.6. *N*-(2-naphthyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (15)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.32 (s, 2H), 3.67 (s, 3H), 3.87 (s, 2H), 3.88–3.90(m, 8H), 3.91 (s, 3H), 3.94 (s, 2H), 7.43–7.45 (m, 2H), 7.51 (d, J =8.4 Hz, 2H), 7.61(d, J =7.2 Hz, 1H), 7.70–7.74(m, 3H), 7.79(d, J =8.4 Hz, 3H) . MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 458.2, (calc) 458.5.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 3.06 (s, 2H), 3.78 (s, 2H), 3.89 (s, 2H), 7.30(d, J =7.8 Hz, 1H), 7.34(s, 1H), 7.41(d, J =7.8 Hz, 1H), 7.47–7.51 (m, 3H), 7.80 (s, 1H), 7.86–7.89 (m, J =7.8 Hz, 1H).

5.3.7. *N*-(ethylmorpholino)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (16)

triester: ^1H NMR (600 MHz, CDCl_3): δ =2.38 (s, 3H), 2.44 (t, J =6.6 Hz, 2H), 2.77 (t, J =6.6 Hz, 2H), 3.39 (s, 2H), 3.64(s, 6H), 3.85–3.86 (m, 8H), 7.49 (d, J =7.8 Hz, 2H), 7.64–7.66 (m, 3H). MS (ESI) m/z : (obs) $[\text{M}+\text{H}]^+$ 408.3, (calc) 408.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ =2.40 (s, 4H), 2.46 (t, J =8.4 Hz, 2H), 2.67 (t, J =7.2 Hz, 2H), 3.09 (s, 2H), 3.64(s, 4H), 3.69 (s, 2H), 7.32 (d, J =6.6 Hz, 2H), 7.40 (d, J =8.4 Hz, 1H).

5.3.8. *N*-(4-biphenyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (17)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.33 (s, 2H), 3.68 (s, 3H), 3.83 (s, 2H), 3.88–3.91(m, 8H), 7.31–7.33 (m, J =7.2 Hz 1H), 7.40–7.43 (m, 4H), 7.54, (d, J =7.2 Hz 2H), 7.58 (d, J =7.2 Hz, 2H), 7.61(d, J =7.8 Hz, 1H), 7.71–7.73(m, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 484.2, (calc) 484.5.

sodium salt: ^1H NMR (600 MHz, D_2O): δ =3.03 (s, 2H), 3.74 (d, J =14.4 Hz 4H), 7.31–7.33 (m, J =7.2 Hz 1H), 7.29 (d, 1H), 7.33–7.37, (m, J =7.2 Hz, 2H), 7.39–7.42 (t, J =8.4 Hz, 3H), 7.44–7.46 (t, J =7.8 Hz, 2H), 7.60 (d, J =7.8 Hz, 2H), 7.73(d, J =7.8 Hz, 2H).

5.3.9. *N*-(2-bromobenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (18)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.31 (s, 2H), 3.67(s, 3H), 3.86–3.88 (s, 6H), 3.92 (s, 4H), 7.08 (t, Hz, 1H), 7.25 (t, J =7.6 Hz, 1H), 7.49(t, J =8.0 Hz, 2H), 7.54 (d, J =8.0 Hz, 1H), 7.68(d, J =7.6 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ (isotopic) 486.2, 488.0 (calc) (isotopic) 486.1, 488.1.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 3.07 (s, 2H), 3.89 (s, 2H), 3.90 (s, 2H), 7.13–7.17 (t, 1H), 7.31–7.36 (m, J =7.2 Hz, 3H), 7.39 (d, J =8 Hz, 2H), 7.56 (d, J =8 Hz, 2H).

5.3.10. *N*-(3-bromobenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (19)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.26 (s, 2H), 3.65(s, 3H), 3.73 (s, 2H), 3.83(s, 2H), 3.86–3.88 (s, 6H), 7.14 (d, J =7.8 Hz, 1H), 7.24 (d, J =7.8 Hz, 1H), 7.33(d, J =7.8 Hz, 1H), 7.48 (s, 1H), 7.54(d, J =7.8 Hz, 1H), 7.68 (d, J =7.8 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ (isotopic) 486.2, 488.0 (calc) (isotopic) 486.1, 488.1.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 3.01 (s, 2H), 3.70(s, 2H), 3.74 (s, 2H), 7.22 (t, J =7.2 Hz, 1H), 7.27–7.31 (m, 3H), 7.41 (d, J =7.8 Hz, 1H), 7.43–7.45 (d, J =7.8 Hz, 1H), 7.51 (d, 1H).

5.3.11. *N*-(4-bromobenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (20)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.25 (s, 2H), 3.65(s, 3H), 3.71 (s, 2H), 3.82 (s, 2H), 3.87–3.89 (s, 6H), 7.20 (d, J =7.8 Hz, 2H), 7.42 (d, J =7.8 Hz, 2H), 7.54(d, J =7.8 Hz, 1H), 7.66–7.69(m, J =7.8 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ (isotopic) 486.2, 488.0 (calc) (isotopic) 486.1, 488.1.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 2.78 (s, 2H), 3.45 (s, 2H), 3.46 (s, 2H), 6.97 (d, J =8.4 Hz, 2H), 7.05–7.07(d, J =7.8 Hz, 1H), 7.12 (s, 1H), 7.21–7.24 (m, J =7.8 Hz, 3H).

5.3.12. *N*-(2-methylbenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (21)

triester: ^1H NMR (600 MHz, CDCl_3): δ =2.31(s, 3H), 3.24 (s, 2H), 3.65(s, 3H), 3.80 (s, 2H), 3.87 (s, 2H), 3.88–3.89 (s, 6H), 7.13 (m, 3H), 7.28 (d, J =6.6 Hz, 1H), 7.52(d, J =7.8 Hz, 1H) 7.64(s, 1H), 7.68 (d, J =7.8 Hz, 1H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 422.2, (calc) 422.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 2.13 (s, 3H), 3.04 (s, 2H), 3.73 (s, 2H), 3.82 (s, 2H), 7.17 (m, 3H), 7.31–7.33 (m, 3H), 7.41 (d, J =7.8 Hz, 1H).

5.3.13. *N*-(3-methylbenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (22)

triester: ^1H NMR (600 MHz, CDCl_3): δ =2.31(s, 3H), 3.28 (s, 2H), 3.66(s, 3H), 3.73 (s, 2H), 3.84 (s, 2H), 3.87–3.88 (s, 6H), 7.03 (d, J =7.8 Hz, 1H), 7.12 (d, J =7.8 Hz, 3H), 7.18(m, J =7.8 Hz, 1H) 7.57(d, J =7.8 Hz, 1H), 7.68 (d, J =7.8 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 422.2, (calc) 422.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 2.28 (s, 3H), 3.01 (s, 2H), 3.70 (s, 2H), 3.74 (s, 2H), 7.12–7.16 (m, J =6 Hz, 2H), 7.16 (s, 1H), 7.24–7.26 (t, J =7.2 Hz, 1H), 7.29–7.31 (m, 2H), 7.41–7.42 (d, J =7.8 Hz, 1H).

5.3.14. *N*-(4-methylbenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (23)

triester: ^1H NMR (600 MHz, CDCl_3): δ =2.31 (s, 3H), 3.27 (s, 2H), 3.66(s, 3H), 3.73 (s, 2H), 3.87–3.89 (s, 6H), 7.11 (d, J =7.8 Hz, 2H), 7.22 (d, J =7.8 Hz, 2H), 7.58(d, J =7.8 Hz, 1H), 7.69–7.70(m, J =7.8 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 422.2, (calc) 422.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 2.27 (s, 3H), 2.96 (s, 2H), 3.70 (s, 2H), 3.72 (s, 2H), 7.18–7.22 (m, J =7.8 Hz, 4H), 7.28–7.30 (m, 2H), 7.40 (d, J =7.8 Hz, 1H).

5.3.15. *N*-(2-trifluoromethylbenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (24)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.28 (s, 2H), 3.66(s, 3H), 3.86(s, 2H), 3.86–3.88 (s, 6H), 3.95 (s, 2H), 7.31 (t, J =7.8 Hz, 1H), 7.51 (t, J =7.8 Hz, 1H), 7.57(t, J =7.8 Hz, 2H) 7.68(m, J =7.8 Hz, 2H), 7.85 (d, J =7.8 Hz, 1H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 476.1, (calc) 476.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 3.05 (s, 2H), 3.79 (s, 2H), 3.91 (s, 2H), 7.33 (m, 2H), 7.36–7.40 (m, 2H), 7.56 (t, J =7.8 Hz, 1H), 7.65 (d, J =7.8 Hz, 1H), 7.78 (d, J =7.8 Hz, 1H).

5.3.16. *N*-(3-trifluoromethylbenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (25)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.29 (s, 2H), 3.66(s, 3H), 3.83 (s, 2H), 3.85 (s, 2H), 3.86–3.89 (s, 6H), 7.40 (t, J =7.8 Hz, 1H), 7.49 (d, J =7.8 Hz, 1H), 7.53(t, J =7.8 Hz, 2H) 7.57(s, J =7.8 Hz, 1H), 7.68 (d, J =7.8 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 476.1, (calc) 476.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 3.03 (s, 2H), 3.75 (s, 2H), 3.80 (s, 2H), 7.29–7.32 (m, J =6 Hz, 2H), 7.16 (s, 1H), 7.41 (d, J =7.8 Hz, 1H), 7.48–7.51 (t, J =7.8 Hz, 1H), 7.55 (d, J =7.8 Hz, 1H), 7.59(d, J =7.8 Hz, 1H), 7.65 (s, 1H).

5.3.17. *N*-(4-trifluoromethylbenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (26)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.28 (s, 2H), 3.66(s, 3H), 3.83 (s, 2H), 3.87 (s, 3H), 3.89 (s, 3H), 7.45 (d, J =7.8 Hz, 2H), 7.54–7.56 (m, J =7.8 Hz, 3H), 7.68–7.70(m, J =7.8 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 476.1, (calc) 476.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 2.89 (s, 2H), 3.61 (s, 2H), 3.66 (s, 2H), 7.16 (m, J =8.4 Hz, 2H), 7.27 (d, J =7.2 Hz, 1H), 7.33 (d, J =6 Hz, 2H), 7.50 (d, J =6.6 Hz, 2H).

5.3.18. *N*-(2-trifluoromethoxybenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (27)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.27 (s, 2H), 3.65(s, 3H), 3.86(s, 2H), 3.87(s, 2H), 3.88 (s, 6H), 7.19 (d, J =7.8 Hz, 1H), 7.25 (m, J =7.8 Hz, 2H), 7.55(m, J =7.8 Hz, 2H) 7.68 (d, J =7.8 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 492.1, (calc) 492.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 3.03 (s, 2H), 3.82 (s, 2H), 3.87 (s, 2H), 7.30–7.36 (m, 5H), 7.40 (d, J =7.2 Hz, 1H), 7.45 (d, J =7.8 Hz, 1H).

5.3.19. *N*-(3-trifluoromethoxybenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (28)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.28 (s, 2H), 3.65(s, 3H), 3.79 (s, 2H), 3.84(s, 2H), 3.86–3.88 (s, 6H), 7.06 (d, J =7.8 Hz, 1H), 7.24 (s, J =7.8 Hz, 1H), 7.30(t, J =7.8 Hz, 2H), 7.57(D, J =7.8 Hz, 1H), 7.68 (d, J =7.8 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 492.1, (calc) 492.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 3.03 (s, 2H), 3.75 (s, 2H), 3.77 (s, 2H), 7.21 (d, J =7.8 Hz, 1H), 7.28–7.32 (m, 4H), 7.39–7.42 (m, J =7.8 Hz, 2H).

5.3.20. *N*-(4-trifluoromethoxybenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (29)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.27 (s, 2H), 3.66(s, 3H), 3.76 (s, 2H), 3.84 (s, 2H), 3.87 (s, 3H), 3.89 (s, 3H), 7.13 (d, J =8.4 Hz, 2H), 7.35 (d, J =8.4 Hz, 2H), 7.54(d, J =8.4 Hz, 1H), 7.67–7.69(m, J =8.4 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 492.1, (calc) 492.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 2.87 (s, 2H), 3.60 (s, 4H), 6.48–6.73(m, 1H), 7.11 (d, J =6 Hz, 2H), 7.16 (m, J =6 Hz, 2H), 7.22 (m, J =6 Hz, 2H), 7.26 (m, J =6 Hz, 1H).

5.3.21. *N*-(4-difluoromethoxybenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (30)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.26 (s, 2H), 3.65(s, 3H), 3.74 (s, 2H), 3.83 (s, 2H), 3.86–3.88 (s, 6H), 6.34–6.59 (m, 1H), 7.02 (d, J =8.4 Hz, 2H), 7.31 (d, J =8.4 Hz, 2H), 7.54(d, J =8.4 Hz, 1H), 7.66–7.69(m, J =8.4 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 474.2, (calc) 474.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 2.86 (s, 2H), 3.57 (s, 2H), 3.60 (s, 2H), 6.48–6.73(m, 1H), 6.95 (d, J =8.4 Hz, 2H), 7.13–7.18 (m, J =8.4 Hz, 4H), 7.24 (m, J =7.8 Hz, 1H).

5.3.22. *N*-(4-carboxybenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (31)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.26 (s, 2H), 3.64(s, 3H), 3.81 (s, 2H), 3.83 (s, 2H), 3.85(s, 3H), 3.86 (s, 3H), 3.87 (s, 3H), 7.39 (d, J =8.4 Hz, 2H), 7.54 (d, J =8.4 Hz, 1H), 7.66–7.68(m, J =8.4 Hz, 2H), 7.94(d, J =8.4 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 466.2, (calc) 466.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 2.83 (s, 2H), 3.54 (s, 2H), 3.58 (s, 2H), 7.11 (d, 2H), 7.17–7.19(m, 3H), 7.25 (d, 1H), 7.61 (d, 2H).

5.3.23. *N*-(4-carboxamidebenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (32)

triester: ^1H NMR (600 MHz, CDCl_3): δ =3.27 (s, 2H), 3.65(s, 3H), δ =4.07 (m, 2H), 3.79–3.82 (m, 4H), 3.85–3.87 (s, 6H), 6.19–6.36 (m, 2H), 7.37 (d, J =7.8 Hz, 2H), 7.53 (d, J =7.2 Hz, 1H), 7.64–7.66(m, 2H), 7.72(d, J =8.4 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 451.3, (calc) 451.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ =3.02 (s, 2H), 3.77–3.79 (m, 4H), 7.31–7.34 (M, 2H), 7.37 (d, J =7.8 Hz, 2H), 7.41(d, J =7.8 Hz, 1H), 7.78(d, J =8.4 Hz, 2H).

5.3.24. *N*-(4-*t*-butylbenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (33)

triester: ^1H NMR (400 MHz, CDCl_3): δ =1.30(s, 9H), 3.31 (s, 2H), 3.67(s, 3H), 3.77 (s, 2H), 3.87 (s, 2H), 3.88–3.91 (s, 6H), 7.27 (d, J =8 Hz, 2H), 7.34 (d, J =8 Hz, 2H), 7.59(d, J =8 Hz, 1H), 7.61(d, J =7.6 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 464.3, (calc) 464.5.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 1.26 (s, 9H), 3.01 (s, 2H), 3.73 (s, 4H), 3.66 (s, 2H), 7.16 (m, J =8.4 Hz, 2H), 7.28–7.31 (m, 3H), 7.34 (s, 1H), 7.41–7.43 (d, J =7.8 Hz, 1H), 7.44–7.46 (d, J =7.8 Hz, 2H).

5.3.25. *N*-(4-(2-perfluoropropyl))-*N*-carboxymethyl-3,4-dicarboxybenzylamine (34)

triester: ^1H NMR (600 MHz, CDCl_3): δ = 3.30 (s, 2H), 3.66(s, 3H), 3.84 (s, 2H), 3.87–3.88 (m, 8H), 7.47 (m, J =7.8 Hz, 2H), 7.52 (m, J =8.4 Hz, 2H), 7.56(d, J =7.8 Hz, 1H), 7.69(m, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 575.9, (calc) 576.4.

sodium salt: ^1H NMR (600 MHz, D_2O): δ = 3.07 (s, 2H), 3.77(s, 2H), 3.83 (s, 2H), 7.33–7.34 (m, 1H), 7.39 (s, 1H), 7.45(d, J =7.8 Hz, 1H), 7.54(d, J =7.8 Hz, 1H), 7.68(d, J =7.8 Hz, 2H).

5.3.26. *N*-(4-bromobenzyl)-*N*-carboxyethyl-3,4-dicarboxybenzylamine (35)—4-bromo-benzyl bromide was added to a solution of compound **4Me**, followed by hydrolysis of the ester product as described above to yield compound **35**.

triester: ^1H NMR (600 MHz, CDCl_3): δ =2.46 (t, 2H), 2.74 (t, 2H), 3.47 (s, 3H), 3.56 (s, 2H), 3.59 (s, 3H), 3.86 (s, 3H), 3.88 (s, 3H), 7.13 (d, J =7.8Hz, 2H), 7.40 (d, J =7.8Hz, 2H), 7.46 (d, J =7.8Hz, 1H), 7.59 (s, 1H), 7.66 (d, J =7.8Hz, 1H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ (isotopic) 500.1, 501.9 (calc) (isotopic) 500.1, 502.1.

sodium salt: ^1H NMR (600 MHz, CDCl_3): δ =2.43 (t, 2H), 2.73 (t, 2H), 3.54 (s, 2H), 3.59 (s, 2H), 7.19 (d, J =7.8Hz, 2H), 7.27–7.31 (m, 2H), 7.41 (d, J =7.2Hz, 1H), 7.48 (d, J =7.8Hz 2H).

5.3.27. *N*-(4-trifluoromethylbenzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (36)—4-trifluoromethylbenzyl bromide was added to a solution of compound **4Me**, followed by hydrolysis of ester product as described above to yield compound **36**.

triester: ^1H NMR (600 MHz, CDCl_3): δ =2.48 (t, 2H), 2.77 (t, 2H), 3.58 (s, 2H), 3.59 (s, 2H), 3.60 (s, 3H), 3.86 (s, 3H), 3.88 (s, 3H), 7.40 (d, J =7.8Hz, 2H), 7.49 (d, J =7.8Hz, 2H), 7.52 (d, J =7.8Hz, 1H), 7.61 (s, 1H), 7.66 (d, J =7.8Hz, 1H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 490.2, (calc) 490.4.

sodium salt: ^1H NMR (600 MHz, CDCl_3): δ = 2.44 (t, 2H), 2.75 (t, 2H), 3.61 (s, 2H), 3.64 (s, 2H), 7.33 (d, J = 7.8 Hz, 2H), 7.41–7.45 (m, 3H), 7.64 (d, J = 7.8 Hz, 2H).

5.3.28. *N*-acetal-*N*-carboxymethyl-3,4-dicarboxybenzylamine (37)—To a 0.8 ml solution of compound **3Me** (0.15 g, 0.5 mmol) in formic acid 0.8 ml of acetic anhydride was added dropwise and the reaction mixture was refluxed at 100 °C for 1 h. The reaction mixture was cooled to r. t. and then concentrated by vacuum and diluted with DCM. The DCM portion was washed 4 times with 1 M NaHCO_3 and twice with water and dried over anhydrous Na_2SO_4 . The concentrated product was dissolved in 0.4 ml of THF and the esters were hydrolyzed as described above to yield compound **37**.

triester: ^1H NMR (600 MHz, CDCl_3): δ = 3.64 (s, 3H), 3.83 (s, 3H), 3.84 (s, 3H), 3.88 (s, 2H), 4.53–4.59 (m, 2H), 7.34 (d, J = 6.6 Hz, 1H), 7.49 (d, J = 8.4 Hz, 1H), 7.63–7.69 (m, 1H), 8.12–8.29 (m, 1H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 364.4, (calc) 364.3.

sodium salt: ^1H NMR (400 MHz, D_2O): δ = 3.0 (s, 2H), 3.55 (s, 2H), 7.15–7.19 (m, 2H), 7.27–7.29 (d, J = 8 Hz, 1H), 8.26 (s, 1H).

5.3.29. *N*-((4-(2-benzyl)vinyl)benzyl)-*N*-carboxymethyl-3,4-dicarboxybenzylamine (38)—Compound **20** (0.48 g, 1.03 mmol) was weighed in a vial and Bis(tri-*tert*-butylphosphine)palladium(0)^{22,23} (0.1 g, 0.19 mmol) was added under inert conditions in a nitrogen bag to the sealed vial. Then, at r. t. and under inert conditions, was sequentially added 3 ml of anhydrous toluene, *N*-cyclohexyl-*N*-methyl cyclohexylamine (0.27 ml, 1.24 mmol) and styrene (0.17 ml, 1.44 mmol). The reaction mixture was allowed for stirring in a microwave reactor at 110 °C for 16–18 h. Water was added to the reaction mixture, followed by extraction with several portions of diethyl ether, dried over anhydrous sodium sulfate and concentrated in vacuum. The product was purified by column chromatography using 0–15% ethyl acetate:hexanes. The resulting ester product was hydrolyzed as described above (section 5.3) to yield compound **38**.

triester: ^1H NMR (600 MHz, CDCl_3): δ = 3.3 (s, 2H), 3.67 (s, 3H), 3.78 (s, 2H), 3.86 (s, 2H), 3.88–3.90 (s, 6H), 7.08 (m, 2H), 7.25 (m, 1H), 7.32–7.35 (m, 4H), 7.45 (d, J = 7.8 Hz, 2H), 7.50 (d, J = 7.8 Hz, 2H), 7.60 (d, J = 7.8 Hz, 2H), 7.71 (d, J = 8.4 Hz, 2H). MS (ESI) m/z : (obs) $[\text{M}+\text{Na}]^+$ 510.2, (calc) 510.5.

sodium salt: ^1H NMR (400 MHz, D_2O): δ = 3.03 (s, 2H), 3.75 (s, 2H), 3.76 (s, 2H), 7.22 (m, 2H), 7.28–7.34 (m, J = 7.2 Hz, 5H), 7.38–7.43 (m, J = 7.8 Hz, 3H), 7.55–7.59 (d, J = 7.8 Hz, 4H).

5.4. Enzyme purification and kinetic assay

ASADHs from *S. pneumoniae* and *V. cholerae* were cloned, expressed and purified following our published procedure.²⁴ The concentrated enzymes were stored at -20 °C in a storage buffer containing 50 mM HEPES (pH 7), 1 mM EDTA and 1 mM DTT. Because of the instability of aspartyl phosphate the reaction was followed in the reverse direction, with the increase in the absorbance of NADPH monitored at 340 nm. Initial velocity kinetics of

ASADH was carried out at r.t. in a reaction buffer composed of 120 mM CHES, pH 8.6 and 200 mM KCl, with ASA at either 0.15 mM (*sp*ASADH) or 0.3 mM (*vc*ASADH), 1.5 mM NADP and 20 mM phosphate in a final volume of 200 μ l. Enzyme (30 μ g ml⁻¹) was added to each well to initiate the reaction. To determine the inhibition constant (K_i) of each compound, inhibitor was added to each well by serial dilution to cover a suitable concentration range. The measured initial velocities were then fitted to a Dixon plot²⁵ that assumes competitive inhibition against ASA to determine the K_i value for each inhibitor.

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Abbreviations

AdoMet	S-adenosyl-L-methionine
ASA	aspartate semialdehyde
DCM	dichloromethane
FBDD	fragment-based drug discovery
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-sulfonic acid
NBS	N-bromosuccinimide
<i>sp</i>ASADH	<i>Streptococcus pneumoniae</i> aspartate semialdehyde dehydrogenase
<i>vc</i>ASADH	<i>Vibrio cholerae</i> aspartate semialdehyde dehydrogenase

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Scheme 1. Synthetic Route to 4-Aminomethylphthalate Derivatives ^a

^a Reagents and conditions: (a) H₂SO₄/MeOH, μ wave, 80 °C, 8 h; (b) NBS/DCM, light, 3 h; (c) NaHCO₃/DMF, r.t., 8 h; (d) 1 N NaOH, r.t., 3 h.

Scheme 2.**Synthetic Route to 3-Aminomethylphthalate Derivatives ^a**

^a Reagents and conditions: (a) H₂SO₄/MeOH, μ wave, 80 °C, 8 h; (b) NBS/DCM, light, 3 h; (c) NaHCO₃/DMF, r.t., 8 h; (d) 1 N NaOH, r.t., 3 h.

Table 1

Synthesis and kinetic evaluation of 4-aminomethylphthalate derivatives

compd	R ₁	R ₂	K _i (μM)	
			<i>sp</i> ASADH	<i>vc</i> ASADH
3	H	H	246 ± 20	528 ± 32
4 ^a	H	H	2400 ± 180	n.i. ^b
5	(<i>S</i>)-CH ₃	H	296 ± 26	609 ± 23
6	(<i>S</i>)-CH ₂ OH	H	324 ± 24	654 ± 22
10	H	CH ₃	296 ± 36	675 ± 54
11	H	allyl	303 ± 22	663 ± 36
12	H	propionitrile	220 ± 14	498 ± 27
13	H	benzyl	297 ± 14	696 ± 33
14	H	1-naphthyl	396 ± 42	749 ± 54
15	H	2-naphthyl	329 ± 22	724 ± 47
16	H	<i>N</i> -ethylmorpholino	176 ± 22	692 ± 39
17	H	4-biphenyl	12 ± 1.2	634 ± 29
37	H	acetaldehyde	276 ± 16	655 ± 51

^a *N*-carboxylmethyl group replaced with *N*-carboxyethyl^b no inhibition observed at concentrations up to 2 mM

Table 2Synthesis and kinetic evaluation of 4-(*N*-substituted)benzylaminoalkylphthalates

compd ^a	R	<i>K_i</i> (μM)	
		<i>sp</i> ASADH	<i>vc</i> ASADH
18	2-bromo	529 ± 38	737 ± 49
19	3-bromo	442 ± 29	665 ± 37
20	4-bromo	57 ± 5	629 ± 53
21	2-methyl	524 ± 27	721 ± 46
22	3-methyl	430 ± 22	690 ± 41
23	4-methyl	63 ± 7	608 ± 42
24	2-trifluoromethyl	509 ± 29	727 ± 60
25	3-trifluoromethyl	441 ± 27	678 ± 36
26	4-trifluoromethyl	24 ± 3	628 ± 48
27	2-trifluoromethoxy	540 ± 32	708 ± 47
28	3-trifluoromethoxy	427 ± 31	684 ± 35
29	4-trifluoromethoxy	36 ± 6	663 ± 39
30	4-difluoromethoxy	54 ± 5	698 ± 44
31	4-carboxy	72 ± 7	635 ± 55
32	4-carboxamide	229 ± 32	649 ± 25
33	4- <i>tert</i> -butyl	16 ± 2	648 ± 32
34	4-(2-perfluoropropyl)	9 ± 0.2	639 ± 37
38	4-((2-benzyl)vinyl)	476 ± 52	1100 ± 200
35*	4-bromo	3600 ± 230	n. i. ^b
36*	4-trifluoromethyl	3800 ± 220	n. i. ^b

^a n = 1 for all entries, except * where n = 2^b no inhibition observed at concentrations up to 4 mM