Department of Chemistry[1] and Department of Biology[2], University of Saskatchewan, Canada

Synthesis and antifungal properties of compounds which target the α-aminoadipate pathway

D. R. J. Palmer,[1] H. Balogh,[1] G. Ma,[1] X. Zhou,[1] M. Marko,[1] S. G. W. Kaminskyj[2]

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Susan G. W. Kaminskyj, Department of Biology, University of Saskatchewan, 112 Science Place, Saskatoon, Saskatchewan Canada S7N 5E2.
Susan.Kaminskyj@usask.ca

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Fungi synthesize lysine via the α-aminoadipate pathway, which is not found in plants or animals. This pathway has been proposed as a target for antifungal agents, but until now no reports have appeared to test this proposal. Hampering studies on the susceptibility of filamentous fungi such as those of the clinically important genus *Aspergillus* is the fact that growth quantitation is notoriously difficult. We have used the recently-reported XTT-based method of biomass quantitation to measure the susceptibility of *Aspergillus nidulans* strain A28 to growth suppression by novel compounds designed to target early steps in the α-aminoadipate lysine biosynthesis pathway, specifically those steps involving (*R*)-homocitrate and (2*R*,3*S*)-homoisocitrate. Three compounds show moderate inhibition of fungal growth, which can be partially restored by the presence of lysine in the growth medium.

1. Introduction

Invasive fungal infections are a severe threat to human health: untreated, mortality rates from systemic fungal infections typically exceed 98%. Fungal spores are ubiquitous; for example, there are 1–100 pathogenic *Aspergillus fumigatus* spores per m^3, worldwide (Latgé 2001). Our main defense against systemic fungal infections is a healthy immune response; thus it is not surprising that these are the primary AIDS-defining infections in HIV-positive people (Walsh and Groll 1999), and that they are the scourge of other immunocompromised individuals including transplant patients and those with autoimmune disease.

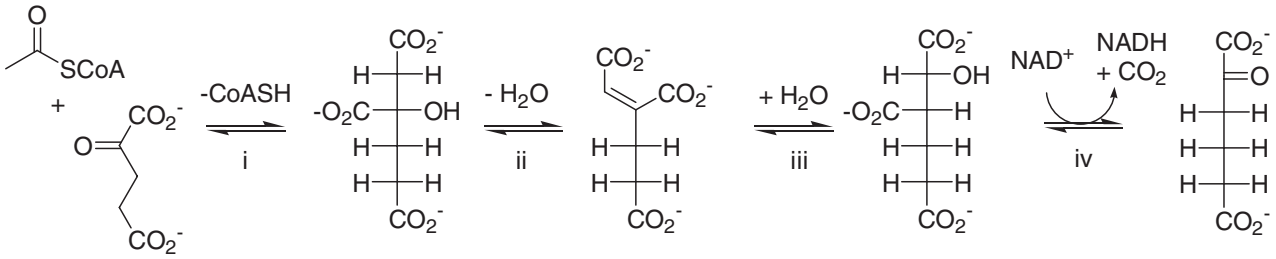
The treatment for systemic fungal infections can be toxic in its own right (Ugur et al. 2002), because most of these drugs target ergosterol or its biosynthesis, and so also affect cholesterol, which is an essential component of mammalian membranes. These treatments are expensive, averaging in excess of US$30,000 per patient per incident (Wilson et al. 2002). Despite intensive treatment with currently-available antifungal agents, permanent cures are uncommon, such that survival/cure rates after systemic fungal infection are often expressed only for the following six-month period, and many patients remain on long-term prophylactic antifungal therapy. In addition, fungi are also severe agricultural pests, causing damage to all crops (e. g. Barnes 1979, Howlett et al. 2001) and stored agricultural products. These facts, coupled with increasing incidence of antibiotic (Alexander and Perfect 1997) and antifungal (Loeffler and Stevens 2003; Vanden Bossche et al. 2003) resistance indicate that new antimicrobial strategies should be pursued constantly.

All higher fungi synthesize lysine via the α-aminoadipate pathway (Zabriskie and Jackson 2000), which is not found in plants or animals. This pathway could therefore serve as a target for antifungal compounds since its disruption should not affect the metabolism of host organisms (Suvarna et al. 1998). However, no studies have appeared reporting the use of this target for the design of antifungal compounds. We now report the synthesis of a series of compounds that target enzymes from the α-aminoadipate pathway, and an investigation of their antifungal behavior.

The early steps of the α-aminoadipate pathway are shown in Scheme 1. While all the metabolites are known, not all of the enzymes responsible for these transformations have been identified (Zabriskie and Jackson 2000). Specifically, the conversion of (*R*)-homocitrate to *cis*-homoaconitate has not been assigned to a gene product. It may be that (*R*)-homocitrate is converted to (2*R*,3*S*)-homoisocitrate by a single enzyme, *in simile* with the reaction catalyzed by aconitase shown in Scheme 2. Indeed, homoaconitate hydratase, or homoaconitase, is clearly related to aconitase in sequence, and most likely in structure (Irvin and Bhattacharjee 1998). However, neither the homoaconitate hydratase from *Saccharomyces cerevisiae* (LYS4) nor *Aspergillus nidulans* (LysF) has been reported to catalyze the dehydration of (*R*)-homocitrate (Gamonet and Laquin 1998, Weidner et al. 1997). This somewhat counter-intuitive phenomenon is a subject of interest in our laboratory.

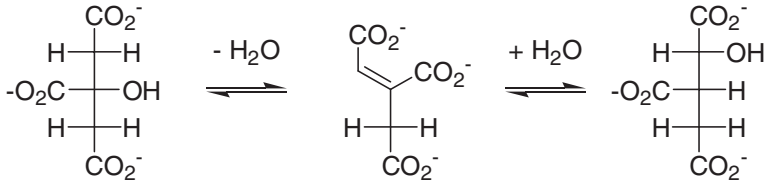
Regardless of the identity of the catalyst that dehydrates (*R*)-homocitrate, a rational approach to the inhibition of steps of the pathway involving (*R*)-homocitrate and (2*R*,3*S*)-

Scheme 1



The first steps of the α-aminoadipate pathway:
i.) homocitrate synthase; ii.) unidentified enzyme; iii.) homoaconitate hydratase (or homoaconitase); iv.) homoisocitrate dehydrogenase

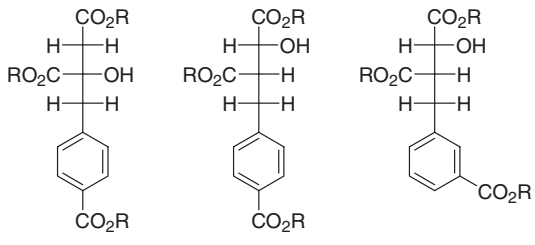
Scheme 2



The reaction sequence catalyzed by aconitase

homoisocitrate can be taken. Specifically, compounds designed to mimic the structure of the metabolites of interest could inhibit homoaconitase and/or homoisocitrate dehydrogenase, resulting in an impairment in lysine biosynthesis. One of the considerations in the design is that the compounds *not* be effective aconitase inhibitors: such compounds are extremely toxic (Peters and Shorthouse 1971, Lauble et al. 1996). Therefore the difference between substrates for aconitase and homoaconitase, namely the presence of a propionate rather than an acetate moiety, should be retained or even exaggerated in any putative antifungal.

Fungal species employ two major growth morphologies: they form colonies of single cells called yeasts, or produce multicellular filamentous mycelia. Some human fungal pathogens, such as *Candida albicans,* are dimorphic, invading as filamentous hyphae but proliferating as yeasts (Gale et al. 1998), whereas others like the molds, *Aspergillus*, are exclusively filamentous (Latgé 2001). Fungi like *Aspergillus* grow by tip growth and form basal cell-like compartments by crosswall insertion (Hamer et al. 1999) creating multicellular colonies. Basal cells in these colonies are quiescent for growth but metabolically active, and reinitiate growth for normal branching or in response to mechanical damage to the mycelium. This leads to a considerable difficulty in accurately estimating viability after anti-fungal treatments because tip growth assays do not accurately reflect the number of potentially viable cells. A colorimetric assay to quantify fungal growth has been developed by Meletiadis *et al*. (2001) that they report correlates well with visible mycelial growth in several *Aspergillus* species.



1a: R = H
1b: R = Me

2a: R = H
2b: R = Me

3a: R = H
3b: R = Me

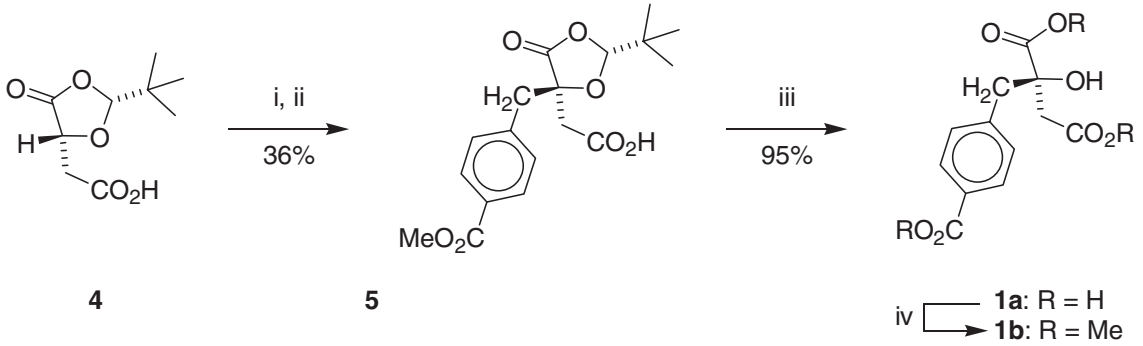
A variety of carboxyalkyl- and carboxyaryl-substituted D-malic acids and their corresponding methyl esters were designed as analogues of (*R*)-homocitrate and (2*R*,3*S*)-homoisocitrate. Syntheses of these compounds rely on a diastereoselective alkylation to give an optically active product. These compounds were tested for their ability to impair the growth of *Aspergillus nidulans* A28 in minimal media (i.e. in the absence of exogenous lysine) and in rich media supplemented with excess lysine. Of these, only methyl esters **1b–3b** showed inhibitory effects on fungal growth.

2. Investigations and results

2.1. Chemistry

As described previously, we have applied the Self-Regenerating Stereocentre (SRS) approach (Seebach et al. 1996) to the synthesis of (*R*)-homocitrate (Ma and Palmer 2000). This approach uses the steric hindrance of the *tert*-butyl group of **4** to control the face of alkylation of the planar enolate intermediate. In this approach, the electrophile added was eventually modified to form the propionate moiety of homocitrate, thus substitution of other electrophiles was an obvious extension of the technique, as illustrated in Scheme 3. Because an equally large or larger moiety was desired in this position, substituted methyl *ortho*-, *meta*-, and *para*-(bromomethyl)benzoates were chosen as electrophiles. The presence of the electron-withdrawing methyl carboxylate substituent would be predicted to result in a less reactive electrophile relative to benzyl bromide, and this was observed in our work. The *para*-substituted compound gave the desired product **5** in moderate yield (36%), about half that obtained when benzyl bromide was used (Seebach et al. 1984). The *ortho*-substituted compound gave the desired product in very low yield (ca. 1%), and reaction with the *meta*-substituted compound resulted in no product in our hands. As reported by Seebach et al. (1984), high stereoselectivity was obtained; the presence of the undesired diastereomer could

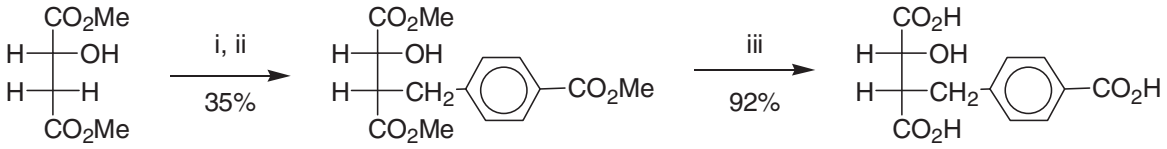
Scheme 3



4 **5**

i.) LiHMDS/THF, methyl *para*-(bromomethyl)benzoate, −78 °C, 3 hr. ii.) 1 N HCl. iii) 50% aqueous TFA, rt, 6 hr. iv.) CH_2N_2 /DMF, 10 min

Scheme 4



2b **2a**

i.) LDA/THF, methyl *para*-(bromomethyl)benzoate, −78 °C, overnight. ii.) 1 N HCl. iii.) 2 N aqueous NaOH, rt, 30 min

not be detected by proton NMR. Hydrolysis of **5** gave the target **1a** in good yield. Subsequent methylation to **1b** with diazomethane proceeded in essentially quantitative yield.

We have reported the synthesis of (2*R*,3*S*)-homoisocitrate by a stereoselective alkylation of dimethyl D-malate (Ma and Palmer 2000), and the same methodology was applied to the preparation of homoisocitrate analogues as shown in Scheme 4. Again, the alkylation using the methyl ester of *para*-(bromomethyl)benzoate gave the desired product **2b** in moderate yield (35%), and the *meta*-substituted electrophile reacted in much lower yield. Hydrolysis to the target carboxylic acids proceeded in >90% yield.

2.2. Fungal susceptibility

Aspergillus nidulans growth was inhibited by compounds **1b**, **2b** and **3b** in a lysine-dependent manner. Esterification was necessary for efficient drug uptake since compounds **1a**, **2a** and **3a** did not have growth inhibitory properties (data not shown). Esterification facilitates uptake of other compounds in higher fungi (Pitt and Barnes 1998; Slaymann et al. 1994), after which a cytoplasmic esterase unmasks the effective compound. Uptake may also be reduced for the acidic forms of these drugs because charged compounds stick to the fungal cell wall and are less efficient at transmembrane uptake in the absence of specific transporter proteins.

Qualitative assessments of visible growth inhibition by compounds **1b, 2b** and **3b** are shown in Fig. 1. Here, replicate colonies of *A. nidulans* were grown from spores in complete medium containing 1% DMSO and 0–10000 μM of each of these compounds. Figure 1A shows the macroscopic colony appearance at 39 h after inoculation, which includes both floating and submerged mycelia. Figure 1B shows the submerged mycelia growing on the bottom of the wells in the 10 mM drug treatments at 24 h after inoculation.

Qualitative results (Fig. 1) were comparable to those obtained with the XTT bioassay (Fig. 2). Due to obvious differences in fungal growth on the complex versus minimal media, critical estimates of compound effectiveness used minimal medium with or without added lysine. To facilitate quantitative comparison of growth on these two defined media, the data in Fig. 2 are normalized with respect to the inoculated-no drug control values. Fifty percent lysine-dependent growth inhibition with compounds **1b**, **2b** or **3b** was typically achieved at about 3 mM drug concentration. Higher levels of drug had additional toxic effects that could not be rescued by exogenous lysine. In contrast, 50% growth inhibition required 1 μM amphotericin B (data not shown). The compounds showed no apparent inhibition of growth of *E. coli*.

3. Discussion

The most notable result of this study is that a rational drug design strategy to inhibit fungal growth in a lysine biosynthesis-dependent manner by generating putative inhibitory compounds has shown proof-of-principle: this is the first report of the development of antifungal compounds that target the α-aminoadipate pathway. It is somewhat surprising that no such reports have appeared before, since this pathway was proposed as a target for the selective control of fungal growth more than ten years ago (Garrad and Bhattacharjee 1992). Perhaps this pathway has been avoided since not all of the steps are completely understood. Indeed, the mystery surrounding the conversion of (*R*)-homocitrate to *cis*-homoaconitate remains, but there is a limited number of expected mechanisms for the dehydration of such a substrate, and therefore inhibitors of this reaction should be accessible. In fact, the discovery of such an inhibitor could lead to identification of the gene product responsible for this transformation. We can envision a mutant screen for colonies that fail to be inhibited by these compounds, followed by cloning the relevant gene by complementation.

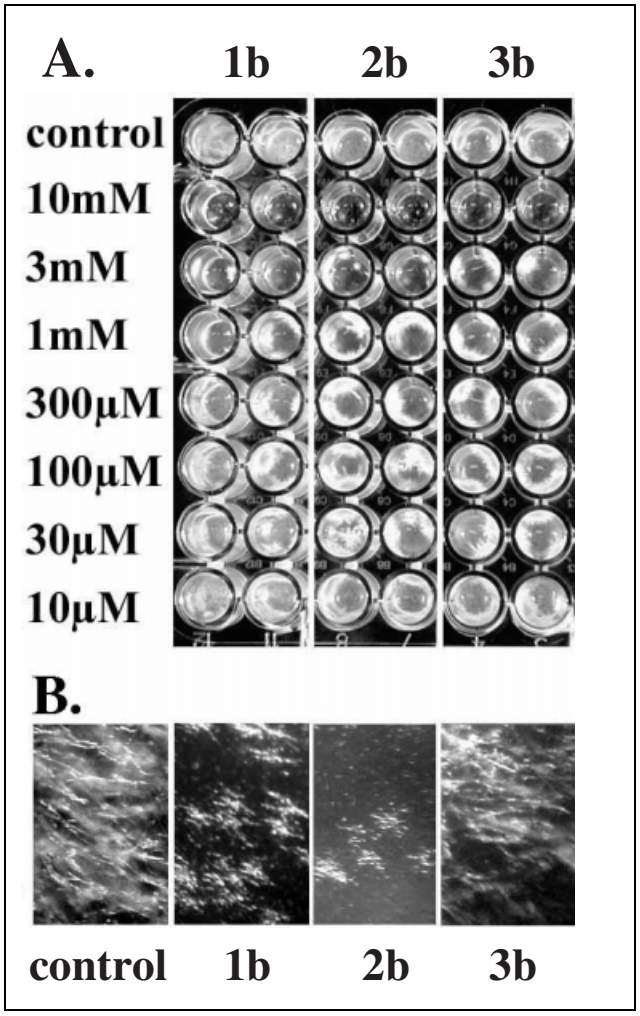


Fig. 1: Qualitative assessment of growth of *Aspergillus nidulans* in CM containing compounds that target the α-aminoadipate pathway. **A**. Qualitative assessment of growth at 39 h after inoculation in a 24-well culture plate. Abundant floating and submerged mycelium is seen in the control and 10 µM–1 mM drug concentrations where there is little inhibition. The relatively dark background in the 10 mM wells is due to sparseness of hyphae growing attached to the bottom of the well. **B**. Representative micrographs of hyphae at 24 h after inoculation growing on the bottom of the wells in the control and 10 mM drug treatments

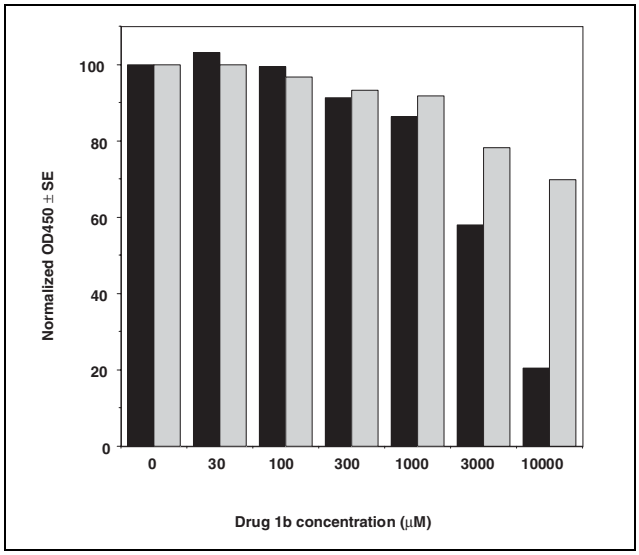


Fig. 2: Quantitative assessment of growth of *Aspergillus nidulans* in MM (dark bars) and CM (light bars) in 0–10000 µM compound **1b** in 1% DMSO, assayed at 24 h after inoculation by production of XTT reduction product. Normalized data, averaged from six replicates for each medium/drug concentration in a single 96-well microtiter plate are presented since growth in the absence of drug is different in MM *versus* MM = lysine

Using stereoselective synthesis, analogues of intermediates in the lysine biosynthetic pathway were synthesized, and their methyl esters shown to inhibit the growth of *A. nidulans* in a lysine-dependent manner. Fifty-percent growth inhibition could only be achieved at relatively high concentrations; nevertheless this may represent a promising first step in the development of antifungal agents. The restoration of growth in the presence of lysine, and the lack of antibacterial activity, supports our conclusion that these compounds have inhibited the α-aminoadipate pathway. The ability of fungi to import lysine may limit the utility of this pathway as a target, but we have demonstrated that growth can be reduced when lysine synthesis is impaired. This approach may be more useful in combination with other therapies.

Unlike the report of Meletiadis et al. (2001), in our experience the quantitative XTT assay results were somewhat variable, although they were always consistent with the qualitative growth estimates. "No-drug" control values between experiments on different days could vary up to 20%, although within a single experiment they provided consistent trends. This suggests that minor variations in the XTT assay protocol might be contributing factors: age of spores used (typically less than a week from harvest), age of cultures being tested (24 h ± 30 min), and mixing and incubation time in the XTT assay mix. Furthermore, even though all spores were inoculated into the liquid medium, by 24 h incubation there was usually some surface growth on the less inhibited treatments. These cultures had often begun aerial, asexual spore development in preference to submerged vegetative hyphal growth (which is more relevant to disease progress). The XTT assay addresses cellular metabolic activity, but inhibited metabolism is not necessarily equivalent to cell death. As noted previously, quantitative assays of viability of filamentous fungal mycelium are notoriously difficult. In future we suggest assaying fungal colonies grown fully submerged in shake cultures (for example in individual Eppendorf tubes to minimize colony volumes), where sporulation is inhibited.

4. Experimental

4.1. Microbiology

4.1.2. Aspergillus nidulans strain and growth conditions

Aspergillus nidulans strain A28 (FGSC, www.fgsc.net) was grown on complete medium (CM) as described in Kaminskyj (2001) at 37 °C. All media contained *p*-aminobenzoic acid and biotin (both Sigma, www.sigma.com) to supplement auxotrophies in A28, and were buffered to pH 6.5 using NaOH. Assays used: fully-defined minimal medium (MM), MM plus lysine (185 mg/L; Sigma), or CM plus lysine.

4.1.3. Solvent testing

Control tests for solvent effects on *A. nidulans* growth used 0–10% v/v acetone, ethanol, methanol, and DMSO. Initial studies used qualitative growth assessment, which were later confirmed quantitatively with the XTT bioassay. 1% DMSO was chosen to minimize growth effects on the fungus in control studies, for compatibility with future testing on animal models, and to ensure drug solubility.

4.1.4. Drug testing

For XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide, (sigma www.sigma-aldrich.com) bioassays, 5 M stock solutions of **1a, 3a, 1b, 2b**, and **3b** were prepared in 100% DMSO. Hundred-fold dilutions in sterile distilled water gave 50 mM drug stocks in 1% DMSO. Then, 5 mM and 500 µM drug stocks were diluted from the 50 mM drug stocks using 1% DMSO. Drug stock solutions and 1% DMSO were mixed to final drug concentrations of 0–20000 µM in 100 µL. Finally, 100 µL of

water was added as an inoculation control, or 100 µL of *A. nidulans* spore suspension in distilled water was added for all other treatments. Growth assays for quantitative XTT studies used 1000 conidia and 200 µL medium per well, in sterile flat-bottomed 96-well microtitre dishes. Qualitative, macroscopic growth assays used 5000 spores in 1 mL of medium in 24-well sterile flat-bottomed culture dishes.
An amphotericin B control study was prepared using drug concentrations of 0.3–300 µg/mL. Plates were inoculated and assayed as described above.

4.1.5. XTT Method of quantitative susceptibility assessment

Fungal metabolic activity was determined colorimetrically using XTT reduction as described by Meletiadis et al. (2001), assayed at 450 nm on a microtitre plate using a kinetic microplate reader from Molecular Devices. XTT reduction values from uninoculated control media were averaged by medium type and subtracted from the respective experimental values before experimental values were averaged. Where appropriate, averaged experimental values were normalized with respect to inoculated, no-drug control replicates. Statistical analyses on data before normalization used Statview SE + Graphics v1.02 (www.statview.com), presented with Cricket Graph 1.3.1 (cjs.cadmus.com). Results are presented as mean ± standard error of the mean (SE).

4.2. Chemistry

4.2.1. General methods

NMR spectra were collected on a Bruker AMX300; chemical shift are reported in ppm downfield of TMS. MS were recorded using a VG 70SE mass spectrometer. All melting points are uncorrected.

*4.2.2. (2R,5R)-2-(tert-Butyl)-5-(carboxymethyl)-5-para-(methylcarboxy)-benzyl-1,3-dioxolan-4-one (**5**)*

The procedure for SRS alkylation (Seebach et al. 1984) was followed. A solution of hexamethyldisilazane (4.5 ml, 21 mM) in anhydrous THF (15 ml) was cooled to −78 °C, and butyllithium (2.5 M, 8 ml, 20 mmol) was added slowly and stirred at −78 °C for 20 min. 2-*tert*-Butyl-5-(carboxymethyl)-1,3-dioxolan-4-one (2.02 g, 10 mmol) was added and the resulting solution stirred at −78 °C for 1 h, after which methyl *para*-(bromomethyl)benzoate (3.44 g, 15 mmol) was added. This solution was stirred at −78 °C for 1 h, then −20 °C for 1 h. The reaction was quenched with HCl (1N, 100 ml) and extracted into CH_2Cl_2 (3 × 150 mL). The crude product was concentrated by rotary evaporation, and isolated by silica gel column chromatography using hexane : EtOAc : HOAc (60 : 12 : 1), yielding compound **5** (1.27 g, 36%). M.p.: 164–165 °C, $[M + 1]^+$: $C_{18}H_{23}O_7$ calculated, 351.1444; found 351.1444. $[\alpha]_D^{25} = -48.37$ (1.02, $CHCl_3$). ^{1}H NMR (dmso-d_6): δ: 7.93 (2 H, d, J = 8.2 Hz, *ortho*-phenyl), 7.42 (2 H, d, J = 8.2 Hz, *meta*-phenyl), 5.27 (1 H, s, H-2), 3.84 (3 H, s, CO_2CH_3), 3.35 (1 H, d, J = 13.6 Hz, $C\underline{H}_2CO_2H$), 3.02 (1 H, d, J = 13.6 Hz, $C\underline{H}_2CO_2H$), 2.92 (1 H, d, J = 16.3 Hz, benzyl), 2.47 (1 H, d, J = 16.3 Hz, benzyl), 0.88 (9 H, s, *t*-butyl). ^{13}C NMR δ: 173.1 ($CH_2\underline{C}O_2H$), 169.5 (benzoate-$\underline{C}O_2Me$, 166.1 (C-4), 140.2 (*ipso*-phenyl), 131.0 (*para*-phenyl), 129.0 (*meta*-phenyl), 128.5 (*ortho*-phenyl), 106.7 (C-2), 79.9 (C-5), 52.1 ($CO_2\underline{C}H_3$), 37.6 ($\underline{C}H_2CO_2H$), 33.7 ($C(\underline{C}H_3)_3$), 23.4 ($\underline{C}(CH_3)_3$).
($C_{18}H_{23}O_7$) (351.1)

4.2.3. (R)-2-para-Carboxybenzylmalic acid (**1a**)

Compound **5** (150 mg) was hydrolyzed at 100 ºC in aqueous 50% 2,2,2-trifluoroacetic acid (10 mL) for 6 h, yielding 110 mg (95%) of crystalline product. M.p. 202–203 °C, $[\alpha]_D^{25} = -9.67$(0.3, MeOH). ^{1}H NMR (CD_3OD) δ: 7.93 (2 H, d, J = 8.2, *ortho*-phenyl), 7.59 (2 H, d, J = 8.2, *meta*-phenyl), 3.03 (3 H, m, H-2, benzyl), 2.62 (1 H, d, J = 16.2, benzyl). ^{13}C NMR δ: 177.3 (C-4), 174.0 (C-1), 170.3 (benzoate-$\underline{C}O_2H$), 142.8 (*ipso*-phenyl), 131.9 (*para*-phenyl), 130.6 (*ortho*-phenyl), 130.5 (*meta*-phenyl) 76.7 (C-3), 46.2 (C-2), 44.2 (benzyl).
($C_{12}H_{12}O_7$)

4.2.4. (R)-2-p-carboxybenzylmalic acid trimethyl ester (**1b**)

Compound **1a** (0.021 g) in 2.0 ml DMF was reacted with diazomethane at room temperature for 10 min. The solvent was evaporated to yield **1b** (0.018, 96%). ^{1}H NMR ($CDCl_3$) δ = 7.94 (2 H, d, J = 8.1 Hz, *ortho*-phenyl), 7.25 (2 H, d, J = 8.1 Hz, *meta*-phenyl), 3.89 (s, 3 H, methyl), 3.72 (s, 3 H, methyl), 3.65 (s, 3 H, methyl), 3.05 (1 H, d, J = 13.3 Hz, H-2), 3.02 (1 H, d, J = 16.1 Hz, benzyl), 2.98 (1 H, d, J = 13.3 Hz, H-2′), 2.70 (d, 1 H, J = 16.1, benzyl'). $[M + 1]^+$.
($C_{15}H_{19}O_7$) (311.1)

4.2.5. (2R,3S)-3-(para-Carboxybenzyl)malate trimethyl ester (**2b**)

Lithium diisopropylamide (2 M, 15 mL, 30 mmol) was added dropwise to a solution of THF (30 mL) containing dimethyl malate (4.12 g, 14 mmol) at −78 °C. After addition, the temperature was raised to −20 °C for 30 min, cooled back to −78 °C, and methyl *p*-(bromomethyl)benzoate (4.12 g, 18 mmol) in anhydrous THF (10 mL) was added. The resulting solution was stirred at −78 °C overnight, then at −20 °C for 3 h, and quenched with aqueous HCl (1 N, 20 ml). The resulting mixture was extracted with CH_2Cl_2 (3 × 50 ml), the organic phase concentrated by rotary evaporation, and the product isolated by silica gel column using an eluent of benzene : acetone (20 : 1), resulting in an oil (1.52 g, 35%) $[\alpha]_D^{25} = -25.37$ (1.65, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ: 7.91 (2 H, d, J = 8.2 Hz, *ortho*-phenyl), 7.28 (2 H, d, J = 8.2 Hz, *meta*-phenyl), 4.06 (1 H, dd, J = 6.6, 2.8 Hz, H-2), 3.83 (3 H, s benzoate methyl), 3.69 (3 H, s), 3.59 (3 H, s), 3.41 (1 H, d, J = 6.6 Hz, O-H), 3.10–3.25 (2 H, m, H-3, benzyl), 2.87(1 H, dd, J = 15.7, 11.4 Hz, benzyl'). ^{13}C δ: 173.9 (C-1), 172.2 (C-4), 167.1 (benzoate-$\underline{C}O_2Me$, 144.1 (*ipso*-phenyl), 130.1 (*para*-phenyl), 129.4 (*meta*-phenyl), 128.8 (*ortho*-phenyl), 69.8 (C-2), 52.9 (C-3), 52.3 (methyl), 52.2 (methyl), 50.2 (methyl), 33.9 (methyl). $[M + 1]^+$.
($C_{15}H_{19}O_7$ (311.1)

4.2.7. (2R,3S)-3-(para-Carboxybenzyl)malic acid (**2a**)

Compound **2b** (510 mg, 1.6 mmol) was added to aqueous NaOH (2 N, 10 ml) and stirred at RT for 30 min. The solution was treated with Dowex 50 W × 8–100 until the pH was between 2–3, and the resin was removed by filtration, washed with water (2 × 2 mL), and the combined aqueous portions evaporated to give a crystalline product (409 mg, 92.5%), m.p. 184–186 °C, $[\alpha]_D^{25} = -9.95$ (0.70, MeOH). ^{1}H NMR (D_2O) δ: 7.55 (1 H, d, J = 7.9 Hz, *ortho*-phenyl), 7.01 (1 H, d, J = 7.9 Hz, *meta*-phenyl), 3.96 (1 H, d, J = 3.3 Hz, H-2), 3.05 (1 H, ddd, J = 8.4, 7.5, 3.3 Hz, H-3), 2.85 (1 H, dd, J = 13.7, 7.5 Hz, benzyl), 2.66 (1 H, dd, J = 8.4, 13.7 Hz, benzyl′). ^{13}C δ: 178.9 (C-1), 177.8 (C-4), 172.6 (benzoate-$\underline{C}O_2H$, 147.1 (*ipso*-phenyl), 132.4 (*para*-phenyl), 131.6 (*meta*-phenyl), 130.2 (*ortho*-phenyl), 72.2 (C-2), 52.3 (C-3), 35.7 (benzyl).
$C_{12}H_{12}O_7$

4.2.8. (2R,3S)-3-meta-Carboxybenzylmalic acid trimethyl ester (**3b**)

The procedure for alkylation of dimethyl malate (synthesis of **2b**) was followed on an 18 mmol scale using methyl *m*-(bromomethyl)benzoate (9.16 g, 40 mmol) as the electrophile, yielding a solid which, after successive silica gel chromatography steps (hexane: EtOAc (20 : 8); benzene : acetone : HOAc (100 : 5 : 1), twice), gave an oil (383 mg, 7%), $[M + 1]^+$. $[\alpha]_D^{25} = -24.24$ (0.82, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ: 7.87 (1 H, s, *ortho*-phenyl), 7.82 (1 H, d, J = 7.8 Hz, *para*-phenyl), 7. 40 (1 H, d, J = 7.6 Hz, *ortho*-phenyl'), 7.29 (1 H, dd, J = 7.8, 7.6 Hz, *meta*-phenyl), 4.05 (1 H, d, J = 2.6, H-2), 3.82 (3 H, s, methyl), 3.67 (3 H, s, methyl), 3.58 (3 H, s, methyl), 3.16 (2 H, m, benzyl), 2.96 (1 H, dd, J = 15.6, 11.1, H-3). ^{13}C δ: 173.9 (C-1), 172.3 (C-4), 167.2 (benzoate-$\underline{C}O_2Me$), 139.1 (*ipso*-phenyl), 134.18 (*meta*-phenyl), 130.6 (*para*-phenyl), 130.3 (*ortho*-phenyl), 128.8 (*meta*-phenyl'), 128.1 (*ortho*-phenyl′), 69.82 (C-2), 52.8 (C-3), 52.3 (methyl), 52.2 (methyl) 50.4 (methyl), 33.7 (benzyl).
$C_{15}H_{19}O_7$ (311.1)

4.2.9. (2R,3S)-3-m-Carboxybenzylmalic acid (**3a**)

Compound **3b** (0.093 g) was hydrolyzed using aqueous NaOH (2M, 4 ml) for 30 min, then treated with Dowex 50W × 8–100 to pH 1–2, filtered, and the resin washed with water (1 mL) and acetone (1 mL). The combined water and acetone solution was evaporated to dryness, then redissolved in minimal H_2O and passed over a Sephadex LH-20 column eluted with water. Lyophilization resulted in a crystalline solid (0.072 g, 90%) m.p. 180–182 °C, $[\alpha]_D^{25} = -8.19$ (0.28, acetone). ^{1}H NMR (CD_3OD) δ: 7.95 (1 H, s, *ortho*-phenyl), 7.88 (1 H, d, J = 7.7 Hz, *ortho*-phenyl)), 7.51 (1 H, d, J = 7.6 Hz, *ortho*-phenyl'), 7.43 (1 H, dd, J = 7.6, 7.7 Hz, *meta*-phenyl), 4.15 (1 H, d, J = 3.5 Hz, H-2), 3.16 (2 H, m, benzyl), 2.99 (1 H, m, H-3). ^{13}C δ: 176.3 (C-1), 175.4 (C-4), 170.1 (benzoate-$\underline{C}O_2H$), 141.2 (*ipso*-phenyl), 135.1 (*meta*-phenyl), 132.2 (*para*-phenyl), 131.6 (*ortho*-phenyl), 129.8 (*meta*-phenyl'), 129.1 (*ortho*-phenyl'), 71.5 (C-2), 52.2 (C-3), 34.9 (benzyl).

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