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Structure–activity relationships of semisynthetic mumbaistatin analogs

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Abstract—Mumbaistatin (**1**), a new anthraquinone natural product, is one of the most potent known inhibitors of hepatic glucose-6-phosphate translocase, an important target for the treatment of type II diabetes. Its availability, however, has been limited due to its extremely low yield from the natural source. Starting from DMAC (**5**, 3,8-dihydroxyanthraquinone-2-carboxylic acid), a structurally related polyketide product of engineered biosynthesis, we developed a facile semisynthetic method that afforded a variety of mumbaistatin analogs within five steps. This work was facilitated by the initial development of a DMAC overproduction system. In addition to reinforcing the biological significance of the anthraquinone moiety of mumbaistatin, several semisynthetic analogs were found to have low micromolar potency against the translocase in vitro. Two of them were also active in glucose release assays from primary hepatocytes. The synergistic combination of biosynthesis and synthesis is a promising avenue for the discovery of new bioactive substances.

1. Introduction

Synthetic chemistry has proven to be a powerful tool for both constructing and derivatizing bioactive natural products.[1] Despite these advances, de novo synthesis of usable quantities of complex natural products remains a formidable challenge. Truly concise and/or high-yielding total syntheses are rare, which underscores the need for new strategies that combine chemical and biosynthetic efforts. Here, we present an example of marrying synthetic and biosynthetic efforts to a challenging problem of medicinal significance.

Type II diabetes, known as non-insulin-dependent diabetes mellitus (NIDDM), represents over 90% of diabetes patients, and is caused by failure to properly use insulin in the body for glucose homeostasis.[2] Recently, efforts to treat this disease through pharmacological approaches have led to some success in regulating the blood glucose levels,[3–5] although the development of new classes of agents to treat type II diabetes remains a priority. Interference of the hepatic glucose production is an attractive target for the therapeutic control of blood glucose levels. An essential enzyme in the process is the glucose-6-phosphatase (G6Pase) complex, which is located in the membrane of endoplasmic reticulum (ER) in liver cells and is therefore a potential therapeutic target.[6]

As shown in Figure 1, the G6Pase complex is composed of four proteins: three translocases and a phosphatase (G6Pase). G6P generated through gluconeogenesis or glycogenolysis is translocated into the lumen of the endoplasmic reticulum of hepatocytes by translocase 1 (G6P T1). The hydrolytic enzyme, G6Pase, then hydrolyzes the substrate into glucose and phosphate which are exported to the cytosol by two other translocases (T2 and T3).[7,8] Because most sugar phosphates cannot cross ER membrane, G6P translocation by G6P T1 represents the rate determining step for G6P hydrolysis. The critical role of G6P T1 as a key enzyme in the regulation of glucose homeostasis[9] is underscored by the fact that patients with von Gierke's disease, who have a genetic defect in G6P T1, are naturally prone to hypoglycemia.[10,11]

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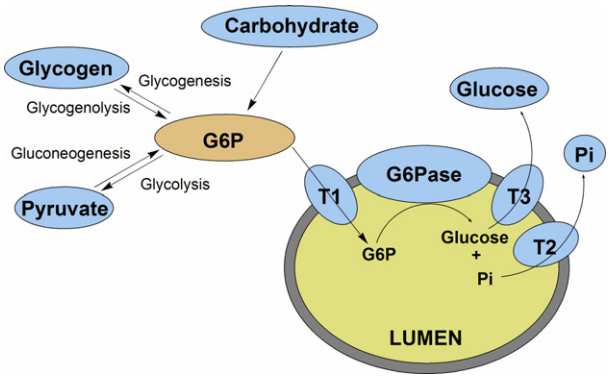

Figure 1. Hepatic glucose production and glucose-6-phosphatase system. Gluconeogenesis and glycogenolysis are two major processes for hepatic glucose production and both involve a common intermediate glucose-6-phosphate (G6P). G6P is finally hydrolyzed to glucose and phosphate at liver by the G6Pase complex. G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; Pi, Phosphate; T1, T2, T3, translocases.

Several non-specific inhibitors of G6Pase complex have been reported, including diethyl pyrocarbonate,[12] phlorizin,[13] and fatty acyl-CoA esters.[14] Complex natural products, such as kodaistatin A (IC_{50} = 80 nM),[15] thielavin (IC_{50} = 0.3–10 μM),[16] CJ-21,164 (IC_{50} = 1.6 μM),[17] and chlorogenic acid (**2**, IC_{50} of 260 μM)[18], have been identified as specific G6P T1 inhibitors. Synthetic analogs of chlorogenic acid, such as S4048 (**3**, IC_{50} = 3 nM), have shown greatly improved potency against G6P T1 (Fig. 2).[19]

Mumbaistatin (**1**, IC_{50} = 5 nM), a recently identified anthraquinone natural product, is the most potent natural G6P T1 inhibitor reported so far.[20,21] It was isolated from cultures of *Streptomyces* sp. DSM 11641, albeit with a very poor yield (70 mg/1000 L culture).[20] A unique structural feature of mumbaistatin is a highly sterically congested tetra-*ortho*-substituted benzophenone moiety.[21] The southern block of the molecule is composed of a 3,8-dihydroxyanthraquinone-2-carboxylic acid, which is attached at C-1 to a 2′,6′-disubstituted benzoyl northern block to form a benzophenone type structure. The diketo form (**1**) of mumbaistatin is not stable and easily cyclized to form a spiroketal lactone (**4**) which loses potency by about three orders of magnitude (Fig. 3a).

We noted that the substituent pattern in the southern block of mumbaistatin is identical to that in 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (DMAC, **5**),[22] an aromatic polyketide produced by a genetically engineered strain of *Streptomyces coelicolor* CH999 (Fig. 3b).[23] Based on this structural similarity, we initially prepared a small number of mumbaistatin analogs modified at C-1 of DMAC by genetic engineering of the aromatic polyketide synthases.[22] Some of these biosynthetic products had extended alkyl chains with 3–6 carbons, yet others had an additional phenolic hydroxyl at C-9. Encouragingly, although DMAC was inactive as a G6P T1 inhibitor, a few analogs showed modest activity (IC_{50} = 100–500 μM). The fact that these analogs were considerably less potent than the natural product suggested that the northern block could potentially be semi-synthetically optimized to obtain analogs with improved activity. In this report, various analogs of mumbaistatin are synthesized from biosynthetic DMAC through chemical modification of methyl group at C-1 position. Some of them have markedly improved activity (IC_{50} ~ 2 μM). This structure–activity relationship analysis of mumbaistatin has therefore yielded a simpler, more stable structure than the natural product for further modification.

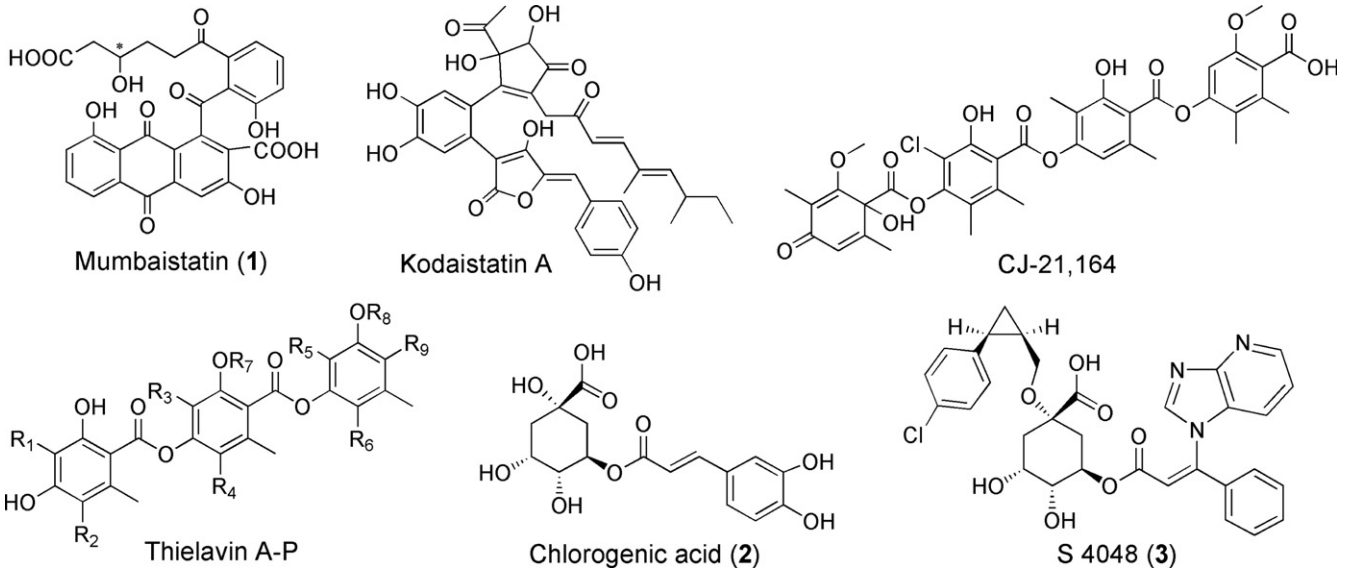

Figure 2. Examples of natural and synthetic inhibitors of G6Pase. Mumbaistatin (**1**) is the most potent known natural inhibitor of G6P T1 with IC_{50} of 5 nM. Kodaistatin A and chlorogenic acid (**2**) are also G6P T1 specific natural inhibitors. CJ-21,164 and thielavin A to P (depending on R groups) are polyphenol natural products and inhibit G6Pase complex. S4048 (**3**) is a synthetic analog of chlorogenic acid which shows the best potency against G6P T1 so far with IC_{50} of 3 nM.

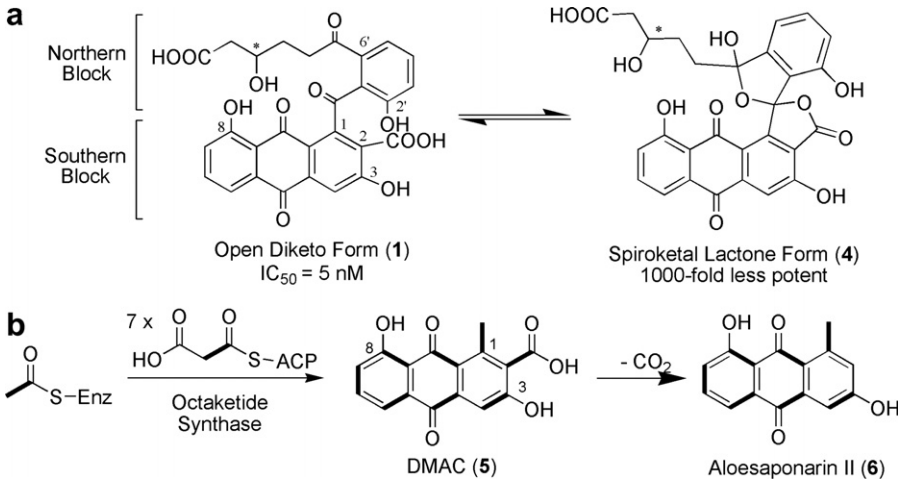

Figure 3. (a) Dynamic equilibrium of mumbaistatin. Diketo form (**1**) of mumbaistatin has an IC_{50} of 5 nM. However, it tends to form a lactone assisted by carboxylic acid at C-2 and eventually form a spiroketal lactone (**4**) which possess much less potency. (b) Biosynthesis of DMAC (**5**) and aloesaponarin II (**6**) from engineered type II polyketide synthases. Octaketide synthase (*act* PKS) with a ketoreductase and cyclase/aromatase enzymes forms DMAC (**5**) and its decarboxylated compound aloesaponarin II (**6**) as major biosynthetic products. The southern block of the open diketo form of mumbaistatin (**1**) has the identical structure to DMAC (**5**).

2. Results and discussion

2.1. Engineering of DMAC producer and preparation of DMAC

In contrast to the extremely poor yield of mumbaistatin from natural producer (70 mg/1000 L culture),[20] DMAC (**5**) was produced in high yield (100 mg/L) from *S. coelicolor* CH999/pRM5, a recombinant strain with an engineered type II polyketide synthase pathway.[24] To further increase polyketide product yield, we developed an improved producer strain by enhancing the copy number of the plasmid-encoded polyketide synthase genes in this host. A high-copy-number cloning vector, pBOOST,[25] was co-transformed with pSEK38,[26] a modified derivative of pRM5, into CH999. The resulting strain CH999/pSEK38/pBOOST was grown on R5 semi-solid media, and the biosynthetic products were extracted after 7 days. The crude extract was injected onto an analytical C18 HPLC column, where DMAC and its decarboxylated analog aloesaponarin II were detected as major biosynthetic products at 280 and 410 nm. Large-scale purification was performed by silica-gel column chromatography. Aloesaponarin II was eluted first as a pure yellow fraction (isolated yield: 70 mg/L of R5 semi-solid media), and then DMAC was obtained when 1% acetic acid was added in the solvent with isolated yield of 250 mg/L. The NMR and MS data of both compounds were identical to previously reported results.[27–29] This improved yield was due to the co-integration of pBOOST, as previously shown from other constructs,[25] and greatly facilitated semisynthetic exploitation of DMAC toward mumbaistatin and its analogs.

2.2. Synthetic manipulation of DMAC

The structure of DMAC includes two phenolic hydroxyl groups and one carboxylic acid along with the characteristic anthraquinone backbone as shown in Figure 3b. For synthetic manipulation of DMAC at the C-1 position, its protection as a methyl ether and ester was attempted. Both dimethyl-protected and trimethyl-protected products were obtained (Fig. 4a). The incomplete protection is presumably due to the strong hydrogen bonding between the phenolic hydroxyl at C-8 and the quinone carbonyl, which likely inhibited methylation at this position as previously observed in similar systems.[29]

With trimethyl-protected DMAC (**7**), oxidation of the benzylic C-1 carbon of DMAC was attempted. Conventional strong oxidizing agents such as selenium dioxide and potassium permanganate, however, did not give the desired oxidation product due to the electron deficient nature of this benzylic position as a result of the neighboring quinone carbonyl and carboxylic acid. As an alternative route to introduce synthetically useful functionality at the C-1 position of DMAC, we attempted benzylic bromination, and successfully obtained only the mono-brominated product (**9**) even with prolonged exposure to an excess of *N*-bromosuccinimide (NBS) (Fig. 4a).

To further manipulate the benzylic position of **9**, we explored nucleophilic substitution reactions as well as cross-coupling reactions. Initial attempts using the cyanide nucleophile, which is a strong and small nucleophile, were unsuccessful under a variety of conditions. Aryl cuprates were therefore used to evaluate whether direct C–C bond formation could occur at this benzylic position. Phenyl cuprate, prepared from phenyl lithium and copper (I) iodide, was added to a solution of **9**. The phenyl derivative of DMAC (**11**) was obtained as expected; however, the yield was very low. To improve the yield of **11**, we explored the Suzuki–Miyaura cross-coupling reaction[30–33]. Several reaction conditions were attempted; the best result was obtained under conditions described by Molander et al. (Fig. 4b).[31] Nucleophilic

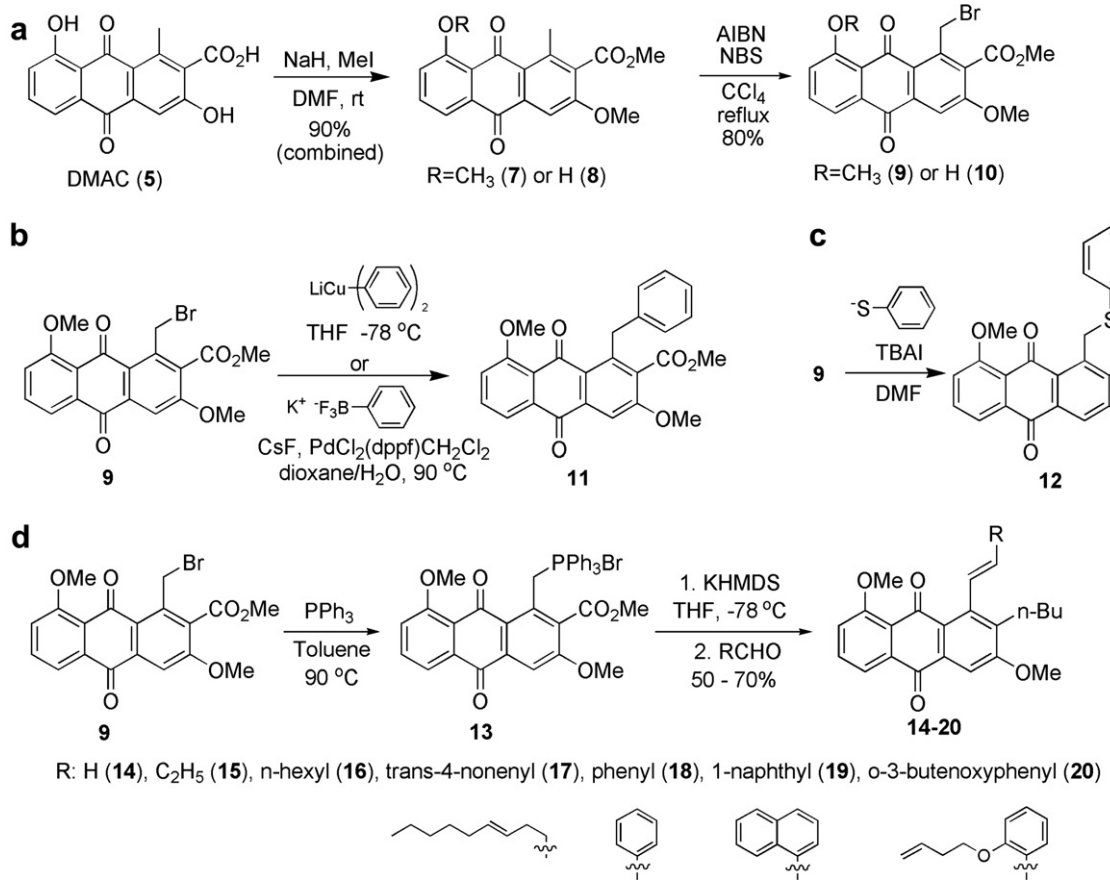

Figure 4. Synthetic manipulation of DMAC. (a) Methyl protection and benzylic bromination of DMAC (b), (c) nucleophilic substitution and Suzuki coupling of monobromide of DMAC (**9**), (d) Wittig salt formation and Wittig reaction with DMAC.

substitution of **9** with thiophenolate was also successful and resulted in the formation of thioether (**12**) in high yield (Fig. 4c).

The benzophenone moiety of mumbaistatin is not stable and readily forms the less potent spiroketal lactone,[21,34] in a manner that is assisted by the C-2 carboxylic acid. To overcome this intrinsic instability of mumbaistatin, analogs lacking reactive functionality at the benzylic position were designed. As shown above, the nucleophilic substitution product **11** with a methylene group at the benzylic position is one such example. As an alternative, we explored the formation of a Wittig salt from **9**. The resulting products were expected to maintain an sp^2 carbon at the benzylic position, as is the case in mumbaistatin. In the reaction with triphenylphosphine and **9**, a pale yellow triphenylphosphonium bromide precipitate (**13**) was obtained with a yield over 80% (Fig. 4d). Various analogs of mumbaistatin with a vinyl group at C-1 position were obtained from the Wittig reaction between **13** and a range of aldehydes. From the simplest analog, obtained by the reaction with formaldehyde (DVAC, **14**), to the longest analog (by the reaction with *trans*-4-decenal, **17**), the analogs with non-aromatic northern blocks were prepared as in Figure 4d. Analogs with aromatic northern blocks were also prepared with benzaldehyde, 1-naphthal, and O-alkylated salicylaldehyde (Fig. 4d). Due to the low reactivity of the stabilized benzylic ylides and the steric hindrance by the methyl ester at the *ortho* position, mostly the (*E*)-isomers were produced.

The next step in mumbaistatin analog preparation involved methyl ether/ester deprotection and was investigated with **7** as a model substrate (Fig. 5a). The ester could be selectively deprotected by saponification to afford **21**. Boron tribromide was tested for the deprotection of the methyl ethers. When three equivalents of boron tribromide were used, only the methyl ester and methyl ether at C-8 were deprotected, whereas the methyl ether at C-3 remained intact to give **22**. Excess reagent, elevated temperature (40 °C) or longer reaction time did not yield the completely deprotected product. Trimethylsilyl iodide was also tested for the deprotection of methyl ether at C-3, but it failed to cleave the methyl ether and disruption of the quinone structure was observed when the reaction temperature was elevated to 50 °C.

This failure was surprising because we expected that the C-3 methyl ether could be easily deprotected through the assistance of the carbonyl oxygen of the neighboring ester that would locate the boron reagent close to the reaction center (as frequently seen in β-keto systems). Therefore, despite our failure with model compound **7**, we proceeded with this deprotection strategy to prepare

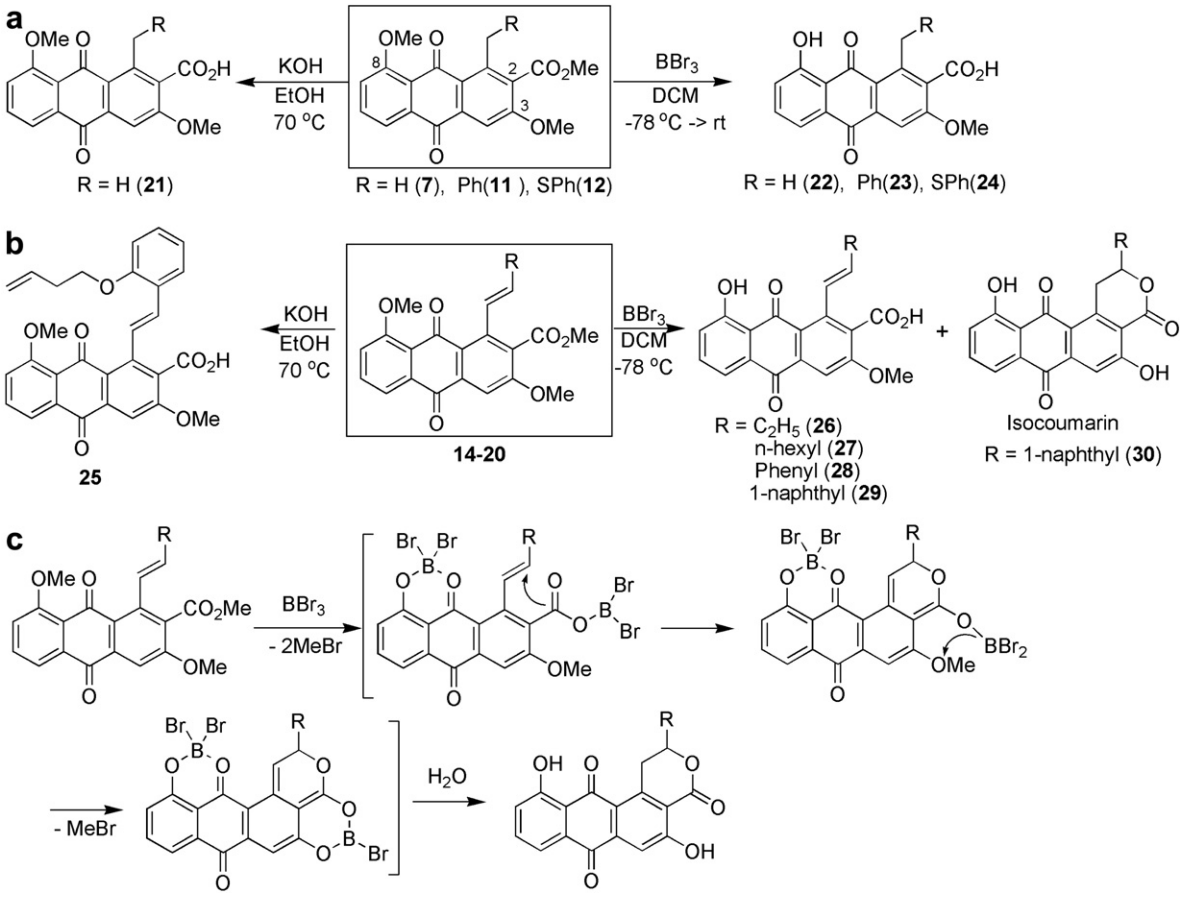

Figure 5. Deprotection of mumbaistatin analogs. (a) Model study with trimethyl-protected DMAC (**7**). Saponification deprotects only methyl ester, and BBr_3 condition deprotects the methyl ester and one of the phenyl methyl ethers. (b) Deprotection of Wittig products. (c) Mechanistic interpretation of isocoumarin formation.

the desired mumbaistatin analogs. First, the analog with O-alkyl-substituted northern block (**20**) was successfully deprotected by saponification to yield **25** (Fig. 5b). For other analogs, three equivalents of boron tribromide was used for deprotection. Interestingly, LC–MS analysis revealed that analogous deprotection attempts with **14**–**19** yielded a mixture of deprotected products including the fully deprotected compounds. The mixture was purified by preparative TLC and individual compounds were characterized by ^{1}H NMR and ESI MS. The most non-polar compound in each product mixture eluted unexpectedly fast on TLC, even with 20% ethyl acetate in hexane without acetic acid. ESI MS results confirmed their masses corresponding to the fully demethylated products (e.g., $[M + 1] = 437.1$, $[M - 1] = 434.7$ for the deprotection product of **19**). The ^{1}H NMR of the isolated compounds, however, did not reveal the vinyl protons but instead had three sets of doublet peaks around 6.4, 4.7, and 3.8 ppm (for deprotection product of **19**). Based on additional NMR and ESI MS data, the product structure was deduced as an isocoumarin type molecule (**30**), in which the carboxylate was added to the vinyl carbon to form a six-membered lactone via electrocyclization (Fig. 5b). The isocoumarin compounds are presumably formed by cyclization through the reversible protonation of the olefinic carbon *ortho* to the carboxyl group.[35,36] The more polar compounds isolated from preparative TLC corresponded to products that retained the C-3 methoxy group.

Deprotection of the methyl ether at C-3 and the favored formation of isocoumarin in the C-3 deprotected compounds can be rationalized analogous to the intramolecular demethylation by the boron reagent. The relatively planar geometry of the extended conjugate system in vinyl-substituted DMAC forced the vinyl moiety toward the opposite side from the quinone carbonyl to relieve steric strain. In turn, this forced the bulky boronate attached to the C-2 carboxyl, whose methyl ester is most vulnerable to deprotection by boron tribromide, toward the C-3 methyl ether as shown in Figure 5c. This molecular conformation favors electrocyclization, and can be stabilized by conjugation through the aromatic ring system. The formation of a six-membered ring presumably forces the C–O in the boronate ester to coordinate to the oxygen of methyl ether at C-3, thereby enabling deprotection of C-3 methyl ether via intramolecular assistance of the boronate ester. The lack of fully demethylated products containing carboxylic acid at C-2 and the failure of demethylation at C-3 with trimethyl-protected DMAC is consistent with this interpretation. However, it is unclear why the C-3 methyl ether remained protected even with 10 equivalents of the reagent, both in trimethyl-protected DMAC (**7**) as well as the Wittig

products. Also, during deprotection of **14**–**19**, the isocoumarin product and monomethyl product were obtained as mixtures with their brominated compounds, which were tentatively identified by mass analysis (data not shown). Further research to find optimal deprotection conditions is warranted.

2.3. Analog with a salicylic acid southern block

The importance of the anthraquinone southern block for the biological activity of mumbaistatin has not been studied. To do so, a simplified analog of mumbaistatin was envisioned in which the anthraquinone is replaced by a simple salicylate. Initial attempts to prepare this salicylate analog were unsuccessful due to the strong tendency of the product to form a five-membered lactone which has been observed from the dynamic equilibrium of mumbaistatin,[21,34] and more recently, in the synthesis of 2′-dealkylmumbaistatin.[37] To overcome this problem, an alternate analog was designed with a vinyl moiety in place of the ketone at the benzylic position (Fig. 6). The structure of this analog was similar to the key intermediate reported in the total synthesis of balanol, and this intermediate was produced through an intramolecular Heck coupling between a benzylic vinyl moiety and aryl iodide.[38]

The acetonide form of 6-vinyl salicylic acid (**33**) was synthesized from the commercially available 2,6-dihydroxybenzoic acid (Fig. 6). Palladium-catalyzed Stille coupling between the triflate **31** and a vinyl tin reagent gave 6-vinyl salicylic acid **34**. The northern block was prepared as a racemic mixture by Grignard reaction of methoxymethyl (MOM) protected 3-hydroxybenzaldehyde and commercially available alkyl magnesium bromide, as in Figure 6. The C-2 position of the northern block was regioselectively iodinated by the *ortho*-directing effect of the MOM group and the benzylic hydroxyl group as previously reported.[39,40] Esterification between a benzoic acid moiety in the southern block and a phenol group in northern block was performed using Furukawa's condition[41] with about 60% yield as shown in Figure 6.[38] With the ester **40**, intramolecular Heck coupling was performed and **41** was obtained as a racemic mixture. Interestingly, between two possible products (7-exo and 8-endo) of Heck coupling, only 7-exo product was isolated as previously observed in the balanol synthesis.[38] Opening of the seven-membered lactone by saponification produced the mono methyl-protected analog of mumbaistatin (**42**).

It should be noted that the overall synthetic strategy toward the analog **42** could be applied to the total synthesis of mumbaistatin from DMAC. There have been several attempts toward the total synthesis of mumbaistatin,[37,42,43] but none of them have been successful, mainly due to the steric hindrance encountered in the coupling reaction.[43] With the intramolecular Heck coupling strategy, the steric hindrance would be overcome using two substituents as a tether.

2.4. Glucose-6-phosphate translocase inhibition assay and structure–activity relationships of mumbaistatin analogs

In a previous report, we observed that several simple biosynthetic analogs of mumbaistatin showed weak but dose-dependent (IC_{50} values varying between 100 and 500 μM) inhibition of G6P T1.[22] In this report, we would provide the first structure–activity relationship (SAR) data on mumbaistatin with several synthetic analogs, which in turn may enable a design strategy toward more potent and drug-like analogs of this natural product.

The assay protocol was verified with chlorogenic acid (CHA, **2**) and the IC_{50} of **2** was determined to be comparable to the earlier reported value (260 μM).[18] Using this assay condition, the inhibition of G6P T1 was mea-

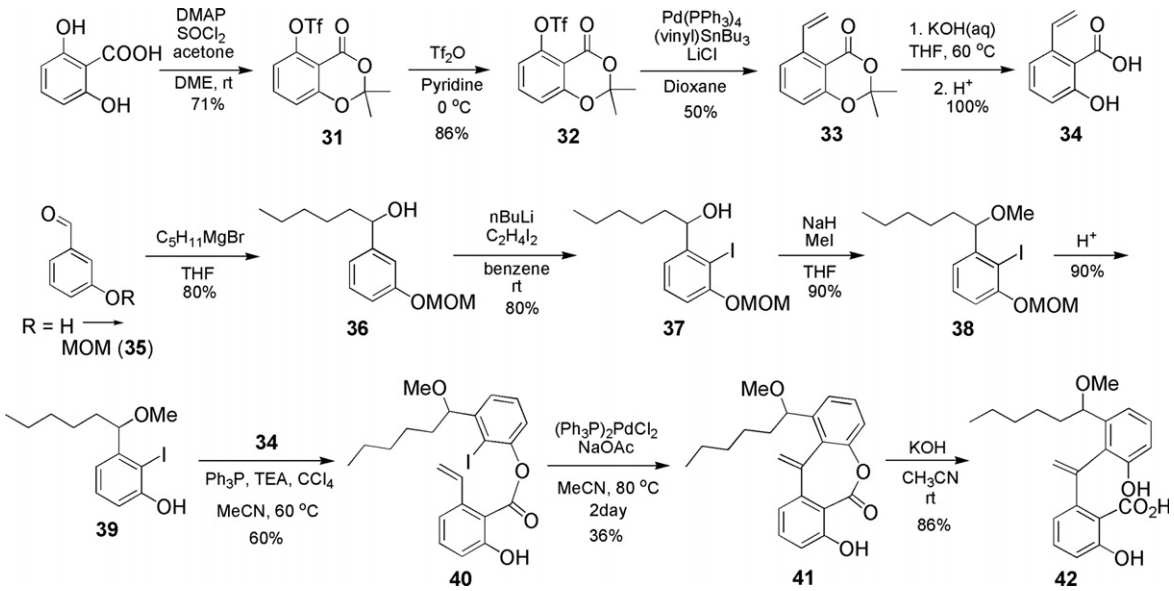

Figure 6. Synthesis of the analog containing salicylic acid southern block.

sured in the presence of a series of biosynthetic and semisynthetic analogs of mumbaistatin. The IC_{50} values are summarized in Figure 7a.

First, the biosynthetic analogs of mumbaistatin, YT127 (**43**)[22] and YT127c (**44**)[44], and synthetic analogs with linear alkyl chains (**26**,**27**) were assayed to confirm the effect of a vinyl moiety at C-1. Even though these synthetic analogs still have the C-3 methyl protecting group, the vinyl containing inhibitors were more potent than compounds with a simple alkyl chain at C-1. This series of molecules, however, did not show clear chain length dependence, and the IC_{50} values were still over 10^3-fold higher than the reported IC_{50} of the natural product. Compounds possessing an aromatic northern block (**25**, **28**, **29**) were also tested. The inhibitors containing aromatic northern blocks (**28**, **29**) showed about 5- to 10-fold better potency than those with linear vinyl alkyl northern blocks. The nature of aromatic northern block has a little effect on the potency, but the methoxy group remaining at C-8 limited the potency of **25** which suggests the importance of deprotection at the position. When the rigid nature of C-1 position in the analog with vinyl northern block (**28**) was removed in the thioether analog **24**, the IC_{50} value changed to 20-fold higher. The Suzuki–Miyaura coupling product **23** has about 10-fold higher IC_{50} value compared to the corresponding vinyl analog (**28**) and 10-fold better potency compared to analogs with a linear northern block (**43**, **44**). These results suggest that a rigid structure at the C-1 position, along with the aromatic nature of northern block, is important for biological activity. Interestingly, the isocoumarin compounds (**30**) showed almost comparable potency to the analog with the vinyl aromatic northern block (**29**), suggesting that the C-2 carboxylic acid may not be necessary for biological activity.

The salicylic acid analog (**42**) was much less potent than several anthraquinone compounds tested here (Fig. 7a). This highlights the importance of the anthraquinone for biological activity, and also reinforces the importance of using DMAC as a starting material for the preparation of mumbaistatin analogs.

Based on these inhibition assay results, the following conclusions were drawn with regard to the structure–activity relationships of mumbaistatin. First, the rigid conformation between the northern and southern blocks has a positive effect on inhibitory activity. Inhibitors containing a rigid conformation (either vinyl or isocoumarin type) at the C-1 substituent showed better potency than **24**, **43**, and **44** which contain flexible

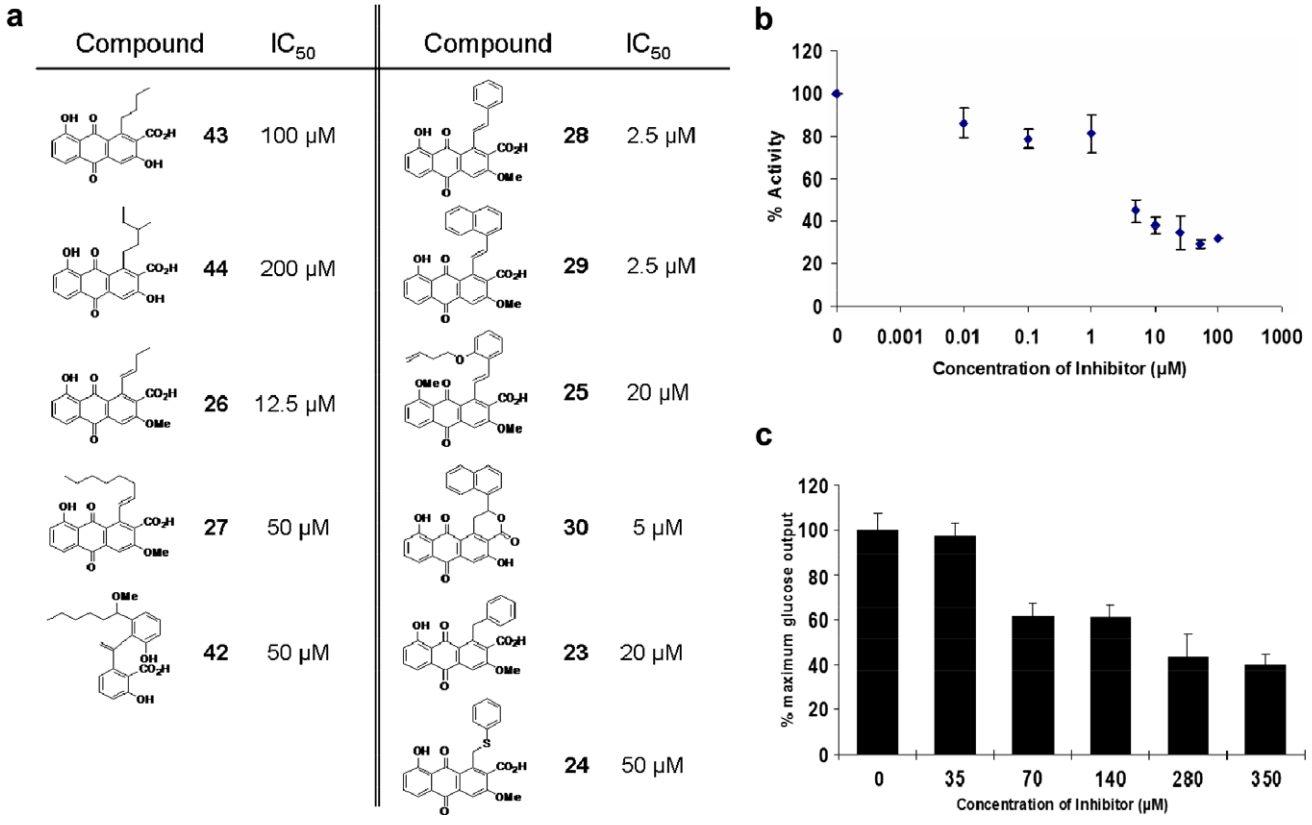

Figure 7. Biological activity of mumbaistatin analogs. (a) Biosynthetic and synthetic analogs of mumbaistatin and their inhibition of G6P T1. Compounds **43** and **44** are biosynthetic analogs previously reported.[22,44] Compounds **26** and **27** are synthetic analogs with linear northern blocks. Compounds **28**, **29**, and **25** are synthetic analogs with aromatic northern blocks. Compound **30** is an analog containing isocoumarin moiety. Compounds **23** and **42** are analogs without oxygen species at the benzylic position. (b) Rat liver microsome assay of compound **29**. (c) Effect of compound **29** on glucose output from rat hepatocytes. Cryopreserved hepatocytes were incubated and prepared for the assay as described in Section 4. Results are expressed as percent output in the presence of 25 nmol/L glucagon. Glucagon-stimulated glucose output was 0.31 ± 0.005 μmol $\cdot 10^6$ cells^{-1} h^{-1}.

substituents at C-1. Second, aromatic northern blocks help the inhibition of G6P T1. It is not clear, however, whether the nature of the aromatic northern block affects the potency dramatically. Third, the phenol at C-8 has important effect on potency. Protection at this position clearly reduced activity. However, it is not clear whether the phenol at C-3 or the carboxylic acid functionality at C-2 affects biological potency.

2.5. Reduction of glucose production from hepatocytes by G6Pase inhibition

To demonstrate the potency of these inhibitors in a physiologically relevant biological assay, we performed cell-based assay using male rat hepatocytes. Cryopreserved hepatocytes were incubated with two representative analogs **28** and **29** in the presence of a maximally stimulatory concentration of glucagon (25 nM). Both compounds showed dose-dependent inhibition on the rate of glucose release by the hepatocytes. Compound **29** inhibited 40% of the glucagon-stimulated glucose production at a concentration of 280 μM (Fig. 7c). The relative difference between the biological assay and the hepatocyte assay is mirrored by the activities of other known biologically active inhibitors of G6P T1.[19]

3. Conclusion

In conclusion, starting from an overproduced polyketide product derived via engineered biosynthesis, we have prepared semisynthetic analogs of mumbaistatin, a potent anti-diabetic natural product lead. Several readily accessible analogs showed low micromolar potency against their therapeutic target, G6P T1; two of them were also active in hepatic glucose release assays. Structure–activity relationship analyses have provided insights toward even more potent anti-diabetic agents. Judicious combination of biosynthetic and synthetic chemistry offers a vast range of new opportunities for accessing bioactive small molecules.

4. Experimental

4.1. General procedures

All chemicals were purchased from Sigma–Aldrich unless otherwise noted. All solvents were dried and freshly distilled under nitrogen prior to use. ^{1}H and ^{13}C NMR spectra were obtained on Varian Inova-500 or Mercury-400 spectrometers. Mass spectra were obtained by electrospray ionization (ESI). HR-ESIMS was performed at the Vincent Coates Foundation Mass Spectrometry Laboratory at Stanford University using a Micromass QToF API US (Manchester, United Kingdom) quadrupole-time of flight mass spectrometer, which was operated in MS-only mode with an internal standard (poly-alanine, Sigma P9003). Unless otherwise stated, all reactions were carried out under nitrogen atmosphere. Reactions were monitored by thin-layer chromatography (TLC) analysis.

4.2. Transformation, culture conditions, extraction, and isolation

The strain (CH999/pSEK38/pBOOST) was prepared by PEG-assisted transformation as described by Hopwood et al.[45] The strain was grown on R5 plates containing 50 mg/L thiostrepton and 100 mg/L apramycin at 30 °C for 7–10 days. The 40 plates from 1 L of R5-agar solid media were chopped into fine pieces and extracted with 1 L of ethyl acetate (EA)/methanol/acetic acid (89:10:1) several times. The crude extracts were dried over sodium sulfate and the organic solvent was removed in vacuo. The residuals were dissolved in 10 mL of EA/methanol (1:1) and adsorbed on silica-gel for column chromatography. Chromatography on silica-gel with hexane–ethyl acetate (7:1 → 4:1 → 1:1 → 1:1 with 2% acetic acid) as an eluent yielded aloesaponarin II (**6**, 70 mg) as yellow powder and DMAC (**5**, 250 mg) as orange powder.

4.2.1. DMAC (5). ^{1}H NMR (500 MHz, DMSO-d_6): 12.86 (s, OH), 7.72 (t, $J = 8$ Hz, 1H), 7.66 (dd, $J = 8$, 1 Hz, 1H), 7.60 (s, 1H), 7.33 (dd, $J = 8$, 1 Hz, 1H), 2.66 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): 189.4, 182.2, 168.5, 161.4, 158.9, 140.8, 136.3, 136.0, 132.5, 131.4, 124.4, 122.5, 118.3, 116.8, 112.3, 19.9. ESI MS: 296.8 ($[M-1]^-$).

4.2.2. Aloesaponarin II (6). ^{1}H NMR (500 MHz, CD_3COCD_3): 7.73 (t, $J = 7.5$ Hz, 1H), 7.71 (dd, $J = 7.5$, 2 Hz, 1H), 7.61 (d, $J = 2.5$ Hz, 1H), 7.31 (dd, $J = 7.5$, 2 Hz, 1H), 7.13 (d, $J = 2.5$ Hz, 1H), 2.79 (s, 3H). ^{13}C NMR (125 MHz, CD_3COCD_3): 190.1, 182.3, 162.5, 162.3, 146.0, 141.7, 137.6, 135.9, 133.2, 127.2, 124.6, 124.3, 118.3, 112.2, 23.3. ESI MS: 253.2 ($[M-1]^-$).

4.3. Methyl protection of DMAC

To a solution of DMAC (0.88 g, 3.0 mmol) in DMF (20 mL), sodium hydride (0.59 g, 14.8 mmol, 60% in mineral oil) was added slowly at 0 °C with vigorous stirring. The mixture was stirred for 30 min at 0 °C, and methyl iodide (0.92 mL, 14.8 mmol) was added dropwise. The reaction mixture was warmed slowly to rt and then stirred overnight. The mixture was poured into cold water and extracted with ethyl acetate. The extract was washed with water and brine, dried over sodium sulfate, and concentrated in vacuo. Chromatography on silica-gel with hexane-ethyl acetate (7:1 → 4:1 → 1:1) as an eluent yielded dimethyl-protected product and trimethyl-protected products as yellow powders with a combined yield of 90% (individual yields vary in a range between 5:1 and 2:1 (**7**:**8**)).

4.3.1. Trimethyl DMAC (7). ^{1}H NMR (500 MHz, $CDCl_3$): 7.83 (dd, $J = 8$, 1 Hz, 1H), 7.63 (t, $J = 8$ Hz, 1H), 7.62 (s, 1H), 7.31 (dd, $J = 8$, 1 Hz, 1H), 4.00 (s, 3H), 3.96 (s, 3H), 3.93 (s, 3H), 2.66 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 184.0, 183.7, 167.9, 159.8, 158.7, 140.2, 136.0, 134.7, 134.2, 131.3, 127.7, 123.4, 119.2, 118.4, 106.4, 56.6, 56.5, 52.8, 19.5. ESI MS: 340.9 ($[M+1]^+$), 338.8 ($[M-1]^-$).

4.3.2. Dimethyl DMAC (8). ^{1}H NMR (500 MHz, $CDCl_3$): 12.9 (s, OH), 7.74 (dd, J = 8, 1 Hz, 1H), 7.72 (s, 1H), 7.59 (t, J = 8 Hz, 1H), 7.26 (dd, J = 8, 1 Hz, 1H), 3.99 (s, 3H), 3.96 (s, 3H), 2.71 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 189.8, 182.5, 167.6, 162.5, 160.0, 141.6, 137.5, 135.9, 132.6, 131.3, 125.1, 124.8, 119.1, 117.0, 107.6, 56.6, 52.9, 20.2. ESI MS: 326.9 ($[M+1]^+$), 294.9 ($[M-OCH_3]^+$).

4.4. Bromination of 7

To a solution of **7** (0.8 g, 2.4 mmol) in carbon tetrachloride (30 mL) were added *N*-bromosuccinimide (NBS) (0.92 g, 5.2 mmol) and 2,2′-azobis(isobutytonitrile) (AIBN) (39 mg, 0.24 mmol). The reaction mixture was heated to reflux under nitrogen for 1 day and then cooled to room temperature, filtered, and concentrated under reduced pressure. The residue was purified by silica-gel chromatography, with hexane–ethyl acetate (4/1) as the eluent, to yield the mono-brominated product (**9**) as yellow-orange powder (0.79 g, 80%).

4.4.1. Bromide of trimethyl DMAC (9). ^{1}H NMR (400 MHz, $CDCl_3$): 7.84 (dd, J = 8, 0.8 Hz, 1H), 7.74 (s, 1H), 7.66 (t, J = 8 Hz, 1H), 7.33 (dd, J = 8, 0.8 Hz, 1H), 5.09 (s, 2H), 4.02 (s, 3H), 3.99 (s, 3H), 3.98 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 183.3, 183.1, 166.6, 160.2, 159.1, 138.7, 136.7, 134.6, 134.5, 131.2, 126.2, 123.2, 119.3, 118.7, 108.8, 56.8, 56.7, 53.0, 26.8. ESI MS: 418.7, 420.8 ($[M+1]^+$). HR-ESIMS: 440.9942 (calcd for $C_{19}H_{15}O_6NaBr$: 440.9950).

4.5. Preparation of 11

(a) Nucleophilic substitution by phenyl cuprate. To an ice-cold suspension of copper (I) iodide (0.014 g, 0.07 mmol) in tetrahydrofuran (1 mL) was added ethereal phenyl lithium (0.080 mL, 0.14 mmol). After stirring for 30 min, **9** (0.0145 g, 0.03 mmol) and *t*-butyl ammonium iodide (0.01 mmol) were added dropwise to the reaction mixture, and the mixture was stirred at room temperature overnight. The reaction mixture was quenched with saturated aqueous NH_4Cl, extracted with ethyl acetate, washed with brine, and dried over sodium sulfate. The solvent was removed in vacuo and the crude product was purified by silica-gel chromatography (eluting with ethyl acetate/hexane = 1:2) to yield **11** as a yellow powder (15 %). *(b) Suzuki–Miyaura coupling.* A solution of potassium phenyltrifluoroborate (0.06 g, 0.33 mmol), CsF (0.148 g, 0.98 mmol), $PdCl_2$(dppf) with CH_2Cl_2 (0.005 g, 0.007 mmol), and **9** (0.137 g, 0.33 mmol) in dioxane/H_2O (10:1) (11 mL) was heated at 90 °C under argon atmosphere for 24 h. The reaction mixture was cooled to room temperature and diluted with water (20 mL) followed by extraction with CH_2Cl_2, washing with brine, and drying over sodium sulfate. The solvent was removed in vacuo, and the crude product was purified by silica-gel chromatography (eluting with ethyl acetate/hexane = 1:2) to yield **11** as yellow powder (0.082 g, 60%).

4.5.1. Suzuki–Miyaura coupling product (11). ^{1}H NMR (500 MHz, $CDCl_3$): 7.82 (dd, J = 15, 2 Hz, 1H), 7.7 (s, 1H), 7.62 (t, J = 16 Hz, 1H), 7.26 (d, J = 16 Hz, 1H), 7.18(dd, J = 14, 4 Hz, 2H), 7.16 (d, J = 14 Hz, 2H), 7.11(t, J = 14 Hz, 1H), 4.57 (s, 2H), 3.99 (s, 3H), 3.90 (s, 3H), 3.74 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 183.9, 183.6, 167.5, 159.5, 158.8, 141.8, 139.9, 136.4, 134.6, 134.0, 131.7, 128.9, 128.9, 128.0, 128.0, 125.8, 119.3, 118.5, 107.0, 56.7, 56.5, 52.5, 36.3. ESI MS: 438.9 ($[M+Na]^+$), 416.4 ($[M+1]^+$), 384.9 ($[M-OCH_3]^+$).

4.6. Preparation of 12

To an ice-cold solution of sodium thiophenolate (0.141 g, 1.07 mmol) in DMF (5 mL), **9** (0.173 g, 0.41 mmol) and *t*-butyl ammonium iodide (0.01 mmol) were added dropwise, and the mixture was stirred to room temperature. The reaction mixture was quenched with saturated aqueous NH_4Cl, extracted with ethyl acetate, washed with brine, and dried over sodium sulfate. The solvent was removed in vacuo and the crude product was purified by silica-gel chromatography (eluting with ethyl acetate/hexane = 1:3) to yield **12** as a yellow powder (98 %).

4.6.1. Thioether 12. ^{1}H NMR (500 MHz, $CDCl_3$): 7.84 (dd, J = 16, 3 Hz, 1H), 7.7 (s, 1H), 7.66 (t, J = 16 Hz, 1H), 7.36 (d, J = 16 Hz, 1H), 7.32 (dd, J = 13, 2 Hz, 2H), 7.19 (d, J = 13 Hz, 2H), 7.16 (t, J = 13 Hz, 1H), 4.72 (s, 2H), 4.02 (s, 3H), 3.97 (s, 3H), 3.66 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 183.9, 183.3, 167.0, 159.7, 159.0, 140.3, 136.3, 136.2, 134.5, 134.3, 132.2, 132.2, 130.5, 128.7, 128.7, 127.3, 127.0, 123.6, 119.1, 118.4, 107.5, 56.7, 52.6, 35.5, 29.7. ESI MS: 449.48 ($[M+1]^+$), 417.49 ($[M-OCH_3]^+$).

4.7. Preparation of 13

A mixture of monobromide (**9**, 0.175 g, 0.42 mmol) and triphenylphosphine (0.109 g, 0.42 mmol) in freshly distilled toluene (10 mL) was heated under reflux for 20 h. The mixture was cooled down and placed in a refrigerator overnight. The precipitate was filtered off and washed with ether. A yellow powder (**13**, 0.18 g, 81%) was obtained and used directly in the next step without further purification.

4.8. General procedure for Wittig reaction

A solution of potassium bis(trimethylsilyl)amide (1.2 equiv, 0.5 M solution in toluene) was added dropwise to a stirred solution of triphenylphosphonium bromide of the methyl-protected DMAC (**13**, 1 equiv) at 0 °C. After complete addition, the mixture was stirred for 30 min and the solution was cooled to −78 °C. To this mixture were added various aldehydes (5 equiv) either directly or as solution in THF dropwise. The reaction mixture was stirred for 2 h at −78 °C and warmed up to room temperature. When TLC showed complete conversion, the reaction mixture was quenched with the saturated solution of NH_4Cl, extracted with ethyl acetate, washed with brine, dried over sodium sulfate, and concentrated in vacuo. The residue was loaded either on the silica gel column or on the preparative TLC plate for chromatographic purification and eluted

with various ratios of ethyl acetate and hexane as solvent.

4.8.1. Wittig product 14. ^{1}H NMR (400 MHz, $CDCl_3$): 7.89 (dd, J = 7.6, 1 Hz, 1H), 7.72 (s, 1H), 7.68 (t, J = 7.6 Hz, 1H), 7.39 (dd, J = 17.2, 11.2 Hz, 1H), 7.34 (dd, J = 7.6, 1 Hz, 1H), 5.42 (dd, J = 11.2, 1.2 Hz, 1H), 5.32 (dd, J = 17, 1.2 Hz, 1H), 4.02 (s, 3H), 4.01 (s, 3H), 3.86 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 183.5, 182.8, 167.3, 160.2, 159.3, 141.2, 136.2, 135.5, 134.7, 134.5, 130.0, 126.5, 122.5, 119.4, 118.5, 117.5, 107.4, 56.6, 56.6, 52.7. ESI MS: 352.8 ($[M+1]^+$), 374.8 ($[M+Na]^+$), 351.7 ($[M-1]^-$). HR-ESIMS: 375.0835 (calcd for $C_{20}H_{16}O_6Na$: 375.0845).

4.8.2. Wittig product 15. ^{1}H NMR (500 MHz, $CDCl_3$): 7.89 (dd, J = 8, 1 Hz, 1H), 7.69 (s, 1H) 7.67 (t, J = 8.5 Hz, 1H), 7.34 (dd, J = 8.5, 1 Hz, 1H), 7.07 (dt, J = 16, 7 Hz, 1H), 5.78 (dt, J = 16, 7 Hz, 1H), 4.03 (s, 3H), 4.00 (s, 3H), 3.86 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 183.7, 181.9, 169.4, 160.1, 155.0, 141.3, 136.3, 135.6, 134.3, 130.4, 129.5, 127.6, 123.2, 120.2, 119.4, 118.5, 107.0, 56.6, 56.6, 52.5, 26.5, 13.8. ESI MS: 381.2 ($[M+1]^+$), 403.2 ($[M+Na]^+$). HR-ESIMS: 403.1153 (calcd for $C_{22}H_{20}O_6Na$: 403.1158).

4.8.3. Wittig product 16. ^{1}H NMR (500 MHz, $CDCl_3$): 7.88 (dd, J = 8, 1 Hz, 1H), 7.68 (s, 1H) 7.67 (t, J = 8 Hz, 1H), 7.34 (d, J = 8 Hz, 1H), 7.08 (d, J = 16 Hz, 1H), 5.78 (dt, J = 16, 7 Hz, 1H), 4.02 (s, 3H), 4.00 (s, 3H), 3.86 (s, 3H), 2.24 (q, J = 7 Hz, 2H), 1.49–1.41 (m, 2H), 1.39–1.25 (m, 6H) 0.89 (t, 7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 183.7, 182.9, 167.6, 160.0, 159.2, 141.2, 135.6, 135.1, 134.7, 134.3, 132.2, 130.2, 128.2, 126.9, 126.6, 122.9, 119.3, 118.5, 106.9, 56.7, 56.5, 52.5, 33.3, 32.2, 31.9, 29.4, 22.7, 14.2. ESI MS: 437.2 ($[M+1]^+$), 459.2 ($[M+Na]^+$). HR-ESIMS: 459.1791 (calcd for $C_{26}H_{28}O_6Na$: 459.1784).

4.8.4. Wittig product 17. ^{1}H NMR (500 MHz, $CDCl_3$): 7.89 (dd, J = 8, 1 Hz, 1H), 7.69 (s, 1H), 7.67 (t, J = 8 Hz, 1H), 7.34 (dd, J = 8, 1 Hz, 1H), 7.10 (dt, J = 11, 1 Hz, 1H), 5.79 (dt, J = 16, 6.5 Hz, 1H), 5.48 (m, 2H), 4.03 (s, 3H), 4.00 (s, 3H), 3.86 (s, 3H), 2.30 (m, 2H), 2.16 (m, 2H), 1.99 (m, 2H), 1.35 (m, 2H), 1.27 (m, 2H), 0.88 (t, 6.5 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 189.8, 181.1, 171.0, 164.9, 159.2, 136.4, 136.0, 134.7, 134.3, 131.1, 130.4, 129.4, 128.6, 126.6, 122.9, 119.4, 118.5, 112.9, 107.0, 56.7, 56.6, 52.5, 33.5, 32.7, 32.4, 31.5, 29.4, 22.7, 14.2. ESI MS: 476.9 ($[M+1]^+$). HR-ESIMS: 499.2098 (calcd for $C_{29}H_{32}O_6Na$: 499.2097).

4.8.5. Wittig product 18. ^{1}H NMR (400 MHz, $CDCl_3$): 7.91(dd, J = 8, 1 Hz, 1H), 7.82 (d, J = 16.4 Hz, 1H), 7.75 (s, 1H), 7.69 (t, J = 8 Hz, 1H), 7.56 (d, J = 7.2 Hz, 2H), 7.37 (t, J = 7.2 Hz, 2H), 7.35 (d, J = 8 Hz, 1H), 7.28 (t, J = 7.2 Hz, 1H), 6.66 (d, J = 16.4 Hz,1H), 4.04(s, 3H), 4.01(s, 3H), 3.79(s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): 182.6, 181.6, 166.5, 159.5, 158.5 138.8, 136.7, 135.2, 134.9, 134.0, 133.1, 133.1, 129.1, 128.0, 127.7, 126.5, 126.5, 126.3, 121.7, 119.4, 118.5, 107.4, 56.6, 56.3, 52.3. ESI MS: 428.8 ($[M+1]^+$), 450.8 ($[M+Na]^+$). HR-ESIMS: 451.1150 (calcd for $C_{26}H_{20}O_6Na$: 451.1158).

4.8.6. Wittig product 19. ^{1}H NMR (500 MHz, $CDCl_3$): 8.17 (d, J = 8.5 Hz, 1H), 8.03 (d, J = 7 Hz, 1H), 7.93 (dt, J = 8, 1 Hz, 1H), 7.86 (d, J = 16 Hz, 1H), 7.89–7.82 (m, 2H), 7.79 (s, 1H), 7.70 (t, J = 7.5 Hz, 1H), 7.65(dd, J = 6.5, 1 Hz, 1H), 7.56 (t, J = 7.5 Hz, 2H), 7.51(dd, J = 8, 1.5 Hz, 1H), 7.48 (dd, J = 7, 1 Hz, 1H), 7.38 (d, J = 16.5 Hz, 1H), 7.35 (dd, J = 8.5, 1 Hz, 1H), 4.07 (s, 3H), 4.02 (s, 3H), 3.78 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 189.2, 182.8, 162.5, 160.2, 159.4, 146.6, 135.8, 134.5, 133.9, 133.7, 131.4, 130.7, 130.4, 130.2, 129.5, 128.6, 128.3, 126.9, 126.2, 126.0, 125.9, 125.8, 124.7, 124.1, 119.5, 118.6, 107.5, 56.8, 56.8, 53.0. ESI MS: 478.9 ($[M+1]^+$). HR-ESIMS: 501.1306 (calcd for $C_{30}H_{22}O_6Na$: 501.1314).

4.8.7. Wittig product 20. ^{1}H NMR (500 MHz, $CDCl_3$): 7.91(dd, J = 7.5, 1 Hz, 1H), 7.84 (d, J = 16.5 Hz, 1H), 7.79 (dd, J = 7.5, 1.5 Hz, 1H), 7.74 (s, 1H), 7.68 (t, J = 7.5 Hz, 1H), 7.34 (d, J = 8.5 Hz, 1H), 7.23 (dd, J = 7.5, 1.5 Hz, 1H), 7.05 (d, J = 16.5 Hz, 1H), 7.00 (t, J = 7.5 Hz, 1H), 6.87 (d, J = 8 Hz, 1H), 5.95 (m, 1H), 5.15 (dd, J = 15, 2 Hz, 1H), 5.07 (dd, J = 10.5, 1 Hz, 1H), 4.04 (t, J = 6.5 Hz, 2H), 4.03 (s, 3H), 4.01 (s, 3H), 3.81 (s, 3H), 2.60 (q, J = 7, 6.5 Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): 189.9, 185.5, 168.4, 164.6, 160.1, 157.0, 156.4, 151.4, 134.6, 134.5, 134.4, 130.4, 129.0, 127.4, 127.3, 127.2, 122.3, 120.8, 120.5, 119.4, 118.5, 117.1, 111.9, 107.2, 67.9, 56.7, 56.6, 53.2, 33.8. ESI MS: 498.9 ($[M+1]^+$). HR-ESIMS: 521.1590 (calcd for $C_{30}H_{26}O_7Na$: 521.1576).

4.9. General procedure for methyl ester deprotection

To the ester dissolved in ethanol was added the same volume of 2 N KOH aqueous solution dropwise at room temperature. The reaction mixture was refluxed at 70 °C overnight and cooled down to room temperature. The resulting solution was diluted with water, acidified to pH 1 using 6 N HCl with vigorous stirring, and extracted with ethyl acetate. The organic phase was washed with water, brine, and dried over sodium sulfate. The solvent was removed in vacuo and the product was purified by column chromatography using 5% methanol in dichloromethane containing 1% acetic acid as eluent. After the removal of solvent, the product was isolated as yellow powder with around 80% yield.

4.9.1. Methyl ester deprotected compound 25. ^{1}H NMR (500 MHz, $CDCl_3$): methyl ethers at 4.06 (s, 3H), 4.02 (s, 3H). ESI MS: 484.8 ($[M+1]^+$), 482.8 ($[M-1]^-$). HR-ESIMS: 507.1406 (calcd for $C_{29}H_{24}O_7Na$: 507.1420).

4.10. General procedure for methyl deprotection with BBr_3

The methyl-protected starting material was dissolved in dry dichloromethane and was cooled down to −78 °C. To this, 3–5 equivalents of boron tribromide was slowly added using microsyringe and the reaction mixture was

stirred at −78 °C for 2 h. The reaction mixture was warmed up and stirred overnight at room temperature. The reaction was quenched by pouring in the ice-cold water, and it was stirred for 30 min before extraction with methylene chloride. The organic phase was washed with brine and dried over sodium sulfate. The solvent was removed in vacuo, and the crude product was purified by preparatory TLC with ethyl acetate/hexane/acetic acid (20:80:1).

4.10.1. Deprotected Suzuki-Miyaura coupling product (23). ^{1}H NMR (500 MHz, CD_3COCD_3): 12.72 (s, OH), 7.86 (s, 1H), 7.71 (dd, J = 10, 3 Hz, 1H), 7.75 (t, J = 10 Hz, 1H), 7.28 (d, J = 10 Hz, 1H), 7.21 (t, J = 14 Hz, 2H), 7.18 (d, J = 14 Hz, 2H), 7.11 (t, J = 14 Hz, 1H), 4.65 (s, 2H), 3.95 (s, 3H). ^{13}C NMR (125 MHz, CD_3COCD_3): 189.1, 181.7, 166.9, 162.3, 159.0, 143.5, 139.6, 138.0, 136.1, 129.6, 128.6, 128.5, 128.5, 127.9, 127.9, 125.7, 124.5, 118.4, 113.0, 51.9, 37.0. ESI MS: 387.3 ([M−1]$^-$).

4.10.2. Demethyl thioether 24. ^{1}H NMR (500 MHz, $CDCl_3$): 10.5 (s, OH), 7.83 (s, 1H), 7.75 (dd, J = 15, 3 Hz, 1H), 7.63 (t, J = 19 Hz, 1H), 7.31 (dd, J = 20, 3 Hz, 1H), 7.24 (m, 2H), 7.18 (m, 3H), 5.28 (s, 2H), 3.80 (s, 3H). ESI MS: 419.4 ([M−1]$^-$).

4.10.3. Demethyl product 26. ^{1}H NMR (500 MHz, $CDCl_3$): 12.9 (s, OH), 9.8 (br, COOH), 7.83 (s, 1H), 7.78 (dd, J = 8, 1 Hz, 1H), 7.63 (t, J = 8 Hz, 1H), 7.32 (d, J = 16.5 Hz, 1H), 5.42 (d, J = 15.6 Hz, 1H), 3.90 (s, 3H), 2.30 (m, 2H), 0.88 (t, J = 6.8 Hz, 3H). ESI MS: 352.9 ([M+1]$^+$), 350.8 ([M−1]$^-$).

4.10.4. Demethyl product 27. ^{1}H NMR (400 MHz, $CDCl_3$): 9.78 (br, COOH), 7.82 (s, 1H), 7.78 (dd, J = 8, 1.2 Hz, 1H), 7.63 (t, J = 8 Hz, 1H), 7.32 (d, J = 15.6 Hz, 1H), 7.31 (dd, J = 8, 1.2 Hz, 1H), 5.40 (dt, J = 15.6, 6.8 Hz, 1H), 3.90 (s, 3H), 2.26 (d, J = 6.8 Hz, 2H), 1.47 (m, 2H), 1.38 (m, 2H), 1.34 (m, 4H), 0.91(t, J = 6.8 Hz, 3H). ESI MS: 407.2 ([M−1]$^-$). HR-ESIMS: 409.1656 (calcd for $C_{24}H_{25}O_6$: 409.1651).

4.10.5. Demethyl product 28. ^{1}H NMR (400 MHz, $CDCl_3$): 12.79 (s, OH, from 500 MHz), 10.07 (br, COOH), 8.03 (d, J = 16.4 Hz, 1H), 7.88 (d, J = 0.4 Hz, 1H), 7.80 (dd, J = 7.6, 1.2 Hz, 1H), 7.64 (t, J = 7.6 Hz, 1H), 7.55 (dd, J = 7.6, 0.4 Hz, 2H), 7.41(t, J = 7.2 Hz, 2H), 7.33 (d, J = 7.2 Hz, 1H), 7.30 (dd, J = 8.4, 1.2 Hz, 1H), 6.28 (d, J = 16.8 Hz, 1H), 3.81 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): 188.4, 181.7, 166.7, 161.4, 159.4, 140.8, 136.6, 136.4, 136.3, 132.4, 130.9, 128.8, 128.8, 128.2, 128.1, 128.0, 126.5, 126.5, 124.5, 122.2, 118.6, 116.5, 112.9, 52.2. ESI MS: 398.7 ([M−1]$^-$). HR-ESIMS: 399.0874 (calcd for $C_{24}H_{15}O_6$: 399.0869).

4.10.6. Demethyl product 29. ^{1}H NMR (500 MHz, $CDCl_3$): 12.82 (s, OH), 10.2 (br, COOH), 8.34 (d, J = 9 Hz, 1H), 8.04–7.83 (6H), 7.66–7.54 (m, 5H), 7.34 (d, J = 3 Hz, 1H), 3.83 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 188.5, 182.0, 170.8, 163.1, 162.6, 146.3, 138.2, 136.1, 134.8, 133.7, 132.9, 132.6, 131.2, 128.3, 128.4, 126.4, 126.2, 126.0, 125.9, 125.2, 124.4, 124.0, 123.5, 120.8, 119.3, 117.0, 115.8, 53.2. ESI MS: 448.7 ([M−1]$^-$). HR-ESIMS: 449.1034 (calcd for $C_{28}H_{17}O_6$: 449.1025).

4.10.7. Isocoumarin 30. ^{1}H NMR (400 MHz, $CDCl_3$): 8.00–7.92 (m, 4H), 7.94 (s, 1H), 7.83 (dd, J = 7.6, 1.2 Hz, 1H), 7.80 (d, 8 Hz, 1H), 7.67(t, J = 8 Hz, 1H), 7.62–7.54 (m, 4H), 7.31 (dd, J = 8.4, 1.2 Hz, 1H), 6.36 (dd, J = 12, 7.2 Hz, 1H), 4.75 (dd, J = 18.4, 3.2 Hz, 1H), 3.83 (dd, J = 18.4, 12 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): 189.2, 181.6, 166.6, 162.8, 159.3, 145.9, 145.1, 140.6, 136.5, 136.0, 132.7, 130.4, 129.9, 129.7, 129.4, 128.4, 127.1, 126.2, 125.5, 125.4, 124.4, 122.4, 119.9, 119.6, 116.5, 89.5. ESI MS: 437.1([M+1]$^+$), 434.7 ([M−1]$^-$). HR-ESIMS: 435.0869 (calcd for $C_{27}H_{15}O_6$: 435.0869).

4.11. Glucose-6-phosphate translocase 1 (G6P T1) inhibition assay

Hepatic microsomes were prepared as described previously[46] and aliquots (120 μL, 2 mg/mL) in 0.25 M sucrose containing 20% glycerol were stored at −80 °C. Compound stock solutions were prepared in DMSO. Glucose-6-phosphate and mannose-6-phosphate were purchased from Sigma, and the stock solution was prepared with 20 mg/mL in phosphate buffer (pH 4.4) and stored at −20 °C. The integrity of microsomes and G6Pase activity was measured based on the colorimetric reaction of inorganic phosphate as previously reported.[47] The enzyme reaction was initiated by adding 6 μL of microsome to the reaction mixture, which contained 42 μL of assay buffer (50 mM HEPES, 100 mM KCl, 2.5 mM EDTA, 2.5 mM $MgCl_2$ and 1 mM DTT at pH 7.2), 6 μL of glucose-6-phosphate or mannose-6-phosphate (final concentration 2 mM), and 6 μL of inhibitor in DMSO. The reaction mixture was incubated at room temperature, and 13 μL of reaction mixture was taken every 10 min and quenched with 117 μL of working solution (6:2:1 mixture of 0.42% ammonium molybdate tetrahydrate in 1 N H_2SO_4, 10% SDS in water, and 10% ascorbic acid in water). The blue reduced phosphomolybdate complex is formed after incubation at 50 °C for 20 min. The absorbance was measured at 820 nm.

4.12. Rat hepatocyte assay

Hepatocyte assay was performed as described previously.[19] Cryopreserved Sprague–Dawley male rat hepatocytes (Cellzdirect) were shown to have 70 % viability when thawed. Cells were resuspended in Krebs–Henseleit bicarbonate buffer (KHBB, pH 7.4) containing 2.5 mmol/L $CaCl_2$ and 1% (wt/vol) gelatin. Hepatocytes were aliquoted at a concentration of $\sim 1 \times 10^6$ cells/mL in a 96-well plate and incubated at 37 °C with a continuous supply of 95% O_2/5% CO_2. Cells were pre-incubated for 10 min with an inhibitor in 1% DMSO or with 1% DMSO alone. After pre-incubation, glucagon (25 nmol/L) or PBS (as a control) was added for 15 min. Aliquots of cell suspension were taken after the 10-min pre-incubation

and after the 15-min incubation. The aliquots were centrifuged, and the glucose concentration of the supernatant was measured using the Amplex Red Glucose/Glucose Oxidase Assay Kit purchased from Invitrogen.

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Supplementary data

Complete experimental procedures and compound characterization data of compounds in Figure 6. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.05.019.

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