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New synthetic inhibitors of fatty acid synthase with anticancer activity

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TITLE RUNNING HEAD: New synthetic FASN inhibitors

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^aAbbreviations: CPT-1, carnitine palmitoyltransferase-1; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EGCG, (-)-epigallocatechin-3-gallate; FASN, fatty acid synthase; FBS, fetal bovine serum; HOBt, 1-hydroxybenzotriazole; i.p., intraperitoneal; MTT, 3-(4,5-dimethyl-1,3-thiazol-2-yl)-2,5-diphenyl-2*H*-tetrazol-3-ium bromide; PARP, poly(ADP-ribose) polymerase; PBS, phosphate buffered solution; PS, polymer supported; SDS, sodium dodecylsulphate; SEM, standard error of the mean; TBS-T, Tris-buffered solution with 0.05% Tween 20; TEA, triethylamine.

ABSTRACT. Fatty acid synthase (FASN) is a lipogenic enzyme that is highly expressed in different human cancers. Here we report the development of a new series of polyphenolic compounds **5-30** that have been evaluated for their cytotoxic capacity in SK-Br3 cells, a human breast cancer cell line with high FASN expression. The compounds with an $IC_{50} < 50 \mu M$ have been tested for their ability to inhibit FASN activity. Among them, derivative **30** blocks the 90% of FASN activity at low concentration (4 μM), is highly cytotoxic in a broad panel of tumor cells, induces apoptosis, and blocks the activation of HER2, AKT and ERK pathways. Remarkably, **30** does not activate carnitine palmitoyltransferase-1 (CPT-1) nor induces in mice weight loss, which are the main drawbacks of other previously described FASN inhibitors. Thus, FASN inhibitor **30** may aid the validation of this enzyme as a therapeutic target for the treatment of cancer.

KEYWORDS. FASN, fatty acid synthase inhibitors, CPT-1, carnitine palmitoyltransferase-1, structure-affinity relationship, polyphenols, anticancer activity, breast cancer

Introduction

Altered lipogenesis is a hallmark of many types of cancer and there is evidence that links overexpression and overactivation of the lipogenic enzymes that are involved in de novo lipid biosynthesis with different human tumors.¹ A key step in elucidating the relationship between cancer and lipogenesis was the identification of the oncogenic antigen-519 (OA-519), a protein linked to poor prognosis in breast cancer patients, as fatty acid synthase (FASN^a).² Since that initial finding, increased FASN expression level has been observed in a wide variety of solid human tumors, including cancers of breast, prostate, colon, lung, bladder, ovary, stomach, endometrium, kidney, skin, esophagus, tongue, and soft tissues. In addition, FASN overexpression occurs early in tumor progression and is associated with the unfavorable outcomes of the most aggressive tumors.³ Thus, FASN has been considered as a metabolic oncogene.⁴ In addition, FASN inhibition induces apoptosis selectively in human cancer cells both in vitro and in vivo, with minimal effect on normal cells.³ This fact, directly related to the high dependence of the cancer cells on de novo fatty acid synthesis and to the differential expression of FASN, makes this enzyme a suitable target for cancer treatment with a potential good therapeutic index. Indeed, pharmacological inhibitors of FASN that block tumor cell proliferation, elicit tumor cell death, and prevent tumor growth in animal models have been identified.^{1,3} These studies confirmed the proposed use of FASN inhibitors as novel antitumor therapeutics. The most representative FASN inhibitors are shown in Chart 1. Cerulenin [(2*R*,3*S*)-3-[(4*E*,7*E*)-nona-4,7-dienoyl]oxirane-2-carboxamide, **1**], a metabolite produced by the fungus *Cephalosporium caerulens*, was the first characterized FASN inhibitor.⁵ It induced selective cytotoxicity in various types of cancer cells and showed promising effects in different cancer xenograft models. However, the presence of the reactive epoxide ring makes cerulenin susceptible to other nonspecific ring-opening reactions, feature that has limited any potential clinical application.^{1,3,6} To overcome this drawback, related analog **C75** (*trans*-4-carboxy-5-octyl-3-methylenebutyrolactone, **2**, Chart 1) was synthesized.⁷ As expected from its capacity to inhibit FASN activity, **2** showed significant antitumor effects on several cancer cell lines and

xenograft models.³ However, it has been suggested that **2** could be sensitive to the presence of endogenous thiols.⁸ In addition, both **1** and **2** induce a profound weight loss, which is far from ideal for cancer patients undergoing chemotherapy. This side effect is apparently due to their activity in increasing fatty acid oxidation through direct activation of carnitine palmitoyltransferase-1 (CPT-1).⁹⁻¹¹ Also, they reduce food intake by blocking the production of hypothalamic neuropeptide-Y.¹¹ These effects clearly hindered the further clinical development of **1** and **2**. In 2004, orlistat [(-)-tetrahydrolipstatin, **3**, Chart 1], marketed as anti-obesity drug, was found to inhibit FASN,¹² and more recently, green tea polyphenols, especially highly abundant catechins such as (-)-epigallocatechin 3-gallate (EGCG, **4**, Chart 1), have received much attention on the grounds of their reported capacity to block the formation and development of tumors in a variety of animal models.¹³ Furthermore, EGCG has been found to inhibit FASN and to induce selective apoptosis in human breast and prostate cancer cells¹⁴⁻¹⁶ without producing concomitant weight loss.¹⁷ Unfortunately, its effectiveness in vivo is quite limited due to its high IC₅₀ value, poor oral bioavailability, and low serum stability.¹⁸ In our research group, we are involved in a project aimed at the development of new FASN inhibitors devoid of CPT-1 stimulation as side effect with the objective of advancing towards the validation of FASN as a new therapeutic target for the treatment of cancer. Based on our promising preliminary results with some structurally simple molecules initially inspired on the polyphenolic nature of EGCG that showed significant in vitro and in vivo antitumor properties,^{19,20} in the present work we report a new series of gallate derivatives in which the A subunit has been modified (compounds **5-30**, Chart 1). Among them, derivative **30** stands out as a highly cytotoxic compound in a broad panel of tumor cells of different tissue origins (IC₅₀ values ranging from 1.4 to 9 μM). Furthermore, compound **30** almost completely abrogates FASN activity at a concentration of 4 μM in breast cancer SK-Br3 cells, induces apoptosis of tumor cells, and interferes with the mitogen-activated protein kinase (MAPK) pathway. Importantly, all these sought-after effects occur without parallel CPT-1 stimulation or weight loss induction. Overall, the possibility of inhibiting FASN without concomitant weight loss induction clearly highlights the potential of this enzyme as a new target in oncology. Accordingly, FASN inhibitors could become a new class of

1 drugs that aid in the challenging endeavor of handling those types of tumors resistant to the current
2 therapies or those lacking adequate treatments.
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8 **Results and Discussion**

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10 **Synthesis of compounds 5-30.** In a previous study, and initially inspired by the polyphenolic nature
11 of EGCG, we designed a small series of compounds structurally simpler than this natural product and
12 with a straightforward synthetic accessibility. Encouraged by the interesting results,²⁰ in the present
13 work we have expanded the structural variability and we have studied the influence of the A subunit in
14 the cytotoxicity and FASN inhibition of compounds **5-30** (Chart 1). Anticipating the need of a synthetic
15 route that enabled us both the quick generation of a variety of compounds as well as their synthesis on
16 the gram-scale needed for in vivo studies, we set up a methodology for the esterification reaction based
17 on the use of polymer supported (PS) reagents (Scheme 1, Method A). For this purpose, different
18 commercially available PS reagents were tested, including Mukaiyama (PS-Mukaiyama), dicyclohexyl-
19 and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (PS-DCC and PS-EDC, respectively), and a *N,N'*-
20 dimethylaminopyridine surrogate bound to polystyrene (PS-DMAP). However, all these reagents led to
21 different and non-desired outcomes, such as the isolation of mixtures of esterification product and the
22 corresponding acid anhydride (with PS-Mukaiyama), of the activated alcohol intermediate (PS-DMAP),
23 or a lack of reactivity of the protected gallic acid with the resin (PS-DCC and PS-EDC). Finally, positive
24 results were obtained with the use of PS-1-hydroxybenzotriazole (PS-HOBt). Thus, protected gallic acid
25 **55** or **56** was loaded on the resin in the presence of diisopropylcarbodiimide (DIC) and DMAP, and the
26 resulting activated acid **57** or **58** linked to the resin was filtered, washed and dried (Scheme 1, Method
27 A). It is important to note that this intermediate can be stored at least one month at 4 °C, without
28 significant reactivity loss. In a second step, the reaction of **57** or **58** with the corresponding diol in the
29 presence of diisopropylethylamine (DIEA) afforded all protected target esters except **38**, **39**, **46**, and **50**
30 that could not be obtained using this PS-based methodology possibly due to steric hindrance. Therefore,
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they were synthesized by classical condensation reaction between the corresponding acyl chloride **59** or **60**²¹ and the adequate diol in the presence of triethylamine (TEA) (Scheme 1, Method B). Final removal of the protecting groups either by catalytic hydrogenation (in the case of benzyl groups) or by treatment with a hydrofluoride acid-pyridine (HF·py) complex (for the TBS-protected intermediates) afforded the desired final compounds **5-30**. In the case of benzyl intermediates **43** and **44**, in which the A subunit is naphthalene, catalytic hydrogenation for 3 to 5 h gave naphthalene compounds **20** and **21**, respectively, whereas exhaustive hydrogenation overnight afforded tetrahydronaphthalene analogues **17** and **18**, respectively.

The synthesis of the starting carboxylic acid **55**²² involved the perbenzylation of the commercial methyl gallate followed by saponification of the ester, whereas **56**²³ was obtained by reaction of gallic acid with *tert*-butyldimethylsilyl chloride (TBSCl) and subsequent selective cleavage of the silyl ester. Non-commercial intermediate diols *trans*- and *cis*-1,2,3,4-tetrahydronaphthalene-1,4-diol,²⁴ naphthalene-1,2-diol,²⁵ methyl 1,4-dihydroxy-2-naphthoate,²⁶ and naphthalene-1,8-diol²⁷ were obtained following previously described procedures.

Cytotoxicity in SK-Br3 cells. All the new final compounds were first screened for their in vitro cytotoxic activity against SK-Br3 human breast cancer cell line, selected because of its high FASN expression. The IC₅₀ values are shown in Table 1, where the reference compound **4** has also been included for comparative purposes. These data have allowed us to carry out a SAR study on the influence of the modifications of the A subunit in the cytotoxic capacity of the compounds. The main conclusions can be summarized as follows: (i) Most of the final compounds are highly cytotoxic in SK-Br3 cells (IC₅₀ < 50 μM), being more potent than **4** (IC₅₀ = 149 ± 20 μM); (ii) in general, the presence of at least one aromatic ring in the A subunit is required for cytotoxicity, as suggested by derivatives **5-7**, inactive in SK-Br3 cells (IC₅₀ > 150 μM); (iii) for those compounds where the A subunit is a phenyl ring (**8-14**), cytotoxicity increases as the distance between the two galloyl moieties augments. Thus, all the compounds with a relative 1,4-substitution in the phenyl ring show lower IC₅₀ values than analogs **8** and **9** (with 1,2- and 1,3-substitution, respectively). Among them, derivative **14** deserves special attention as

1 being the most cytotoxic one ($IC_{50} = 8 \mu M$); (iv) replacement of the benzene ring in the A subunit for
2 different tetrahydronaphthalenes (derivatives **15-18**) did not yield any significant improvement in
3 cytotoxicity, with compounds showing IC_{50} values between 22 and 42 μM ; (v) introduction of a
4 naphthalene ring as A subunit (compounds **19-29**) yielded some interesting derivatives with good
5 cytotoxicity values. Among them, polyphenols **20**, **23**, **28** and **29** with IC_{50} values between 5 and 26 μM ,
6 deserve special attention. Importantly, this naphthalene series suggests that, as observed for benzene
7 derivatives **8-14**, cytotoxicity increases as the distance between the galloyl moieties augments, being **28**
8 the most potent compound (relative 2,6-orientation of galloyl groups and $IC_{50} = 5 \mu M$), and compounds
9 **19**, **26** and **27** the less active ones (relative 1,2-, 1,8- and 2,3-orientation of galloyl groups, and IC_{50}
10 values of 74, 78 and 76 μM , respectively). These data prompted us to introduce a biphenyl system as A
11 subunit with the two galloyl groups as distant as possible (i.e. in a relative 4,4'-position, see Table 1).
12 This modification led to the identification of analog **30**, the most potent derivative in the series in terms
13 of cytotoxicity, with an IC_{50} value of 4 μM .
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32 **FASN inhibition.** Considering our objective of identifying FASN inhibitors with antitumor
33 properties, those compounds that inhibited more effectively the proliferation of the SK-Br3 cells (IC_{50}
34 value lower than 50 μM) were assessed for their capacity to block FASN activity. Towards this end, SK-
35 Br3 cells were incubated for 24 h in the presence of a concentration of the compound equal to its
36 cytotoxic IC_{50} value in these cells, and FASN activity was measured in particle-free supernatants. The
37 inhibition is expressed as the percentage of inactivation compared to a 100% activity control sample
38 (Table 1). The obtained results show that some of the cytotoxic compounds (**13**, **16**, **20**, **21**, **23**, **27** and
39 **30**) are indeed FASN inhibitors able to block between the 30% and the 90% of its total activity. Among
40 them, derivatives **20** and **30** stand out as being not only highly cytotoxic in SK-Br3 cells (IC_{50} values of
41 21 and 4 μM , respectively) but also the most potent FASN inhibitors identified within the series (90% of
42 FASN inhibition, Table 1). Prompted by these results, we previously disclosed the pharmacological
43 profile of derivative **20**, which turned out to induce apoptosis in SK-Br3 cells without affecting CPT-1
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activity.²⁰ Considering that both **20** and **30** block FASN activity in a similar extent (up to 90%) but **30** does it at a five-fold lower concentration (21 μ M for **20** vs 4 μ M for **30**), this latter compound is clearly worthy of an in-depth pharmacological study since it could be a good tool-compound for ensuing in vivo studies and validation of FASN as a target with therapeutic interest. Among the obtained results it is important to note that compound **28** had a cytotoxic potency in SK-Br3 cells comparable to compound **30** but did not inhibit FASN significantly (Table 1), which suggests that there are additional mechanisms apart from inhibition of FASN activity that may derive in cytotoxic effects. Conversely, an effect of derivative **30** on an additional target different from FASN cannot be ruled out. Accordingly, we sought to rule out general cytotoxicity of compound **30** and to confirm that FASN was the main mechanism mediating the cell death induced by this derivative. With respect to the former, we determined the capacity of compound **30** to inhibit cell proliferation in non cancer human mammary epithelial HMEpC and hepatic HL-7702 cells. In both cell lines, derivative **30** induced little cytotoxicity, with IC₅₀ values from eight to ten-fold higher than in SK-Br3 cells [IC₅₀ (**30**, SK-Br3) = 4 μ M; IC₅₀ (**30**, HMEpC) = 31 μ M; IC₅₀ (**30**, HL-7702) = 41 μ M]. To study the actual contribution of FASN to the cytotoxic effects, its expression was knocked down in SK-Br3 cells using a FASN specific siRNA and cellular viability was determined in parallel for transfected and non-transfected cells. Our results show that decreasing FASN expression in an 80% (quantified by western blot band intensity densitometry) eliminates the cytotoxicity of compound **30**, which does not induce any significant cell death up to a concentration of 10 μ M (Figure 1). Hence, derivative **30** was selected for a comprehensive biological characterization including the study of its cytotoxicity in a panel of different tumor cell lines, its molecular mechanism of action as well as its selectivity vs CPT-1 enzyme.

Cytotoxicity of FASN inhibitor 30 in a panel of tumor cells. The capacity of derivative **30** to induce cytotoxicity was further evaluated in a panel comprising a variety of tumor cells of different tissue origins, including colon, pancreas, skin, liver, ovary, breast, and lung. The IC₅₀ values obtained for the different cell lines are shown in Table 2. In general, compound **30** showed a remarkable cytotoxicity in the tumor cell line panel, being especially relevant its potency in the HCT116, PANC-1, A431, SKOV3,

1 MCF-7, ZR-75-1, MDA-MB-231, and H460 cells in which the IC_{50} values were below 10 μ M. In
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3 general, breast cancer cells with high FASN levels, such as SK-Br3, MCF-7, ZR-75-1, and MDA-MB-
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5 231 cells (Figure 2A), are more sensitive to compound **30**, with IC_{50} values between 2 and 5 μ M (Table
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7 2). Lower FASN levels decrease the sensitivity of cells towards compound **30**, as observed in BT-20 and
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9 AU565 breast cancer cell lines (IC_{50} values of 17 and 24 μ M, respectively, Table 2 and Figure 2A). This
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11 correlation between FASN levels and cytotoxicity of derivative **30** is also observed in the hepatic cancer
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13 cells Bel-7402, SK-Hep1 and HepG2 (Figure 2B) with IC_{50} values of 40, 56 and 70 μ M, respectively
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15 (Table 2). Taken together, these results clearly support the potential of the new FASN inhibitor **30** as an
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17 antitumor compound of wide application and prompted us to gain insights on the cell death mechanism
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19 and molecular pathways involved in this effect.
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24 **Cell death mechanism and signaling pathways.** In order to establish the mechanism of action of
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26 compound **30**, we first evaluated whether it induced apoptosis and caspase activity by western blotting
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28 analysis showing cleavage of poly(ADP-ribose) polymerase (PARP). Treatment of SK-Br3 cells for 48 h
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30 induced a marked increase in the levels of the PARP cleavage product (89 kDa band) in a time-
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32 dependent manner (Figure 3A). In the light of the previously reported effect of FASN inhibitors not only
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34 in apoptosis but also in the HER2 receptor and its associated pathways,^{17,20,28} we assessed the effects of
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36 **30** on HER2 activation as well as on its downstream signal transduction pathways ERK1/2 and
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38 PI3K/AKT. Incubation of cells with **30** dramatically decreased the levels of the phosphorylated form of
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40 HER2 (p-HER2), that occurred as soon as 2 h after treatment (data not shown), and became complete at
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42 6 h after treatment (Figure 3B). During this period, there was no significant change in the total level of
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44 HER2 protein, as assessed by either western blotting analysis (Figure 3B) or HER2-specific ELISA (data
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46 not shown). Phosphorylated forms of ERK1/2 (p-ERK1/2) and AKT (p-AKT) were also noticeably
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48 decreased after 6 h of exposure to compound **30** (Figure 3B). Overall, the derivative **30** induced a
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50 sustained blockade of the HER2 signaling pathway, at least throughout the whole time interval analyzed
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52 (24 h). During this period, no significant changes in the total level of the corresponding proteins (HER2,
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54 AKT, and ERK1/2) were detected. Remarkably, under the same culture conditions, EGCG did not
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1 induce apoptosis nor blocked the activation of HER2 oncogene and its downstream signal transduction
2 pathways, ERK1/2 and PI3K/AKT.²⁰
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5 **CPT-1 activation and in vivo weight loss.** Given the importance of inhibition of FASN without a
6 cross-activation of CPT-1, which is relevant to avoid the side effect of weight loss, compound **30** was
7 analyzed both in vitro and in mice. Importantly, **30** did not stimulate CPT-1 activity (Figure 4A), in a
8 similar manner to what it had been previously described for the natural FASN inhibitor **4** and in sharp
9 contrast to compound **2** which, as previously reported, produced a significant activation of CPT-1
10 activity. Both reference FASN inhibitors **2** and **4** are included in Figure 4A for comparative purposes.
11 Consistently with these in vitro results, healthy mice treated with a single intraperitoneal (i.p.) dose of
12 30, 50 or 75 mg/kg of compound **30** did not show any significant weight loss vs the control group
13 (Figure 4B). Food intake was similar to controls and altered behavior or signs of suffering or distress
14 were not observed in mice treated with **30** (data not shown). These effects are especially significant
15 when compared with animals treated with a 30 mg/kg i.p. dose of **2**, in which a 21% weight loss, as well
16 as signs of suffering, were observed. Altogether, our findings indicate that the new synthetic FASN
17 inhibitor **30** does not induce weight loss in vivo.
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38 Conclusion

39 In this work we report the synthesis of a new series of polyphenolic derivatives that have been
40 evaluated for their cytotoxic capacity in SK-Br3 cells, a human breast cancer cell line with high FASN
41 expression. The most cytotoxic compounds have been further assessed as FASN inhibitors. Among
42 them, derivative **30** stands out as a potent FASN inhibitor which blocks the 90% of FASN activity at
43 low concentration (4 μ M) and is highly cytotoxic in a broad panel of tumor cells of different tissue
44 origins, with IC₅₀ values below 10 μ M. At the molecular level, compound **30** induces apoptosis of tumor
45 cells and blocks activation of the HER2 receptor and its downstream proteins AKT and ERK1/2.
46 Importantly, these effects occur without parallel CPT-1 stimulation or in vivo weight loss. Overall, our
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1 results clearly indicate that compound **30** is a promising new FASN inhibitor that could contribute to the
2 validation of this enzyme as a therapeutic target for the treatment of different types of cancer.
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6 7 **Experimental Section**

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9 **Chemistry.** Melting points (mp, uncorrected) were determined on a Stuart Scientific electrothermal
10 apparatus. Infrared (IR) spectra were measured on a Shimadzu-8300 or Bruker Tensor 27 instrument
11 equipped with a Specac ATR accessory of 5200-650 cm^{-1} transmission range; frequencies (ν) are
12 expressed in cm^{-1} . Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker Avance 300-
13 AM (^1H , 300 MHz; ^{13}C , 75 MHz) or Bruker 200-AC spectrometer (^1H , 200 MHz; ^{13}C , 50 MHz) at the
14 UCM's NMR facilities. Chemical shifts (δ) are expressed in parts per million relative to internal
15 tetramethylsilane; coupling constants (J) are in hertz (Hz). The following abbreviations are used to
16 describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), m (multiplet), br (broad).
17 Mass spectrometry (MS) was carried out on a Bruker LC-Esquire in electrospray mode (ESI). High
18 Pressure Liquid Chromatography-Mass Spectrometry (HPLC-MS) analysis was performed using an
19 Agilent 1200LC-MSD VL. LC separation was achieved with an Eclipse XDB-C18 column (5 μm , 4.6
20 mm x 150 mm) together with a guard column (5 μm , 4.6 mm x 12.5 mm). The gradient mobile phases
21 consisted of A (95:5 water/MeOH) and B (5:95 water/MeOH) with 0.1% ammonium hydroxide and
22 0.1% formic acid as the solvent modifiers. MS analysis was performed with an ESI source. The capillary
23 voltage was set to 3.0 kV and the fragmentor voltage was set at 72 eV. The drying gas temperature was
24 350 $^\circ\text{C}$, the drying gas flow was 10 L/min, and the nebulizer pressure was 20 psi. Spectra were acquired
25 in negative ionization mode from 200 to 800 m/z and in UV-mode at four different wavelengths (210,
26 230, 254, and 280 nm). Satisfactory HPLC-MS traces confirmed a purity of at least 95% for all tested
27 compounds and their retention times are provided on the supporting information. Hydrogenations were
28 carried out in a Parr Hydrogenator under an initial pressure of hydrogen of 40-50 psi at room
29 temperature for 1 h. Analytical thin-layer chromatography (TLC) was run on Merck silica gel plates
30 (Kieselgel 60 F-254) with detection by UV light (254 nm), ninhydrin solution, or 10% phosphomolybdic
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acid solution in ethanol. Flash chromatography was performed on glass column using silica gel type 60 (Merck, particle 230-400 mesh) or on a Varian 971-FP flash purification system using silica gel cartridges (Varian, particle size 50 μm). Unless stated otherwise, starting materials, reagents and solvents were purchased as high-grade commercial products from Sigma-Aldrich, Lancaster, Scharlab or Panreac, and were used without further purification. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl and used immediately. Dichloromethane (DCM) was distilled from calcium hydride.

Compounds **55**,²² **56**,²³ **59**,²¹ and **60**²¹ were synthesized according to described procedures, and their spectroscopic data are in agreement with those previously reported. Spectral characterization data of all described compounds were consistent with the proposed structures. Satisfactory HPLC chromatograms were also obtained for final compounds **5-30**. We include here the data of protected esters **39**, **40**, and **53**, and final compounds **16**, **17**, **23**, **29**, and **30**. All other compounds are fully characterized in the Supporting Information.

General procedure for the preparation of the resin-bound intermediates 57 and 58. According to a previously optimized methodology,²⁹ the following anhydrous DCM solutions were sequentially added to one equiv of PS-HOBt: 0.6 equiv of DMAP in 20 mL/mmol, 1.5 equiv of **55** or **56** in 2.5 mL/mmol, and 4.4 equiv of DIC in 0.6 mL/mmol. The reaction was stirred under an argon atmosphere at room temperature for 3 h and the resulting solid was filtered and washed with DMF, DCM, and THF. The residue was dried under vacuum to give **57** or **58** in quantitative yield.

3,4,5-Tris(benzyloxy)benzoic acid bound to PS-HOBt (57) was prepared from 475 mg of PS-HOBt, 39 mg of DMAP dissolved in 6.4 mL of DCM, 350 mg of **55** dissolved in 2.0 mL of DCM and 1.2 mL of DMF, and 0.36 mL of DIC dissolved in 1.4 mL DCM.

3,4,5-Tris(*tert*-butyldimethylsilyloxy)benzoic acid bound to PS-HOBt (58) was prepared from 150 mg of PS-HOBt, 12 mg of DMAP dissolved in 2 mL of DCM, 128 mg of **56** dissolved in 0.6 mL of DCM and 0.3 mL of DMF, and 0.11 mL of DIC dissolved in 0.44 mL DCM.

General procedure for the synthesis of protected esters 31-37, 40-45, 47-49, and 51-54 (Method A). A suspension of **57** or **58** (2.14 equiv), the adequate diol (1 equiv) and DIEA (2.86 equiv) in anhydrous THF (20 mL/mmol of diol) was stirred at room temperature under an argon atmosphere overnight. The reaction mixture was filtered through a plug of silica gel, washed with saturated NH₄Cl, water and brine, dried over Na₂SO₄ and evaporated under vacuum. The title compound was obtained as a white solid and was used in the following reaction without further purification.

1,2-Bis([3,4,5-tris(benzyloxy)benzoyl]oxy)ethane (31) was prepared from **57** and ethylene glycol. Yield 71%.

cis-1,2-Bis([3,4,5-tris(benzyloxy)benzoyl]oxy)cyclohexane (32) was prepared from **57** and *cis*-cyclohexane-1,2-diol. Yield 64%.

trans-1,2-Bis([3,4,5-tris(benzyloxy)benzoyl]oxy)cyclohexane (33) was prepared from **57** and *trans*-cyclohexane-1,2-diol. Yield 50%.

1,2-Bis([3,4,5-tris(benzyloxy)benzoyl]oxy)benzene (34) was prepared from **57** and cathechol. Yield 37%.

1,3-Bis([3,4,5-tris(benzyloxy)benzoyl]oxy)benzene (35) was prepared from **57** and resorcinol. Yield 68%.

1,4-Bis([3,4,5-tris(benzyloxy)benzoyl]oxy)benzene (36) was prepared from **57** and hydroquinone. Yield 24%.

2-Methyl-1,4-bis([3,4,5-tris(benzyloxy)benzoyl]oxy)benzene (37) was prepared from **57** and 2-methylbenzene-1,4-diol. Yield 52%.

2-Methoxy-1,4-bis([3,4,5-tris(benzyloxy)benzoyl]oxy)benzene (40) was prepared from **57** and 2-methoxybenzene-1,4-diol. Yield 30%; mp 197-198 °C; IR (KBr) ν 1735, 1585, 1506, 1115; ¹H NMR (200 MHz, CDCl₃) δ 3.90 (s, 3H, CH₃), 5.24 (s, 4H, 4CH₂ Bn), 5.26 (s, 8H, 4CH₂ Bn), 6.88-6.96 (m, 2H, benzene), 7.25 (d, *J* = 8.4, 1H, benzene), 7.34-7.57 (m, 30H, Bn), 7.62 (s, 2H, gal), 7.65 (s, 2H, gal); ¹³C NMR (50 MHz, CDCl₃) δ 56.1 (CH₃), 71.2, 71.3 (4CH₂), 75.1 (2CH₂), 106.8 (CH), 109.7, 109.8 (4CH), 113.5, 123.1 (2CH), 124.2 (2C), 127.5, 127.6, 128.0, 128.2, 128.5 (30CH), 136.5, 137.3, 137.4,

137.6 (7C), 143.0, 143.1, 149.3, 151.9 (4C), 152.6, 152.7 (4C), 164.1, 164.5 (2C=O); ESI-MS 1007.1 (M + Na)⁺.

cis-1,4-Bis[(3,4,5-tris(*tert*-butyldimethylsilyloxy)benzoyl)oxy]-1,2,3,4-tetrahydronaphthalene (**41**) was prepared from **58** and *cis*-1,2,3,4-tetrahydronaphthalene-1,4-diol. Yield 20%.

trans-1,4-Bis[(3,4,5-tris(*tert*-butyldimethylsilyloxy)benzoyl)oxy]-1,2,3,4-tetrahydronaphthalene (**42**) was prepared from **58** and *trans*-1,2,3,4-tetrahydronaphthalene-1,4-diol. Yield 53%.

1,3-Bis[(3,4,5-tris(benzyloxy)benzoyl)oxy]naphthalene (**43**) was prepared from **57** and naphthalene-1,3-diol. Yield 47%.

1,4-Bis[(3,4,5-tris(benzyloxy)benzoyl)oxy]naphthalene (**44**) was prepared from **57** and naphthalene-1,4-diol. Yield 64%.

1,2-Bis[(3,4,5-tris(*tert*-butyldimethylsilyloxy)benzoyl)oxy]naphthalene (**45**) was prepared from **58** and naphthalene-1,2-diol. Yield 60%.

1,5-Bis[(3,4,5-tris(benzyloxy)benzoyl)oxy]naphthalene (**47**) was prepared from **57** and naphthalene-1,5-diol. Yield 42%.

1,6-Bis[(3,4,5-tris(*tert*-butyldimethylsilyloxy)benzoyl)oxy]naphthalene (**48**) was prepared from **58** and naphthalene-1,6-diol. Yield 24%.

1,7-Bis[(3,4,5-tris(*tert*-butyldimethylsilyloxy)benzoyl)oxy]naphthalene (**49**) was prepared from **58** and naphthalene-1,7-diol. Yield 24%.

2,3-Bis[(3,4,5-tris(benzyloxy)benzoyl)oxy]naphthalene (**51**) was prepared from **57** and naphthalene-2,3-diol. Yield 37%.

2,6-Bis[(3,4,5-tris(benzyloxy)benzoyl)oxy]naphthalene (**52**) was prepared from **57** and naphthalene-2,6-diol. Yield 39%.

2,7-Bis[(3,4,5-tris(*tert*-butyldimethylsilyloxy)benzoyl)oxy]naphthalene (**53**) was prepared from **58** and naphthalene-2,7-diol. Yield 39%; mp 66-67 °C; IR (neat) ν 1741, 1578, 1082; ¹H NMR (300 MHz, CDCl₃) δ 0.19 (s, 12H, 2Me₂SiO), 0.29 (s, 24H, 4Me₂SiO), 0.99 (s, 36H, 4*t*-BuSiO), 1.03 (s, 18H, 2*t*-BuSiO), 7.36 (dd, *J* = 8.9, 2.2, 2H, naph), 7.42 (s, 4H, gal), 7.67 (d, *J* = 2.2, 2H, naph), 7.92 (d, *J* = 7.9,

2H, naph); ^{13}C NMR (75 MHz, CDCl_3) δ -3.9, -3.6 (12 CH_3), 18.5, 18.8 (6C), 26.1, 26.2 (18 CH_3), 116.1 (4CH), 118.4 (2CH), 121.1 (2C), 121.2, 129.1 (4CH), 129.3, 134.4 (2C), 144.0, 148.7 (6C), 149.5 (2C), 164.8 (2 C=O); ESI-MS 1171.6 (M + Na) $^+$.

4,4'-Bis([3,4,5-tris(benzyloxy)benzoyl]oxy)-1,1'-biphenyl (54) was prepared from **57** and 1,1'-biphenyl-4,4'-diol. Yield 77%.

General procedure for the synthesis of protected esters 38, 39, 46, and 50 (Method B). To a solution of **59** or **60** (2.2 equiv) and TEA (2.2 equiv) in anhydrous THF (10 mL/mmol), the corresponding diol (1 equiv) dissolved in THF (2 mL/mmol) was added. The reaction was stirred under an argon atmosphere at room temperature overnight. Afterwards, the reaction mixture was washed with saturated NaHCO_3 , dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column chromatography using the appropriate eluent to give pure title compound as a white solid.

2,5-Bis([3,4,5-tris(benzyloxy)benzoyl]oxy)-1,1'-biphenyl (38) was prepared from **59** and 1,1'-biphenyl-2,5-diol. Chromatography DCM/hexane 7:3 (R_f 0.26); yield 57%.

Methyl 2,5-bis[**(3,4,5-tris(*tert*-butyldimethylsilyloxy)benzoyl)oxy**]benzoate (**39**) was prepared from **60** and methyl 2,5-dihydroxybenzoate. Chromatography DCM/hexane 2:8 (R_f 0.27, DCM/hexane 4:6); yield 53%; mp 191-192 °C; IR (neat) ν 1741, 1576, 1081; ^1H NMR (300 MHz, CDCl_3) δ 0.18 (s, 12H, 2 Me_2SiO), 0.28 (s, 24H, 4 Me_2SiO), 0.98 (s, 36H, 2 t -BuSiO), 1.02 (s, 18H, 2 t -BuSiO), 3.73 (s, 3H, CH_3), 7.28 (d, J = 8.9, 1H, benzene), 7.38 (s, 2H, gal), 7.41 (s, 2H, gal), 7.45 (dd, J = 8,7, 2,9, 1H, benzene), 7.89 (d, J = 2.8, 1H, benzene); ^{13}C NMR (75 MHz, CDCl_3) δ -3.5, -3.4, -3.2 (12 CH_3), 18.9, 19.2 (6C), 26.5, 26.6 (18 CH_3), 52.6 (CH_3), 116.5, 116.7 (4CH), 121.1, 121.4 (2C), 124.9 (C), 125.3, 125.4, 127.4 (3CH), 144.4, 144.6 (2C), 148.6, 148.7 (2C), 149.0, 149.1 (4C), 164.8, 164.9, 165.4 (3C=O); ESI-MS (ESI) 1179.6 (M + Na) $^+$.

Methyl 1,4-bis[**(3,4,5-tris(*tert*-butyldimethylsilyloxy)benzoyl)oxy**]-2-naphthoate (**46**) was prepared from **60** and methyl 1,4-dihydroxy-2-naphthoate. Chromatography DCM/hexane 2:8 (R_f 0.42); yield 41%.

1,8-Bis[(3,4,5-tris(*tert*-butyldimethylsilyloxy)benzoyl)oxy]naphthalene (50) was prepared from **60** and naphthalene-1,8-diol. Chromatography DCM/hexane 1:1 (R_f 0.24); yield 24%.

General procedure for the synthesis of final compounds 5-12, 14, 17, 18, 20, 21, 23, 27, 28, and 30. To a solution of benzyl derivative **31-38, 40, 43, 44, 47, 51, 52, or 54** in a mixture of DCM/ethanol (270 mL/mmol), 20% Pd(OH)₂ on carbon (980 mg/mmol) was added, and the mixture was hydrogenated (initial hydrogen pressure: 50 psi) at room temperature for 3 to 5h or overnight (for compounds **17** and **18**). Then, the catalyst was filtered, the solvents were evaporated under reduced pressure, and the resulting solid was recrystallized from DCM/methanol, to afford pure title compound.

1,2-Bis[(3,4,5-trihydroxybenzoyl)oxy]ethane (5) was prepared from intermediate **31**. Yield 95%.

cis-1,2-Bis[(3,4,5-trihydroxybenzoyl)oxy]cyclohexane (6) was prepared from intermediate **32**. Yield 70%.

trans-1,2-Bis[(3,4,5-trihydroxybenzoyl)oxy]cyclohexane (7) was prepared from intermediate **33**. Yield 75%.

1,2-Bis[(3,4,5-trihydroxybenzoyl)oxy]benzene (8) was prepared from intermediate **34**. Yield 60%.

1,3-Bis[(3,4,5-trihydroxybenzoyl)oxy]benzene (9) was prepared from intermediate **35**. Yield 60%.

1,4-Bis[(3,4,5-trihydroxybenzoyl)oxy]benzene (10) was prepared from intermediate **36**. Yield 80%.

2-Methyl-1,4-bis[(3,4,5-trihydroxybenzoyl)oxy]benzene (11) was prepared from intermediate **37**. Yield 67%.

2,5-Bis[(3,4,5-trihydroxybenzoyl)oxy]-1,1'-biphenyl (12) was prepared from intermediate **38**. Yield 75%.

2-Methoxy-1,4-bis[(3,4,5-trihydroxybenzoyl)oxy]benzene (14) was prepared from intermediate **40**. Yield 77%.

1,3-Bis[(3,4,5-trihydroxybenzoyl)oxy]-5,6,7,8-tetrahydronaphthalene (17) was prepared from intermediate **43**. Yield 92%; mp 213 °C (dec); IR (neat) ν 3356, 1705, 1615, 1200; ¹H NMR (200 MHz, CD₃OD) δ 1.80 (m, 4H, tetrahydronaph), 2.59 (m, 2H, tetrahydronaph), 2.83 (m, 2H, tetrahydronaph), 6.78 (d, J = 2.3, 1H, tetrahydronaph), 6.84 (d, J = 2.3, 1H, tetrahydronaph), 7.18 (s, 2H, gal), 7.21 (s,

2H, gal); ^{13}C NMR (50 MHz, CD_3OD) δ 23.6, 23.8, 24.3, 30.6 (4 CH_2), 110.7 (4CH), 114.4 (CH), 120.4, 120.6 (2C), 120.8 (CH), 128.4 (C), 140.6, 140.7, 141.1 (3C), 146.7, 146.8 (4C), 150.3, 151.0 (2C), 166.6, 167.0 (2C=O); ESI-MS 466.9 ($\text{M} - \text{H}$) $^-$.

1,4-Bis[(3,4,5-trihydroxybenzoyl)oxy]-5,6,7,8-tetrahydronaphthalene (18) was prepared from intermediate **44**. Yield 88%.

1,3-Bis[(3,4,5-trihydroxybenzoyl)oxy]naphthalene (20) was prepared intermediate **43**. Yield 65%.

1,4-Bis[(3,4,5-trihydroxybenzoyl)oxy]naphthalene (21) was prepared from intermediate **44**. Yield 88%.

1,5-Bis[(3,4,5-trihydroxybenzoyl)oxy]naphthalene (23) was prepared from intermediate **47**. Yield 30%; mp 265 °C (dec); IR (KBr) ν 3404, 1718, 1618, 1209; ^1H NMR (300 MHz, CD_3OD) δ 7.35 (s, 4H, gal), 7.40 (d, $J = 7.5$, 2H, naph), 7.58 (t, $J = 7.9$, 2H, naph), 7.84 (d, $J = 8.2$, 2H, naph); ^{13}C NMR (50 MHz, $\text{DMSO}-d_6$) δ 109.2 (4CH), 117.5 (2CH), 119.3 (2C), 127.6, 127.8 (4CH), 128.4 (2C), 139.6 (2C), 145.8 (2C), 146.7 (4C), 164.5 (2C=O); ESI-MS 462.7 ($\text{M} - \text{H}$) $^-$.

2,3-Bis[(3,4,5-trihydroxybenzoyl)oxy]naphthalene (27) was prepared from intermediate **51**. Yield 60%.

2,6-Bis[(3,4,5-trihydroxybenzoyl)oxy]naphthalene (28) was prepared from intermediate **52**. Yield 54%.

4,4'-Bis[(3,4,5-trihydroxybenzoyl)oxy]-1,1'-biphenyl (30) was prepared from intermediate **54**. Yield 70%; mp 268 °C (dec); IR (KBr) ν 3396, 1701, 1608, 1192; ^1H NMR (300 MHz, CD_3OD) δ 7.24 (s, 4H, gal), 7.24-7.28 (m, 4H, biphenyl), 7.67-7.71 (m, 4H, biphenyl); ^{13}C NMR (50 MHz, CD_3OD) δ 110.8 (4CH), 120.8 (2C), 123.5, 129.2 (8CH), 139.4 (2C), 140.6 (2C), 146.9 (4C), 152.4 (2C), 167.2 (2C=O); ESI-MS 488.8 ($\text{M} - \text{H}$) $^-$.

General procedure for the synthesis of final compounds 13, 15, 16, 19, 22, 24-26, and 29. To a solution of *tert*-butyldimethylsilyl derivative **39**, **41**, **42**, **45**, **46**, **48-50**, and **53** in a mixture of pyridine (5.8 mL/mmol) and THF (11.5 mL/mmol), $\text{HF}\cdot\text{py}$ complex (5.8 mL/mmol) was added, and the reaction

1 was stirred at room temperature. After the complete disappearance of starting material (TLC) the
2 reaction mixture was diluted with water (50 mL/mmol) and the crude was extracted with ethyl acetate.
3 The organic layers were then combined, washed with saturated aqueous CuSO₄, dried over MgSO₄, and
4 concentrated under reduced pressure. The obtained off-white solid was recrystallized from
5 DCM/methanol, to afford pure title compound.
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10 **Methyl 2,5-bis[(3,4,5-trihydroxybenzoyl)oxy]benzoate (13)** was prepared from intermediate **39**.
11 Yield 70%.

12 **cis-1,4-Bis[(3,4,5-trihydroxybenzoyl)oxy]-1,2,3,4-tetrahydronaphthalene (15)** was prepared from
13 intermediate **41**. Yield 88%.

14 **trans-1,4-Bis[(3,4,5-trihydroxybenzoyl)oxy]-1,2,3,4-tetrahydronaphthalene (16)** was prepared
15 from intermediate **42**. Yield 62%; mp 225 °C (dec); IR (neat) ν 3351, 1683, 1612, 1214; ¹H NMR (300
16 MHz, acetone-*d*₆) δ 2.09-2.15 (m, 2H, tetrahydronaph), 2.43-2.47 (m, 2H, tetrahydronaph), 6.23 (s, 2H,
17 tetrahydronaph), 7.12 (s, 4H, gal), 7.37-7.47 (m, 4H, tetrahydronaph), 8.07 (br s, 2H, OH), 8.20 (br s,
18 4H, OH); ¹³C NMR (75 MHz, acetone-*d*₆) δ 25.8 (2CH₂), 69.8 (2CH), 109.9 (4CH), 122.0 (2C), 129.6,
19 130.5 (4CH), 136.6, 139.9 (4C), 146.1 (4C), 166.2, 167.0 (2C=O); ESI-MS 467.0 (M – H)⁻.

20 **1,2-Bis[(3,4,5-trihydroxybenzoyl)oxy]naphthalene (19)** was prepared from intermediate **45**. Yield
21 80%.

22 **Methyl 1,4-bis[(3,4,5-trihydroxybenzoyl)oxy]-2-naphthoate (22)** was prepared from intermediate
23 **46**. Yield 70%.

24 **1,6-Bis[(3,4,5-trihydroxybenzoyl)oxy]naphthalene (24)** was prepared from intermediate **48**. Yield
25 60%.

26 **1,7-Bis[(3,4,5-trihydroxybenzoyl)oxy]naphthalene (25)** was prepared from intermediate **49**. Yield
27 69%.

28 **1,8-Bis[(3,4,5-trihydroxybenzoyl)oxy]naphthalene (26)** was prepared from intermediate **50**. Yield
29 78%.

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2,7-Bis[(3,4,5-trihydroxybenzoyl)oxy]naphthalene (29) was prepared from intermediate **53**. Yield 55%; mp 273 °C (dec); IR (KBr) ν 3389, 1687, 1610, 1146; ^1H NMR (300 MHz, CD_3OD) δ 7.26 (s, 4H, gal), 7.35 (dd, $J = 8.9, 2.1$, 2H, naph), 7.70 (d, $J = 2.1$, 2H, naph), 7.99 (d, $J = 8.9$, 2H, naph); ^{13}C NMR (75 MHz, CD_3OD) δ 109.6 (4CH), 118.6 (2CH), 119.5 (2C), 121.5, 129.3 (4CH), 129.8, 134.9 (2C), 139.6, 145.7 (6C), 150.0 (2C), 166.1 (2C=O); ESI-MS 487.0 (M + Na) $^+$.

Biochemistry. EGCG, 3-(4,5-dimethyl-1,3-thiazol-2-yl)-2,5-diphenyl-2*H*-tetrazol-3-ium bromide (MTT), EDTA, dithiothreitol, acetyl-CoA, malonyl-CoA, and NADPH were purchased from Sigma (St. Louis, MO, USA), and **2** from Alexis Biochemicals (San Diego, CA, USA). Monoclonal anti- β -actin mouse antibody (C-4), monoclonal anti-FASN, polyclonal anti- β -actin and polyclonal Her-2 (Neu-C18) rabbit antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against phospho-Her-2 (C-erb-2 Ab18) was mouse monoclonal from Thermo Fisher (Fremont, CA, USA). Rabbit polyclonal antibodies against PARP, AKT, phospho-AKT^{Ser473}, ERK-1 and -2, and mouse monoclonal antibodies against phospho-ERK-1 and -2 were from Cell Signaling Technology (Denver, MA, USA). Peroxidase-conjugated secondary antibodies were from Calbiochem (San Diego, CA, USA). CPT-1 activity assay was done using palmitoyl-CoA lithium salt from Sigma (St. Louis, MO, USA), fatty acid-free bovine serum albumin from Roche (Manheim, Germany), L-Carnitine hydrochloride from Sigma (St. Louis, MO, USA), and L-[methyl- ^3H] carnitine hydrochloride (82 Ci/mmol) from Amersham Biosciences.

Cell lines and cell culture. Cells were routinely incubated at 37 °C with 5% CO_2 . MCF-7 and MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and were routinely grown in Dulbecco's modified Eagle's medium (Gibco, Berlin, Germany) containing 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, UT, USA), 1% L-glutamine, 1% sodium pyruvate, 50 U/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin (Gibco). SK-Br3 breast cancer cells were obtained from Eucellbank (Barcelona, Spain), and were passaged in

McCoy's 5A medium containing 10% FBS, 1% L-glutamine, 1% sodium pyruvate, 50 U/mL penicillin, and 50 µg/mL streptomycin.

Cytotoxicity assay. Drug sensitivity was determined using a standard colorimetric MTT assay. Briefly, cells were plated out at a density of 5 or 7 x 10³ cells/100 µL/well in 96-well microtitre plates, and allowed an overnight period for attachment. Then the medium was removed and cells were incubated for 48 h with fresh medium containing different concentrations of EGCG or the corresponding compound **5-30**. Following treatment, drug-free medium (100 µL/well) and 10 µL of a 5 mg/mL MTT solution were added and cells were incubated for 3 h at 37 °C. After careful removal of the supernatants, the MTT-formazan crystals formed by metabolically viable cells were dissolved in DMSO (100 µL/well) and absorbance was measured at 570 nm in a multi-well plate reader (Model Anthos Labtec 2010 1.7) or at 540 nm on a FlexStation 3 instrument.

FASN activity assay. After 6, 12 or 24 h of exposure to compound under study, cells were harvested by treatment with trypsin-EDTA solution, pelleted by centrifugation, washed twice, and resuspended in cold phosphate buffered solution (PBS). Cells were sonicated during 30 min at 4 °C (PSelecta Ultrasons) and centrifuged for 15 min at 4 °C to obtain particle-free supernatants. FASN activity, expressed in nmol NADPH oxidized min⁻¹·protein mg⁻¹, was assessed in particle-free supernatant samples of equal protein content (measured by the Lowry-based BioRad assay) by spectrophotometrically monitoring oxidation of NADPH at 340 nm as described.¹⁷

RNA interference-mediated silencing of the FASN gene. FASN siRNA (h), a pool of 3 target-specific 19-25 nt siRNAs, commercially available from Santa Cruz Biotechnology, was used following the manufacturer's instructions to knock down FASN gene expression in SK-Br3 cells. To determine cytotoxicity in these cells, the MTT protocol indicated above was followed except for the fact that the percentage of cell viability was measured after 24 h incubation in the presence of compound **30**.

Immunoblot analysis of p185^{HER2/neu}, phospho-p185^{HER2/neu}, ERK1/2, phospho-ERK1/2, AKT, phospho-AKT^{Ser473} and PARP. Following treatment of SK-Br3 cells with EGCG or FASN inhibitor **30** at the corresponding concentration and for the indicated time, cells were harvested using trypsin-EDTA,

1 washed twice with PBS, and stored at -80 °C. Cells were lysed in lysis buffer (1 mM EDTA, 150 mM
2 NaCl, 100 µg/mL phenylmethylsulfonyl fluoride 50 mM Tris-HCl, pH 7.5) and kept at 4 °C while they
3 were routinely mixed every 2 min on the vortex during 30 min. A sample was taken for measurement of
4 protein content by the Lowry-based BioRad assay. Equal amounts of protein were heated in sodium
5 dodecylsulphate (SDS) sample buffer (Laemmli) for 5 min at 95 °C, separated on a 3-8% SDS-
6 polyacrylamide gel (p185^{HER2/neu}, phospho-p185^{HER2/neu}) or 4-12% SDS-polyacrylamide gel (AKT,
7 phospho-AKT, ERK1/2, phospho-ERK1/2, and PARP), and transferred onto nitrocellulose membranes.
8 Membranes were incubated for 1 h at room temperature in blocking buffer (2.5% powdered-skim milk
9 in tris-buffered solution with 0.05% Tween-20; TBS-T [10 mM Tris-HCl pH 8.0, 150 mM NaCl, and
10 0.05% Tween-20]) to prevent non-specific antibody binding, and incubated with the corresponding
11 primary antibody diluted in blocking buffer overnight at 4 °C. After 3x5 min washes in TBS-T, blots
12 were incubated for 1 h with corresponding peroxidase conjugated secondary antibody and developed
13 employing a commercial kit (West Pico chemiluminescent substrate). Blots were re-probed with an
14 antibody against β-actin as control of protein loading and transfer.
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34 **Measurement of CPT-1 activity.** CPT-1 activity was assayed by the forward exchange method using
35 L-[³H]carnitine as we previously described.¹⁰ Briefly, reactions (total volume of 0.5 mL) consisted in the
36 standard enzyme assay mixture with 200 µM of the corresponding FASN inhibitor, 2 mM L-
37 [³H]carnitine (~5000 dpm/nmol), 80 µM palmitoyl-CoA, 20 mM HEPES (pH 7.0), 1% fatty acid-free
38 albumin, and 40-75 mM KCl. Reactions were initiated by addition of isolated intact yeast mitochondria.
39 The reaction was linear up to 4 min and all incubations were done at 30 °C for 3 min. Reactions were
40 stopped by addition of 6% perchloric acid and were then centrifuged at 2500 rpm for 5 min. The
41 resulting pellet was suspended in water and the product [³H]palmitoylcarnitine was extracted with
42 butanol at low pH. After centrifugation at 2300 rpm for 3 min, an aliquot of the butanol phase was
43 transferred to a vial and counted by liquid scintillation. To perform the assay, mitochondria from GS115
44 *Pichia pastoris* transformants that express Rat CTPIA were isolated by disrupting the yeast cells with
45 glass beads as previously described.¹⁰
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In vivo experiments. Male mice C57BL/6J (12 weeks, 23-25 g) were purchased from Harlan Laboratories (France), fed *ad libitum* with a standard rodent chow and housed in a light/dark 12 h/12 h cycle at 22 °C for one week. Animals were randomized into four groups (treated with vehicle or compounds 2, 4 or 30) of 4 animals each. All experiments were done in accordance with guidelines on animal care and use established by University of Barcelona School of Farmacia Institutional Ethic and Scientific Committee (Barcelona, Spain). Treatments were done as previously described.^{11,30} Briefly, mice were fasted for 12 h during the dark cycle before treatment. Each group received a single i.p. injection (0.5 mL) of FASN inhibitor at the corresponding dose or vehicle alone (DMSO), dissolved in RPMI 1640 medium. After i.p. injections, mice were given free access to rodent chow for 24 h. At this time, the experiment finished and body weight was registered.

Statistical analysis for in vivo studies. Results were analyzed by Student's t test or by one-way ANOVA using a Tukey test as a post-test. $P < 0.05$ was considered statistically significant. All data are means \pm SD. All observations were confirmed by at least three independent experiments.

Supporting Information Available. Spectral characterization data of protected esters **31-38, 41-52, and 54** and final compounds **5-15, 18-22, 24-28**, as well as HPLC-MS analysis of purity for all final compounds **5-30**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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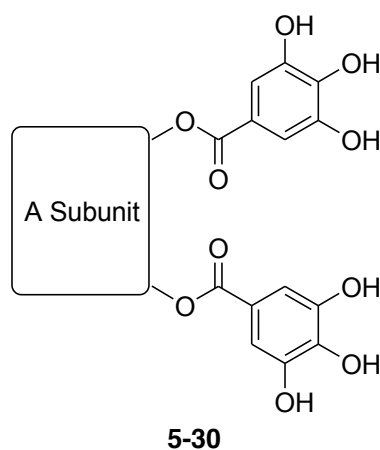
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Table 1. Cytotoxicity in SK-Br3 breast cancer tumor cells and inhibition of FASN activity of compounds **5-30**



Comp.	A Subunit	IC ₅₀ (μM) ^a	FASN inhib. (%) ^{a,b}	Comp.	A Subunit	IC ₅₀ (μM) ^a	FASN inhib. (%) ^{a,b}
5		>150	N.D.	18		41±3	N.I.
6^c		>150	N.D.	19		74±2	N.D.
7^c		>150	N.D.	20^c		21±7	90%
8^c		125±5	N.D.	21^c		29.0±0.3	69%
9^c		42±7	N.I.	22		28±6	N.I.
10^c		25±4	N.I.	23		26±3	30%

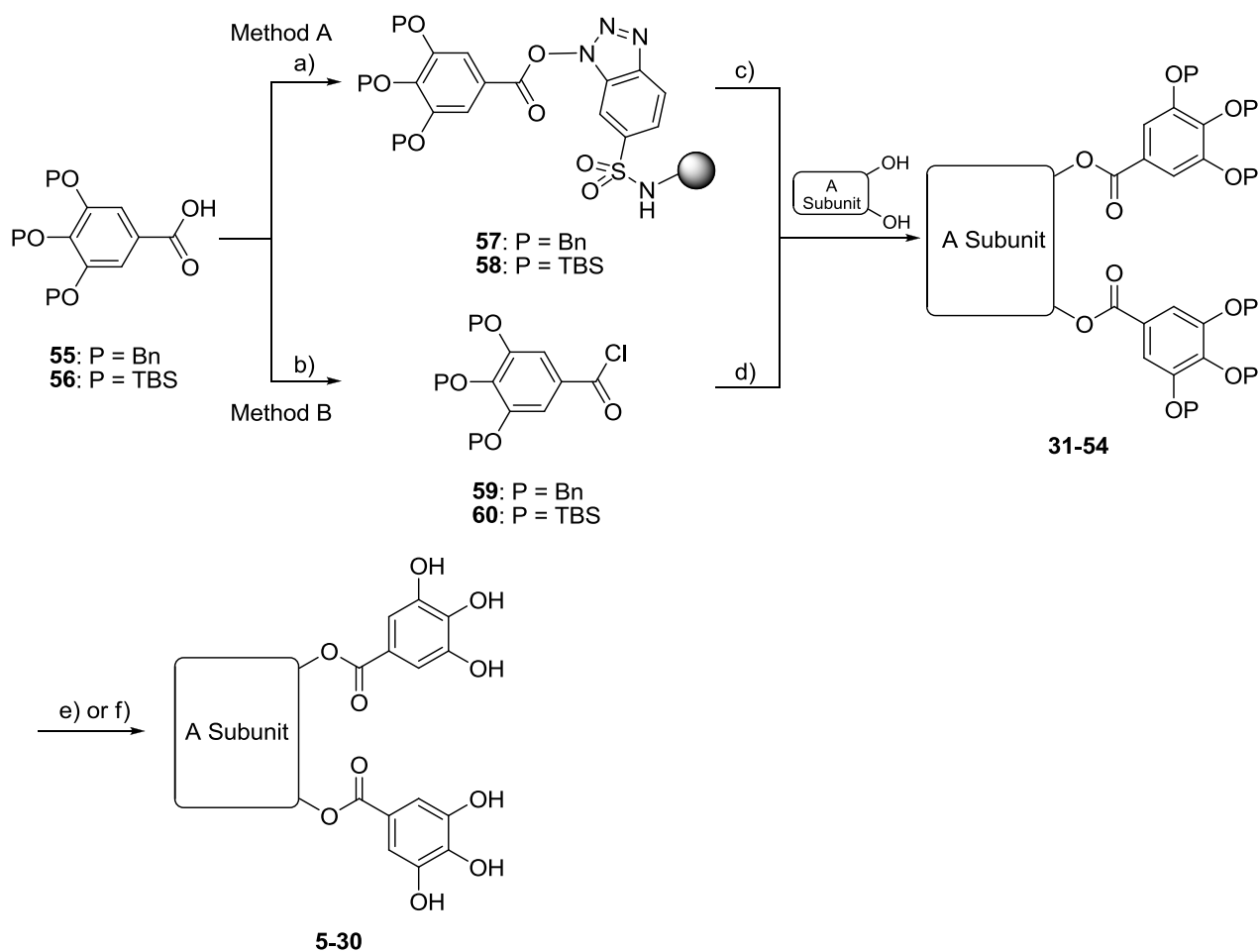
11		23±2	N.I.	24		39±8	N.I.
12		17±2	N.I.	25		47±8	N.I.
13		36±7	43%	26		78±4	N.I.
14		8±1	N.I.	27 ^c		76±9	31%
15		22±4	N.I.	28		5±1	N.I.
16		42±5	41%	29		26±10	N.I.
17		31±3	N.I.	30		4±1	90%
EGCG (4)						149±20	18%

^aThe values are the mean±SEM from two independent experiments performed in triplicate. ^bFASN inhibition values are determined at a concentration equal to the IC₅₀ value of the cytotoxicity of the compound in SK-Br3 cells. ^cData are from reference 20. N.D., not determined. N.I., no significant inhibition of FASN activity at the tested concentration.

Table 2. Cytotoxicity of compound **30** in different human cancer cell lines

Tissue origin	Cell line	IC ₅₀ (μM) ^a
Colon	HCT116	4 ± 2
Pancreas	PANC-1	2.1 ± 0.5
Skin	A431	1.4 ± 0.1
	Bel-7402	40 ± 3
Liver	SK-Hep1	56 ± 5
	HepG2	70 ± 21
Ovary	SKOV3	5 ± 2
	SK-Br3	4 ± 1
Breast	MCF-7	3.8 ± 0.8
	ZR-75-1	1.9 ± 0.3
	MDA-MB-231	5 ± 3
	BT-20	17 ± 2
	AU565	24 ± 5
Lung	HCC827	14 ± 8
	H460	9 ± 1
	A549	17 ± 10

^aThe values are the mean ± SEM from at least two independent experiments performed in duplicate or triplicate.

Scheme 1. Synthesis of final compounds 5-30.^a

^aReagents and conditions: a) PS-HOBT, DIC, DMAP, DCM, rt, quantitative; b) (COCl)₂, DMF, toluene, 55 °C, quantitative; c) DIEA, THF, rt, 20-77%; d) TEA, THF, rt, 24-53%; e) H₂, Pd(OH)₂/C, EtOH, DCM (P = Bn), rt, 54-95%; f) HF·py, pyridine, THF (P = TBS), rt, 55-88%.

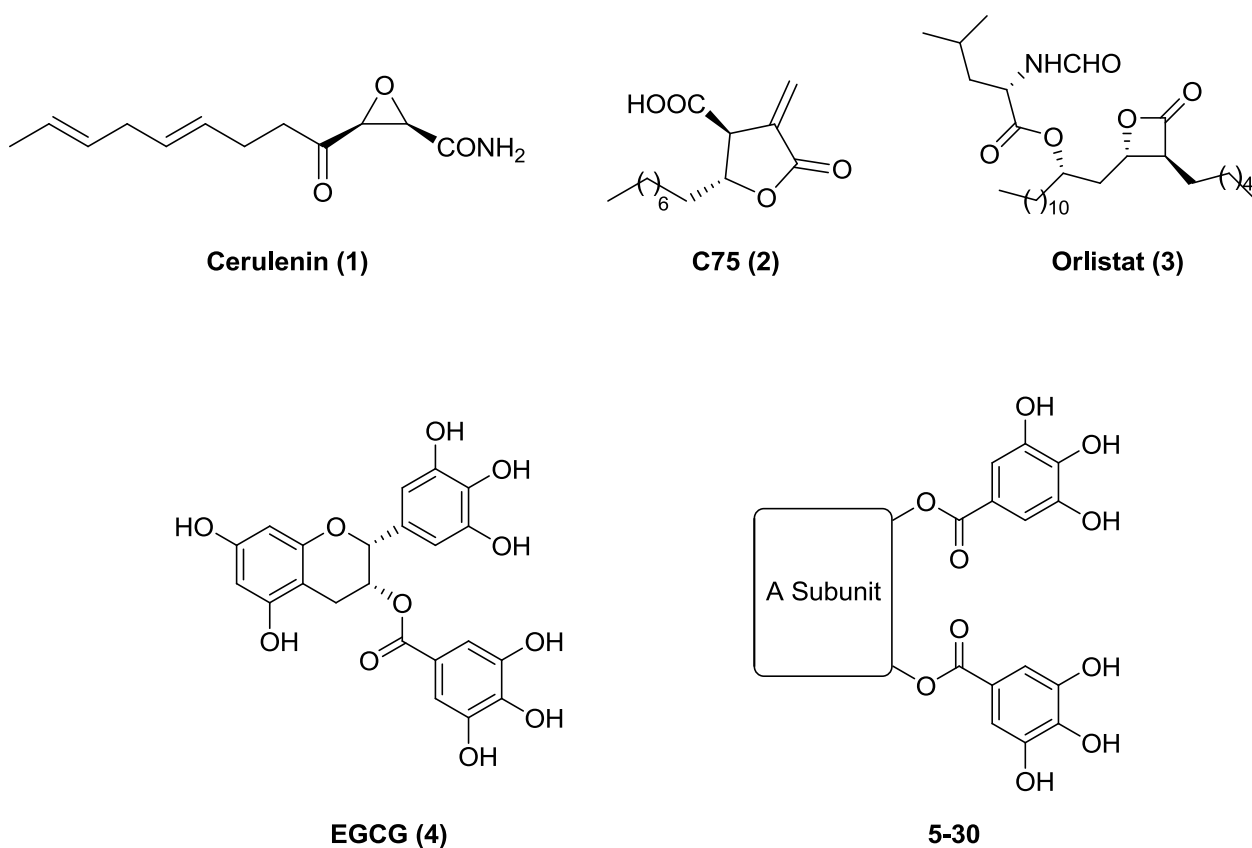


Chart 1. Reported FASN inhibitors cerulenin (1), C75 (2), orlistat (3), and (-)-epigallocatechin 3-gallate (EGCG, 4), and new compounds 5-30.

LEGENDS FOR FIGURES

Figure 1. (A) Effect of siRNA-induced silencing of FASN gene expression on cytotoxicity induced by compound **30**. Cytotoxicity of compound **30** at different concentrations (white bars) is eliminated by siRNA-induced reduction of FASN expression levels (black bars). (B) SK-Br3 breast cancer cells were transfected with siRNA-targeting FASN for 72 h. Equal amounts of total protein from SK-Br3 cells transfected with siRNA-targeting FASN or with the control were subjected to immunoblotting analyses with antibodies against FASN or β -actin. Results shown in panel A are the average \pm SEM of three different experiments performed in duplicate. Gels shown in panel B are representative of those obtained in two independent experiments. * $p < 0.01$ (Student's t-test) vs the same concentration of compound **30** in control-transfected cells.

Figure 2. FASN expression levels in different breast (A) and liver (B) cancer cell lines. Equal amounts of total protein were subjected to immunoblotting analysis with a monoclonal antibody against FASN. Immunoreactive bands in the figure are from one representative experiment. Equivalent results were found in two to four independent experiments. Blots were re-probed with an antibody for β -actin to control for protein loading and transfer (not shown for the sake of clarity).

Figure 3. Compound **30** (A) induces apoptosis as assessed by PARP cleavage (note intact PARP at 116 kDa and its cleavage product at 89 kDa); and (B) blocks the activation of HER2, AKT and ERK1/2 signaling pathways. SK-Br3 cells were treated with **30** for 6, 12 or 24 h, and equal amounts of lysates were immunoblotted with the corresponding antibody. Blots were re-probed for β -actin as loading control. In all cases, shown gels are representative of those obtained from at least two independent experiments.

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7 **Figure 4.** Compound **30** (A) does not modulate CPT-1 activity in vitro and (B) does not induce weight
8 loss in vivo in a similar manner to EGCG (**4**) and in sharp contrast to **2**, both included in the Figure for
9 comparative purposes. CPT-1 activity was determined in intact yeast mitochondria as a source of
10 enzyme in the absence or presence of the corresponding compound. For determination of the effect of
11 the compounds in body weight, mice were fasted for 12 h, treated with a single i.p. (30, 50 or 75 mg/kg)
12 dose of the compound under study or vehicle as indicated, refed for 24 h, and body weight was
13 registered. Results shown are the average \pm SEM of a least three different experiments with independent
14 mitochondrial preparations (panel A) or the average \pm SEM, n=4 (panel B).
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Figure 1

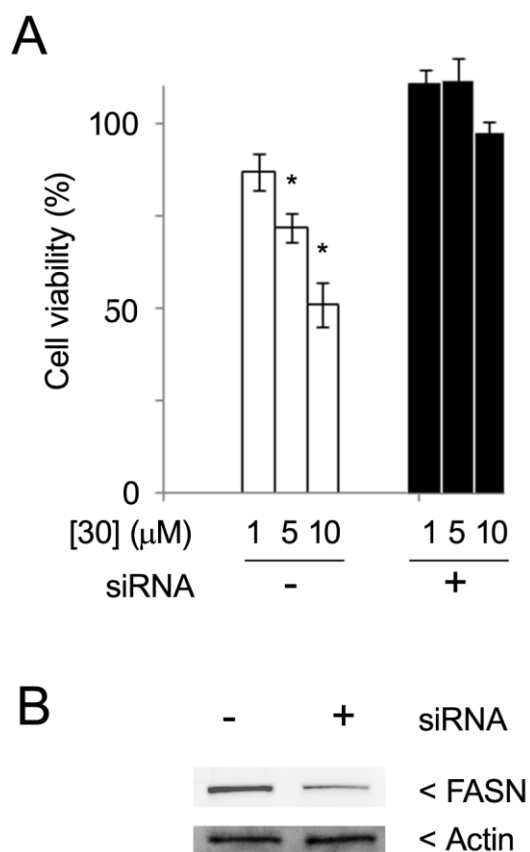


Figure 2

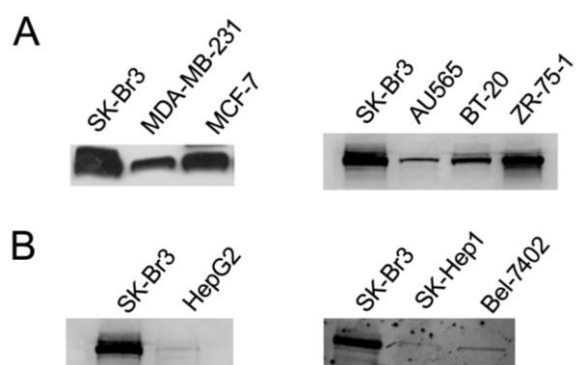


Figure 3

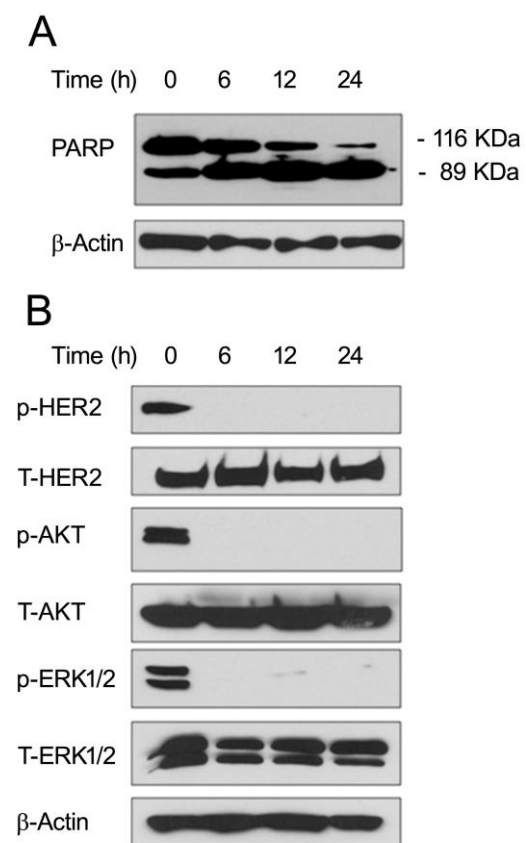
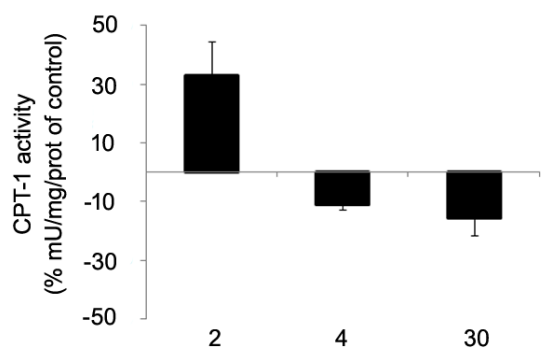


Figure 4

A



B

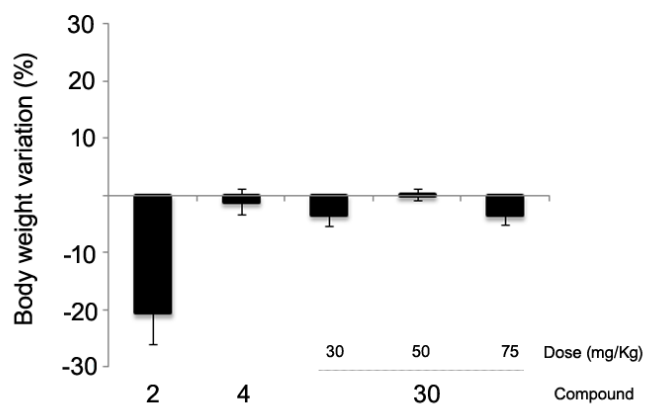


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