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Synthesis and Antiangiogenic Properties of Tetrafluorophthalimido and Tetrafluorobenzamido Barbituric Acids

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Abstract

The development of novel thalidomide derivatives as immunomodulatory and anti-angiogenic agents has revived over the last two decades. Herein we report the design and synthesis of three chemotypes of barbituric acids derived from the thalidomide structure: phthalimido-, tetrafluorophthalimido-, and tetrafluorobenzamidobarbituric acids. The latter were obtained by a new tandem reaction, including a ring opening and a decarboxylation of the fluorine-activated phthalamic acid intermediates. Thirty compounds of the three chemotypes were evaluated for their anti-angiogenic properties in an ex vivo assay by measuring the decrease in microvessel outgrowth in rat aortic ring explants. Tetrafluorination of the phthalimide moiety in tetrafluorophthalimidobarbituric acids was essential, as all of the nonfluorinated counterparts lost anti-angiogenic activity. An opening of the five-membered ring and the accompanying increased conformational freedom, in case of the corresponding tetrafluorobenzamidobarbituric acids, was well tolerated. Their activity was retained, although their molecular structures differ in torsional flexibility and possible hydrogen-bond networking, as revealed by comparative X-ray crystallographic analyses.

Keywords

angiogenesis; barbituric acids; phthalimides; tetrafluorobenzamides; tetrafluorophthalimides

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Introduction

Angiogenesis, the precisely controlled generation of new blood vessels from pre-existing vessels, is a complex and important process. In solid tumor formation, undesired angiogenesis is required to sustain the nutrient and oxygen supply for continued tumor growth and invasion. A direct correlation has been shown between tumor microvessel density and the incidence of metastasis. Anti-angiogenic drugs have been successfully used to restrict vascular supply to the tumor, and are therefore established drugs for anticancer therapy.^[1] Following the findings that the immunomodulatory drug thalidomide (**I**, Figure 1) inhibited the production of tumor necrosis factor- α (TNF- α) by activated monocytes and macrophages,^[2] and possessed anti-angiogenic properties,^[3] interest in thalidomide's biological activities was renewed, leading to the identification of a primary target—cereblon—a substrate receptor of the CRL4 ubiquitin ligase complex.^[4] Several analogues of thalidomide have been developed, sharing and augmenting the antiproliferative, anti-angiogenic, and TNF- α reducing properties of the parent compound. They were designated as immunomodulatory drugs (IMiDs). Lenalidomide (**II**), for example, also interacts with cereblon, leading to differential effects on T- and B-cells, as compared to thalidomide.^[5]

One successful strategy in the course of the development of further phthalimide derivatives was the introduction of fluorine atoms into the molecular structure.^[6] For example, the chirally stable α -fluoro-4-aminothalidomide (**III**) was found to be a highly potent inhibitor of TNF- α synthesis.^[7] An exchange of the $-\text{CH}-\text{CH}_2-\text{CH}_2-$ unit in **I** by $-\text{C}=\text{CH}-\text{NH}-$ resulted in the achiral azathalidomide derivative **IV**;^[8] in the course of an isosteric replacement,^[9] the nonpolar analogue **V** was obtained.^[8] Moreover, in compound **VI**, the fused benzene ring was fully fluorinated, leading to a strongly enhanced inhibition of both TNF- α production by monocytes^[8] and microvessel outgrowth in an angiogenesis model.^[10,11] Compound **VI** was further characterized with regard to its antimyeloma capacity, activation of the stress kinase p38 pathway, and the effect on blood vessels depending on their state of maturity.^[12] The direct connection of a cyclic substituent to the phthalimide nitrogen atom was not requisite for bioactivity, as could be concluded from biodata of *N*-alkylphthalimide derivatives.^[13]

A structural modification within the N-substituent of compound **IV**, i.e., replacement of the uracil by barbituric acid moieties, led to a further class of thalidomide analogues. We designed a small library of barbituric acids in which the barbituric acid is either connected to a phthalimide or tetrafluorophthalimide core, or linked through a carboxamide unit to tetrafluorobenzene. We prepared ten compounds of each chemotype with a consistent substitution pattern. To obtain the tetrafluorophthalimides, a synthetic procedure had to be applied that differed somewhat from that for phthalimides. Moreover, tetrafluorobenzamides could be obtained in the course of a new tandem ring opening/decarboxylation sequence. Both fluorinated chemotypes were characterized by X-ray diffraction analyses. This report details the syntheses and structural chemistry of the three chemotypes. Recently, we described the anticancer properties of two selected tetrafluorophthalimides and two tetrafluorobenzamides from this library.^[14] Herein the antiangiogenic properties of the entire library^[15] are collated and reported.

Results and Discussion

The envisaged method for the preparation of (tetrafluoro)phthalimido barbiturates relied on the condensation of (tetrafluoro)phthalic anhydride with free 5-aminobarbituric acids in glacial acetic acid.^[8,16] A new protection–deprotection route to the desired 5-aminobarbituric acids **12** is outlined in Scheme 1. The preparation of the 5-acetylaminobarbituric acids **10** included the facile alkylation to diethyl acetylaminomalonates **1** and **2**,^[17] and condensation with appropriate ureas. Some 5-acetylaminobarbituric acids have already been prepared that way.^[18,19] The condensation reaction to **10a–i** was performed under comparable mild conditions to avoid deacetylation and instantaneous base-catalyzed ring contraction to hydantoins.^[19,20] Prolonged heating of **10a–i** in HCl (8 M) was found to be suitable to provide the corresponding hydrochlorides **11a–i**, which, after cooling, were obtained directly from the reaction mixtures in pure form. Ensuing treatment with aqueous NaOH (2 M) led to the free aminobarbituric acids **12 f–i**. However, conversion of the obtained acetylmino derivatives **10** into the aminobarbituric acids **12** under the above-described conditions was limited. On the one hand, 1-phenyl-5-acetylmino derivatives **10** ($R^1 = \text{Me, Et}$, $R^2 = \text{Ph}$, $X = \text{Ac}$) could not be transformed into the corresponding salts **11** (data not shown). On the other hand, isolation of the bases **12** liberated from the parent salts **11 a–e** was unsuccessful. However, both **11** and **12** were shown to be suitable substrates for the next reaction step. An alternative approach was developed for the preparation of 5-amino-5-ethyl-1-phenylbarbituric acid (**12 k**). Diethyl [(*tert*-butoxycarbonyl)amino]malonate was alkylated with ethyl bromide,^[21] and the resulting malonic ester **3** was reacted with phenylurea to give the N-protected barbituric acid **10k**. After removal of the Boc group upon treatment with an HCl solution in 1,4-dioxane at room temperature, the desired phenyl derivative **12 k** was liberated from the hydrochloride **11 k**.

With compounds **11 a–e** and **12 f–k** in hand, their condensation with both phthalic anhydride and tetrafluorophthalic anhydride was studied (Scheme 2). The use of acetic acid as solvent for the reaction of **12** with phthalic anhydride gave undesired N-acetylated barbituric acids to various extents. In contrast, boiling DMF proved to be the most suitable solvent for the preparation of phthalimides **13**, which was accomplished in the presence of additional triethylamine to convert the salts **11 a–e**.

To synthesize the tetrafluorophthalimides **14 f–k**, aminobarbituric acids **12 f–k** were condensed with a slight excess of tetrafluorophthalic anhydride in glacial acetic acid, according to a published procedure.^[8] This methodology was then applied to hydrochlorides **11 a–e**, which were transformed into **14 a–e** in glacial acetic acid in the presence of triethylamine. The increased reactivity of the fluorinated anhydride accelerated the cyclocondensation leading to **14 a–k** without the competing acetylation, as observed in reactions with the non-fluorinated phthalic anhydride.

In view of the aforementioned acetylation, initial attempts were made to replace acetic acid with DMF in the reactions with tetrafluorophthalic anhydride as well. Surprisingly, under conditions analogous to those established for compounds **13 f–k**, a spontaneous decarboxylation of intermediate tetrafluorophthalamic acids took place instead of

cyclization. This led to the formation of tetrafluorobenzamides **15 f–k**. A similar approach to provide tetrafluorobenzamides **15 a–e** from hydrochloride substrates **11 a–e** in DMF and the presence of triethylamine failed due to an undesired reaction of **15** with dimethylamine (data not shown). Dimethylamine is known to be formed via thermal decomposition of DMF.^[22] In the presence of triethylamine, and also other tertiary amines, this process occurred to such an extent that the consecutive nucleophilic substitution of **15** with dimethylamine gave 4-dimethylamino-2,3,5-trifluorobenzamides as side products or as the main product in the case of **11a**. In the search for an appropriate reaction medium to obtain sufficient solubility of both substrates and to avoid these side reactions, the use of DMSO at 153 °C and equimolar amounts of triethylamine were found to afford **15 a–e** from the corresponding hydrochloride substrates **11a–e**.

So far, the decarboxylation of fluorine-activated phthalamic acids has not been used for the synthesis of specific compounds. It was, however, observed as an undesired reaction in the purification of solution-phase reactions using tetrafluorophthalic anhydride,^[23] and also observed when tetrafluorophthalic anhydride was reacted with an aminoporphyrine to give the corresponding porphyrin–tetrafluorophenylcarboxamide in 3% yield.^[24]

Use of the ring opening-decarboxylation reaction enabled us to access the novel tetrafluorobenzamides **15** with the complete substitution pattern of compounds **13** and **14**. The three series of barbituric acid derivatives were provided for comparative biological investigations.

The structures of **13–15** were unambiguously assigned by means of ¹H and ¹³C NMR spectroscopy, elemental analyses, and mass spectrometry. In particular, the ¹³C NMR spectra of **14** showed three distinct signals—two multiplets and one doublet—for the carbon atoms of the fused tetrafluorobenzene ring. Tetrafluorobenzamides **15**, on the other hand, gave six distinct shifts of the carbon resonances, which appear as five multiplets and one doublet. The carbon–fluorine coupling constant of directly bound nuclei was observed between 240–268 Hz. The proton at position 6 of tetrafluorobenzamides **15** gave a multiplet in the range of 7.51–7.61 ppm.^[25]

To confirm the structures of tetrafluorophthalimides and tetrafluorobenzamides and to characterize the different structural features, single-crystal X-ray analyses were carried out on the representative compounds **14c** and **15d**. In the case of the tetrafluorophthalimide derivative **14c** (Figure 2), a mean plane calculated through all barbituric acid atoms except H, R¹ and R² makes an angle of 86° with the nearly planar phthalimide moiety. This perpendicular orientation is similar to the crystal structure of thalidomide ^[26] The maximum deviation from planarity in the barbituric acid part of **14c** was 0.368 Å. Its four shortened CO–N bonds vary in their lengths between 1.368 and 1.407 Å corresponding to the triketo tautomer with contributing resonance structures. In compound **14c** with all hydrogen atoms of the phthalimide being replaced by fluorine, further intermolecular interactions in the crystal unit are expected. Pairs of centrosymmetrically related molecules are associated into hydrogen bonded dimers through N–H...O=C(2) interactions and make additional C–H...F and C–H...O contacts; the non-*peri*-substituted fluorines interact with the isopropyl moiety which is linked to the O=C(4) oxygen atom of a third molecule.

The molecular structure of **15d** was also revealed by X-ray diffraction (Figure 3). As expected, the amide group is not co-planar with the tetrafluorobenzene ring, and the dihedral angle of both is 27.3° a value similar to that of other aromatic carboxamides.^[27] The amide oxygen atom is oriented toward the aromatic hydrogen, while the amide hydrogen is incorporated in an intramolecular hydrogen bond of type N–H...F–C(2) with a H...F distance of 2.24 Å. In a related 2,3,4,5-tetrafluorobenzamide, the *ortho*-fluorine also acted as hydrogen bond acceptor.^[28] We observed a significant deviation from planarity for the barbituric acid part, but the maximum deviation of 0.174 Å was smaller than in the case of **14c**. The four CO–N bond lengths vary from 1.366 to 1.392 Å. The planes of the carboxamide and barbituric acid part are twisted with respect to each other by 89.9°. As noted for **14c**, face-to-face dimerizaion of the barbituric acid motifs of **15d** occurs via N–H...O=C(2') contacts. Besides the intramolecular hydrogen bond to the acceptor fluorine F–(2), the amide hydrogen atom forms an intermolecular contact to carbonyl oxygen O=C(4'). This three-center (bifurcated) hydrogen bond accounts for the extended stacking network of **15d**. Some structures with unsymmetrical bifurcated hydrogen bonds to fluorine and oxygen have been reported.^[29]

The ten representatives of each chemotype, phthalimidobarbituric acids **13**, tetrafluorophthalimidobarbituric acids **14**, and tetrafluorobenzamidobarbituric acids **15**, were evaluated to determine their anti-angiogenic activities. The compounds were tested in the rat aortic ring assay at a concentration of 50 µM. Their inhibitory potency is reflected by a decrease in vascular outgrowth. 5-Amino-1-[[3,5-dichloro-4-(4-chlorobenzoyl)phenyl]methyl]–1 *H*-1,2,3-triazole-4-carboxamide carboxyamidotriazole (CAI), a synthetic, cytostatic inhibitor of non-voltage-operated calcium channels and calcium channel-mediated signaling pathways and known anti-angiogenic agent, was used as positive control. The vascular outgrowth was quantified as percentage of outgrowth compared with the outgrowth from control ring explants (Table 1).

Examination of the biological data clearly indicated that nearly all phthalimides **13** were inactive as inhibitors of angiogenesis, independently from substituents at positions CS and NI. Out of this subseries, only **13b** was weakly active and showed more than 40% inhibition of the microvessel outgrowth. In contrast, the fluorinated counterparts **14**, without exception, were identified as excellent inhibitors of the vascular outgrowth. The majority of the compounds **14** exhibited more than 90% inhibition, with methyl, propyl, and isopropyl compounds being particularly potent. The activity of the 1-phenyl derivative **14k** was improved due to the introduction of the cycloaliphatic residue present at this position in compound **14i**. Among the new benzamides **15**, the most potent compounds were **15c**, **15d**, **15e**, **15g**, and **15i**, all leading to 10% or less vascular growth.

The anti-angiogenic activity of the tetrafluorophthalimide derivative **14k** has already been confirmed by the HUVEC tube formation assay, in which **14k**, at a concentration of 12.5 µM, inhibited the tube formation of human umbilical vein endothelial cells by 93%.^[11] A preselection of four tetrafluoro inhibitors was previously investigated in further assays to characterize the anti-angiogenic and antiproliferative activities in greater detail.^[14] Using the NCI60 high-throughput screen (NCI, Frederick, MD, USA) for anticancer activity, phthalimides **14e** and **14f** were shown to be more potent than the benzamide derivatives **15d**

and **15g** in 59 tumor cell lines.^[31] The GI₅₀ (50% growth inhibition) values of **14e** and **14f** in the NCI60 screen were 2.21 and 5.12 μ M, respectively. Among the four compounds, the tetrafluorophthalimide **14e** was most efficient in inhibiting HUVEC cell proliferation. The anti-angiogenic activity in transgenic *flil*:EGFP zebrafish embryos was investigated, and the number of intersomitic blood vessels was decreased upon treatment with **14e**, **14f**, and **15g**. Notably, the tetrafluorobenzamide derivative **15d** significantly decreased the tumor size in a human prostate cancer mouse xenograft model.

Moreover, the tetrafluorophthalimide derivative **14k** was subjected to a study aimed at assessing its antileukemic activity.^[32] Compound **14k** was described as a member of a class of redox-reactive thalidomide analogues with the capacity to upregulate nuclear factor of activated T-cells (NFAT) transcriptional pathways.

Based on a comparison of phthalimide derivatives,^[14] it has already been suspected that the chemotype of tetrafluorophthalimidobarbituric acids is privileged not only because of the fluorine introduction, but also due to the presence of adequate substituents at the phthalimide nitrogen atom. The complete data presented in this report clearly confirm the suitability of the structural assembly of chemotype **14**. It is likely that the barbiturate substructure provides their heteroatoms for potential hydrogen bonding to contribute to protein–ligand interactions.

Overall, the representatives of chemotype **15** share the biological activity of derivatives **14**. These tetrafluorobenzamide derivatives, obtained by a formal ring opening and the concomitant loss of a CO unit, have an increased degree of conformational freedom which might explain certain differences in some assays. Tetrafluorophthalimide derivatives **14** exhibit a molecular structure similar to that of thalidomide as could be concluded from the X-ray analysis of **14c** reported herein. The X-ray crystal structure of one tetrafluorobenzamide representative, **15d**, evidencing a deviant molecular geometry, has also been solved in the course of this study. However, because of the structural flexibility of compounds **15**, they might adopt a bioactive conformation different from that of the crystalline form when bound to a protein target. Hence, both chemotypes are capable of making use of the tetrafluorobenzene as well as the barbiturate substructure.

Conclusions

In general, the results summarized in Table 1 clearly show the presence of the tetrafluoro substitution pattern to be an essential feature for bioactivity. The structurally analogous non-fluorinated compounds **13** lost the anti-angiogenic properties. Several studies have underlined that the introduction of fluorine in place of hydrogen results in enhanced biological activities, and the identification of fluorophilic binding areas in protein targets has emerged as a powerful strategy in medicinal chemistry.^[33] Further investigations to identify the targets of the tetrafluorinated IMiDs **14** and **15** are underway in our research groups.

Experimental Section

Melting points were determined on a Gallenkamp capillary melting point apparatus or on a Rapido Boetius apparatus, and are uncorrected. The purity of the obtained substances and the composition of the reaction mixtures were controlled by thin-layer chromatography (TLC) using Merck aluminum silica gel plates with 60 F₂₅₄ indicator. Preparative column chromatography was performed using Merck silica gel 60 (63–200 mesh). Petroleum ether (PE) used was a mixture of alkanes boiling between 40–60 °C, according to the supplier's declarations. NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer at 500 MHz (¹H NMR) and 125 MHz (¹³C NMR) in [D₆]DMSO. Chemical shifts (δ) are reported in ppm. Spin multiplicities are indicated by the following symbols: s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), s (sextet), sept (septet), m (multiplet). All multiplets related with ¹J_(C,F) coupling in ¹³C NMR spectra are centered. Mass spectra were recorded on an MS-50 A.E.I. spectrometer under electron impact ionization (EI) at 70 eV. Elemental analyses were performed with a Vario EL apparatus. Compound **13 k** was available from a previous study.^[10] Isopropylurea (11.15 g, 109.16 mmol) was obtained by bubbling gaseous NH₃ through a solution of isopropylisocyanate (10.22 g, 120 mmol) in anhydrous CH₂Cl₂ (200 ml). The resulting precipitate was collected by suction filtration and washed with CH₂Cl₂.

Chemistry

Diethyl 2-acetylamino-2-methylmalonate (1). Diethyl acetylaminomalonate (21.72 g, 100 mmol) and methyl iodide (6.9 mL, 15.61 g, 110 mmol) were added to a solution of NaOEt in EtOH (200 mL, 0.55 M, 1.1 equiv). The reaction mixture became orange and was stirred at reflux for 12 h. EtOH was removed under reduced pressure. After the addition of CH₂Cl₂ (300 mL) the insoluble material was separated by filtration. The filtrate was evaporated to dryness and the residue was recrystallized from water to give **1** as colorless crystals (13.35 g, 58%); mp: 79–85 °C, lit. mp: 88–90 °C.^[17]

Diethyl 2-acetylamino-2-ethylmalonate (2). Diethyl acetylaminomalonate (21.72 g, 100 mmol) and ethyl bromide (11.99 g, 110 mmol) were added to a solution of NaOEt in EtOH (200 mL, 0.55 M, 1 equiv). The reaction mixture was stirred at reflux for 12 h. EtOH was removed under reduced pressure. After the addition of CH₂Cl₂ (300 mL) the insoluble material was separated by filtration. Silica gel (4 g), Na₂SO₄ (10 g) and charcoal (4 g) were added to the filtrate. The mixture was filtered to obtain a colorless solution. Evaporation of the solvent and drying over CaCl₂ gave **2** as a colorless solid (22.12 g, 82%), which was used without further purification; mp: 64–68 °C, lit. mp: 83 °C.^[17]

Diethyl 2-[(*tert*-butyloxycarbonyl)amino]-2-ethylmalonate (3). A solution of diethyl 2-[(*tert*-butyloxycarbonyl)amino]malonate (27.53 g, 100 mmol) in EtOH (50 mL) was added dropwise to a solution of NaOEt in EtOH (122 mL, 0.90M, 1.1 equiv). After 30 min, ethyl bromide (11.99 g, 110 mmol) was added and the reaction mixture was held at reflux for 7 h. After removal of the precipitated NaBr, the resulting filtrate was poured into brine (250 mL) and the aqueous layer was extracted with EtOAc (5 × 100 mL). The combined organic extracts were washed with water (2 × 100 mL) and brine (2 × 100 mL), dried over Na₂SO₄, and filtered after addition of charcoal. Evaporation to dryness under reduced pressure

afforded **3** (11.12 g, 40 %) as a yellow oil which was used for the next step without further purification.

Synthesis of barbituric acids 10a–k: General Procedure. Diethyl 2-acetylamino-2-methylmalonate **1** (9.25 g, 40 mmol) or diethyl 2-acetylamino-2-ethylmalonate **2** (9.81 g, 40 mmol) or diethyl 2-[(*tert*-butyloxycarbonyl)amino]-2-ethylmalonate **3** (12.13 g, 40 mmol) and 40 mmol of the appropriate urea (methylurea 2.96 g; ethylurea 3.52 g; *n*-propylurea 4.09 g; isopropylurea 4.09 g; cyclohexylurea 5.69 g; phenylurea 5.45 g) were added to a solution of NaOEt in EtOH (200 ml, 0.24 M, 1.2 equiv). The resulting mixture was stirred at 78 °C for 3 h. The clear, yellow solution was then evaporated in vacuo. The oily residue was dissolved in a minimum amount of water, subsequently acidified to pH 2–3 by dropwise addition of HCl (2 M) and kept overnight at 5 °C. The precipitated product was isolated by filtration and dried under reduced pressure. If no precipitation occurred, the solution was extracted with EtOAc (5 × 20 ml). The organic layer was separated, dried (Na₂SO₄), filtered, and evaporated to dryness. Pure products were used in the next step without further purification. Otherwise, further treatment or recrystallization was performed as indicated below.

5-Acetylamino-1,5-dimethylbarbituric acid (10 a) was prepared from **1** and methylurea (**4**); yield 50% (4.80 g); mp: 123–129 °C.

5-Acetylamino-5-methyl-1-propylbarbituric acid (10 b) was prepared from **1** and *n*-propylurea (**6**); yield 37% (3.84 g); mp: 207–209 °C.

5-Acetylamino-1-isopropyl-5-methylbarbituric acid (10 c) was obtained from **1** and isopropylurea (**7**); yield 58% (5.60 g); mp: > 250 °C, lit. mp: > 230 °C.^[19]

5-Acetylamino-1,5-diethylbarbituric acid (10 d) was obtained from **2** and ethylurea (**5**); yield 21 % (2.03 g); mp: 189–194 °C.

5-Acetylamino-5-ethyl-1-propylbarbituric acid (10 e). Reaction of **2** and *n*-propylurea **6** following the General Procedure gave **10e**, which was purified by recrystallization from EtOH; yield 22 % (2.25 g); mp: 165–168 °C.

5-Acetylamino-1-ethyl-5-methylbarbituric acid (10 f) was prepared from **1** and ethylurea (**5**); yield 51 % (4.82 g); mp: 138–141 °C.

5-Acetylamino-5-ethyl-1-isopropylbarbituric acid (10 g). Compound **2** was reacted with isopropylurea (**7**) using the General Procedure. Extraction with EtOAc (5 × 20 ml) gave a yellow oil. Crystallization from EtOAc/hexane afforded **10g**; yield 13% (1.33 g); mp: 195–198 °C.

5-Acetylamino-1-cyclohexyl-5-methylbarbituric acid (10 h). This compound was obtained from **1** and cyclohexylurea (**8**) using the General Procedure to give **10 h**, which was purified by recrystallization from EtOH; yield 45 % (5.06 g); mp: > 250 °C.

5-Acetylamino-1-cyclohexyl-5-ethylbarbituric acid (10 i). Reaction of **2** and cyclohexylurea (**8**) following the General Procedure yielded **10i**, which was purified by recrystallization from EtOH; yield 24% (2.84 g); mp: > 250 °C, lit. mp: 282–288 °C.^[34]

5-[(*tert*-Butyloxycarbonyl)amino]-5-ethyl-1-phenylbarbituric acid (10 k). This compound was obtained from **3** and phenylurea (**9**) using the General Procedure except that the reaction was run for 5 h to give **10 k**, which was purified by recrystallization from EtOH; yield 26% (3.61 g); mp: 195–200 °C.

Synthesis of 5-aminobarbituric acid hydrochlorides 11 a-i: General Procedure. A mixture of the corresponding 5-acetylamino-1-cyclohexyl-5-ethylbarbituric acid **10** (3.5 mmol) and HCl (25 ml, 8 M) was stirred under reflux for 7 h. The hot, colorless solution was then allowed to cool down to room temperature, the resulting colorless solid was filtered off and dried under reduced pressure. The filtrate was concentrated in vacuo to less than half of its volume and kept at 5 °C overnight. The precipitate that formed was collected by filtration, dried and combined with the first fraction of the product. The crude material was pure and was used for the next step without further purification.

5-Amino-1,5-dimethylbarbituric acid hydrochloride (11 a). Yield 66% (0.48 g); mp: > 250 °C.

5-Amino-5-methyl-1-propylbarbituric acid hydrochloride (11 b). Yield 73% (0.65 g); mp: > 250 °C.

5-Amino-1-isopropyl-5-methylbarbituric acid hydrochloride (11 c). Yield 50% (0.41 g); mp: > 250 °C.

5-Amino-1,5-diethylbarbituric acid hydrochloride (11 d). Yield 75 % (0.62 g); mp: > 250 °C.

5-Amino-5-ethyl-1-propylbarbituric acid hydrochloride (11 e). Yield 79% (0.69 g); mp: > 250 °C.

5-Amino-1-ethyl-5-methylbarbituric acid hydrochloride (11 f). Yield 65 % (0.50 g); mp: > 250 °C.

5-Amino-5-ethyl-1-isopropylbarbituric acid hydrochloride (11 g). Yield 42% (0.37 g); mp: 248–252 °C.

5-Amino-1-cyclohexyl-5-methylbarbituric acid hydrochloride (11 h). Yield 64 % (0.62 g); mp: > 250 °C.

5-Amino-1-cyclohexyl-5-ethylbarbituric acid hydrochloride (11 i). This compound was prepared according to the General Procedure except that the starting **10i** was dissolved in EtOH (25 mL) and then HCl (200 ml, 8 M) was added; yield 64% (0.65 g); mp: > 250 °C.

5-Amino-5-ethyl-1-phenylbarbituric acid hydrochloride (11 k). Compound **10 k** (1.22 g, 3.5 mmol) was dissolved in a solution of HCl in 1,4-dioxane (14 ml, 4 M) and stirred at

room temperature for 2 h. The colorless solid that formed was filtered off, washed with PE and dried under reduced pressure to give **11 k** (0.93 g, 88%); mp: 227–233 °C.

Synthesis of 5-aminobarbituric acids 12 f-k: General Procedure. The corresponding 5-aminobarbituric acid hydrochloride **11** (3.5 mmol) was dissolved in a minimum volume of water. The insoluble material was separated by filtration and the acidic filtrate (pH 2–3) was adjusted to pH 6 by dropwise addition of aqueous NaOH (2 M) under stirring in an ice-bath. The resulting colorless solid was collected by filtration and dried under reduced pressure. The obtained material was pure and was directly used in the next step unless stated otherwise.

5-Amino-1-ethyl-5-methylbarbituric acid (12 f). Yield 39% (0.25 g); mp: 185–191 °C.

5-Amino-5-ethyl-1-isopropylbarbituric acid (12 g). Yield 52 % (0.39 g); mp: 140–142 °C.

5-Amino-1-cyclohexyl-5-methylbarbituric acid (12 h). Yield 96 % (0.80 g); mp: 202–203 °C.

5-Amino-1-cyclohexyl-5-ethylbarbituric acid (12 i). Yield 97% (0.86 g); mp: 160–164 °C, lit. mp: 171–173 °C.^[19]

5-Amino-5-ethyl-1-phenylbarbituric acid (12 k). The crude product was recrystallized from EtOH; yield 37% (0.32 g); mp: 214–217 °C, lit. mp: 192–193 °C.^[19]

Preparation of 5-phthalimidobarbituric acids 13a–e from 5-aminobarbituric acid hydrochlorides 11a–e: General Procedure. A mixture of the corresponding 5-aminobarbituric acid hydrochloride **11** (1.50 mmol), phthalic anhydride (0.22 g, 1.50 mmol) and Et₃N (0.15 g, 1.50 mmol) in DMF (11 ml) was stirred under reflux for 5 h. The reaction mixture was then allowed to cool down to room temperature and poured into water (50 mL). The colorless precipitate that formed was collected by filtration, washed with water and dried under reduced pressure. The obtained material was pure unless stated otherwise.

5-(1,3-Dihydro-1,3-dioxo-2H-isoindol-2-yl)-1, 5-dimethyl-2,4,6(1H,3H,5H)-pyrimidinetrione (13 a). Yield 40% (0.18 g); mp: > 250 °C.

5-(1,3-Dihydro-1,3-dioxo-2H-isoindol-2-yl)-5-methyl-1-propyl-2,4,6(1H,3H,5H)-pyrimidinetrione (13b). Yield 40% (0.20 g); mp: 188–190 °C.

5-(1,3-Dihydro-1,3-dioxo-2H-isoindol-2-yl)-1-isopropyl-5-methyl-2,4,6(1H,3H,5H)-pyrimidinetrione (13c). Yield 36% (0.18 g); mp: 221–224 °C.

1,5-Diethyl-5-(1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)-2,4,6(1H,3H,5H)-pyrimidinetrione (13 d). Yield 28% (0.14 g). An analytical sample was obtained by column chromatography using PE/EtOAc (4:1); mp: 201–205 °C.

5-(1,3-Dihydro-1,3-dioxo-2H-isoindol-2-yl)-5-ethyl-1-propyl-2,4,6(1H,3H,5H)-pyrimidinetrione (13 e). Yield 21 % (0.11 g); mp: 185–187 °C.

Preparation of 5-phthalimidobarbituric acids 13 f–i from 5-aminobarbituric acids 12 f–i: General Procedure. A mixture of the corresponding 5-aminobarbituric acid **12** (1.50 mmol), phthalic anhydride (0.22 g, 1.50 mmol) in DMF (11 mL) was stirred under reflux for 5 h. The reaction mixture was then cooled to room temperature and poured into water (50 mL). The colorless precipitate that formed was collected by filtration, washed with water and dried under reduced pressure. The obtained material was pure unless stated otherwise.

5-(1,3-Dihydro-1,3-dioxo-2H-isoindol-2-yl)-1-ethyl-5-methyl-2,4,6(1H,3H,5H)-pyrimidinetrione (13 f). Yield 48 % (0.23 g); mp: 235–238 °C.

5-(1,3-Dihydro-1,3-dioxo-2H-isoindol-2-yl)-5-ethyl-1-isopropyl-2,4,6(1H,3H,5H)-pyrimidinetrione (13 g). Yield 23% (0.12 g). An analytical sample was obtained by column chromatography using PE/EtOAc (4:1); mp: 212–215 °C.

1-Cyclohexyl-5-(1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)-5-methyl-2,4,6(1H,3H,5H)-pyrimidinetrione (13 h). The crude material was recrystallized from PE/EtOAc (4:3); yield 49% (0.27 g); mp: 241–244 °C.

1-Cyclohexyl-5-(1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)-5-ethyl-2,4,6(1H,3H,5H)-pyrimidinetrione (13 i). This compound was prepared according to the General Procedure from **12i** (0.38 g, 1.50 mmol) and phthalic anhydride (0.22 g, 1.50 mmol). The crude product was recrystallized from EtOH to give 81 mg of **13 i**. After the removal of the crude product, *N*-(1-cyclohexyl-5-ethylhexahydro-2,4,6-trioxypyrimidin-5-yl)phthalamic acid precipitated from the DMF/H₂O filtrate after standing at room temperature for three days. The isolated compound (0.45 g, 1.12 mmol) was transformed into the desired phthalimidobarbituric acid **13 i** by heating at 85 °C for 10 min in Ac₂O (7 mL). After cooling, the precipitated material was collected and washed with a mixture of hexane/Et₂O (1:1) to give 0.34 g of the final product **13i**. Total yield 73%; mp: 245–250 °C, lit. mp: 248–253 °C.^[8]

Preparation of 5-(tetrafluorophthalimido)barbituric acids 14a–e from 5-aminobarbituric acid hydrochlorides 11 a–e: General Procedure. A mixture of the corresponding 5-aminobarbituric acid hydrochloride **11** (1.50 mmol), tetrafluorophthalic anhydride (0.40 g, 1.80 mmol) and Et₃N (0.15 g, 1.50 mmol) in glacial AcOH (11 mL) was stirred under reflux for 3 h. The yellow solution was then allowed to cool down to room temperature and evaporated to dryness under reduced pressure. The oily residue was recrystallized from 70% EtOH to give 5-tetrafluorophthalimides **14a–e** as colorless crystals.

1,5-Dimethyl-5-(4,5,6,7-tetrafluoro-1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)-2,4,6-(1H,3H,5H)-pyrimidinetrione (14 a). Yield 47 %
(0.22 g); mp: 168–172 °C.

5-Methyl-1-propyl-5-(4,5,6,7-tetrafluoro-1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)-2,4,6-(1H,3H,5H)-pyrimidinetrione (14 b). Yield 42% (0.25 g); mp: 173–177 °C.

1-Isopropyl-5-methyl-5-(4,5,6,7-tetrafluoro-1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)-2,4,6(1H,3H,5H)-pyrimidinetrione (14 c). Yield 42% (0.25 g); mp: 184–187 °C.

1,5-Diethyl-5-(4,5,6,7-tetrafluoro-1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)-2,4,6-(1H,3H,5H)-pyrimidinetrione (14d). Yield 23 % (0.14 g); mp: 152–154 °C.

5-Ethyl-1-propyl-5-(4, 5,6,7-tetrafluoro-1,3-dihydro-1, 3-dioxo-2H-isoindol-2-yl)-2,4,6(1H,3H,5H)-pyrimidinetrione (14 e). Yield 47% (0.29 g); mp: 137–140 °C.

Preparation of 5-(tetrafluorophthalimido)barbituric acids 14 f–k from 5-aminobarbituric acids 12 f–k: General Procedure. A mixture of the corresponding 5-aminobarbituric acid **12** (1.50 mmol) and tetrafluorophthalic anhydride (0.40 g, 1.80 mmol) in glacial AcOH (11 mL) was stirred under reflux for 3 h. The yellow solution was then cooled and evaporated to dryness under reduced pressure. The oily residue was recrystallized from EtOH to give 5-tetrafluorophthalimides **14 f–k** as colorless crystals.

1-Ethyl-5-methyl-5-(4,5,6,7-tetrafluoro-1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)-2,4,6(1H,3H,5H)-pyrimidinetrione (14f). Yield 55 % (0.32 g); mp: 178–184 °C.

5-Ethyl-1-isopropyl-5-(4,5, 6,7-tetrafluoro-1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)-2,4,6(1H,3H,5H)-pyrimidinetrione (14g). Yield 56% (0.35 g); mp: 134–139 °C.

1-Cyclohexyl-5-methyl-5-(4,5,6,7-tetrafluoro-1,3-dihydro-1, 3-dioxo-2H-isoindol-2-yl)-2,4,6(1H,3H,5H)-pyrimidinetrione (14h). Yield 46% (0.31 g); mp: 208–211 °C.

1-Cyclohexyl-5-ethyl-5-(4,5,6,7-tetrafluoro-1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)-2,4,6(1H,3H,5H)-pyrimidinetrione (14i). Yield 73% (0.50 g); mp: 172–176 °C.

5-Ethyl-1-phenyl-5-(4,5, 6,7-tetrafluoro-1,3-dihydro-1,3-dioxo-2H-isoindol-2-yl)-2,4,6(1H,3H,5H)-pyrimidinetrione (14 k). Yield 59% (0.40 g); mp: 209–215 °C, lit. mp: 218–220 °C.^[8]

Preparation of 5-(2,3,4,5-tetrafluorobenzamido)barbituric acids 15a–e from 5-aminobarbituric acid hydrochlorides 11 a–e: General Procedure. A mixture of the corresponding 5-aminobarbituric acid hydrochloride **11** (2 mmol), tetrafluorophthalic anhydride (0.44 g, 2 mmol), Et₃N (0.20 g, 2 mmol) and DMSO (3 mL) was stirred and heated on an oil bath at 153 °C for 5 h. The reaction mixture was then allowed to cool down to room temperature and poured into water (10 mL). The oil that immediately formed was carefully removed from the solution, from which a solid precipitated after four days standing at room temperature. The precipitate was collected by filtration, washed with water and dried under reduced pressure. The obtained material was pure unless stated otherwise.

N-(Hexahydro-1,5-dimethyl-2,4, 6-trioxo-5-pyrimidinyl)-2,3,4,5-tetrafluorobenzamide (15a). Yield 29% (0.21 g); yellow crystals; mp: 219–222 °C.

N-(Hexahydro-5-methyl-2,4,6-trioxo-1-propyl-5-pyrimidinyl)-2,3,4,5-tetrafluorobenzamide (15b). Yield 17% (0.14 g); yellow crystals; mp: 83–87 °C.

***N*-(Hexahydro-1-isopropyl-5-methyl-2,4,6-trioxo-5-pyrimidinyl)-2,3,4,5-tetrafluorobenzamide (15c).** The crude material was recrystallized from PE/EtOAc (1:1); yield 7% (54 mg); colorless crystals; mp: 225–229 °C.

***N*-(1,5-Diethyl-hexahydro-2,4,6-trioxo-5-pyrimidinyl)-2,3,4,5-tetrafluorobenzamide (15d).** The crude material was recrystallized from PE/EtOAc (3:1); yield 13 % (0.10 g); light brown crystals; mp: 163–167 °C.

***N*-(5-Ethyl-hexahydro-2,4, 6-trioxo-1-propyl-5-pyrimidinyl)-2,3,4,5-tetrafluorobenzamide (15 e).** Yield 12% (0.10 g); colorless crystals; mp: 79–82 °C.

Preparation of 5-(2,3,4,5-tetrafluorobenzamido)barbituric acids 15 f–k from 5-aminobarbituric acids 12 f–k: General Procedure. A mixture of the corresponding 5-aminobarbituric acid **12** (2 mmol), tetrafluorophthalic anhydride (0.44 g, 2 mmol) and DMF (14 ml) was stirred under reflux for 5 h. The yellow solution was then allowed to cool down to room temperature and poured into water (50 mL). The precipitate that formed was collected by filtration and dried under reduced pressure. The obtained material was pure unless stated otherwise.

***N*-(1-Ethyl-hexahydro-5-methyl-2,4,6-trioxo-5-pyrimidinyl)-2,3,4,5-tetrafluorobenzamide (15 f).** Yield 87 % (0.63 g); colorless crystals; mp: 207–212 °C.

***N*-(5-Ethyl-hexahydro-1-isopropyl-2,4,6-trioxo-5-pyrimidinyl)-2,3,4,5-tetrafluorobenzamide (15 g).** Yield 91 % (0.71 g), colorless crystals; mp: 166–171 °C.

***N*-(1-Cyclohexyl-hexahydro-5-methyl-2,4,6-trioxo-5-pyrimidinyl)-2,3,4,5-tetrafluorobenzamide (15 h).** Yield 92 % (0.76 g); yellow crystals; mp: 232–234 °C.

***N*-(1-Cyclohexyl-5-ethyl-hexahydro-2,4,6-trioxo-5-pyrimidinyl)-2,3,4,5-tetrafluorobenzamide (15 i).** Yield 72 % (0.62 g); colorless crystals; mp: 210–212 °C.

***N*-(5-Ethyl-hexahydro-2,4, 6-trioxo-1-phenyl-5-pyrimidinyl)-2,3,4,5-tetrafluorobenzamide (15 k).** The crude material was recrystallized from PE/EtOAc (2:1); yield 32% (0.28 g); yellow crystals; mp: 210–215 °C.

Biological investigations

Rat aortic ring assay: To determine the extent of the anti-angiogenic effects of the test compounds, the rat aortic ring assay was carried out as described elsewhere.^[10,14] Briefly, 12-well tissue culture plates were covered with 250 µL of Matrigel (Becton-Dickinson) and allowed to gel for 30 to 45 min at 37 °C and 5 % CO₂. Sections of thoracic aorta were removed from 8- to 10-week-old male Sprague–Dawley rats. Following excision of fibroadipose tissue, the aortic sections were cut into 1-mm-long cross-sections, placed on Matrigel-coated wells, and layered with additional Matrigel (250 µl). These were then allowed to set, after which the cross-sectional rings were covered with endothelial cell growth media (EGM-II) and incubated under 5 % CO₂ at 37 °C overnight. EGM-II consists of endothelial cell basal medium (EBM-II) and endothelial cell growth factors. After 24 h, the medium was removed and replaced with EBM-II (1 mL), supplemented with fetal bovine

serum (2%), amphotericin **B** (0.25 $\mu\text{g mL}^{-1}$), and gentamicin (1 $\mu\text{g mL}^{-1}$). The aortic rings were treated daily with vehicle (0.5 % DMSO), CAI (12.5 $\mu\text{g mL}^{-1}$; positive control), test compounds, each at a dose of 50 μM , for four days. This was replicated four times using aortas from four different rats. The area of angiogenic sprouting was quantified using Adobe Photoshop.

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- [30]. The X-ray crystallographic data collection for compounds **14c** and **15d** was performed on a Bruker D8 Venture diffractometer at 100(2) K. The diffractometer was equipped with a low-temperature device (Cryostream 800er series, Oxford Cryosystems) and used $\text{CuK}\alpha$ radiation ($\lambda = 1.54178 \text{ \AA}$). Intensities were measured by fine-slicing ϕ - and ω -scans and corrected for background, polarization, and Lorentz effects. Semi-empirical absorption corrections were applied for all data sets by using Bruker's SADABS program. The structures were solved by direct methods and refined anisotropically by the least-squares procedure implemented in the ShelX-2014/7 program system. Hydrogen atoms were included isotopically using the riding model on the bound carbon atoms. CCDC 1506731 (**14c**) and 1506732 (**15d**) contain the supplementary crystallographic data for this paper. These data are provided free of charge by The Cambridge Crystallographic Data Centre.
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Figure 1.
Structures of thalidomide and selected IMiDs.

Figure 2.

Top: Molecular plot of 1-isopropyl-5-methyl-5-(4,5,6,7-tetrafluoro-1,3-dihydro-1,3-dioxo-2*H*-isoindol-2-yl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione (**14c**) showing the displacement ellipsoids at the 50% probability level for non-hydrogen atoms. Hydrogen atoms are depicted as white spheres of arbitrary radii. Bottom: Packing diagram of **14c** along the *y*-axis. The C–H...F, C–H...O and N–H...O contacts are shown as broken lines.^[30]

Figure 3.

Top: Molecular plot of *N*-(1,5-diethylhexahydro-2,4,6-trioxo-5-pyrimidinyl)-2,3,4,5-tetrafluorobenzamide (**15d**) showing the displacement ellipsoids at the 50 % probability level for non-hydrogen atoms. The non-aliphatic hydrogen atoms are depicted as white spheres of arbitrary radii. Bottom: Packing diagram of **15d** along the z-axis. The N–H...F and N–H...O contacts are shown as broken lines.^[30]

Scheme 1.

Synthesis of 5-aminobarbituric acids **11** and **12**. *Reagents and conditions:* a) NaOEt, EtOH, reflux, 3 h; b) HCl (8 M), reflux, 7 h for **10a–i**, HCl/dioxane (4 M), RT, 2 h; c) NaOH, 0 °C.

Scheme 2.

Synthesis of 5-phthalimidobarbituric acids **13**, 5-tetrafluorophthalimidobarbituric acids **14**, and 5-(2,3,4,5-tetrafluorobenzamido)barbituric acids **15**. *Reagents and conditions*: a) phthalic anhydride, Et_3N , DMF, 153 °C, 5 h; b) phthalic anhydride, DMF, 153 °C, 5 h; for **13 k**, see ref. [8]; c) tetrafluorophthalic anhydride, Et_3N , AcOH, reflux, 3 h; d) tetrafluorophthalic anhydride, AcOH, reflux, 3 h; e) tetrafluorophthalic anhydride, Et_3N , DMSO, 153 °C, 5 h; f) tetrafluorophthalic anhydride, DMF, 153 °C, 5 h.

Table 1.

Inhibitory effects of 5-phthalimidobarbituric acids **13**, 5-tetrafluorophthalimidobarbituric acids **14**, and 5-(2,3,4,5-tetrafluorobenzamido)barbituric acids **15** on angiogenesis.

<p>13 X = H 14 X = F</p>			15		
Substitution pattern	R ¹	R ²	Inhibition [%] ^[a]		
			13	14	15
a	Me	Me	n.i. ^[b]	>95	42
b	Me	<i>n</i> Pr	51	>95	77
c	Me	<i>i</i> Pr	n.i.	>95	94 ^[c]
d	Et	Et	n.i.	>95	91 ^[d]
e	Et	<i>n</i> Pr	n.i.	94 ^[d]	>95
f	Me	Et	n.i.	94 ^[d]	83 ^[e]
g	Et	<i>n</i> Pr	n.i.	>95	90 ^[d]
h	Me	<i>c</i> Hex	n.i.	88	84 ^[f]
i	Et	<i>c</i> Hex	n.i.	89	90
k	Et	Ph	n.i. ^[g]	76 ^[g]	82 ^[h]

^[a] Percent inhibition of microvessel outgrowth in the rat aortic ring assay. Compounds were administered daily at a concentration of 50 μ M for four days. The control was measured with 0.5 % DMSO in the absence of test compounds and was set as 100 % growth (0 % inhibition). CAI, at a concentration of 12.5 μ g mL⁻¹, gave 96 $\pm$ 8 % inhibition of the vascular outgrowth. Standard deviations were < 4 % unless stated otherwise.

^[b] n.i.: no inhibitory activity (< 40 % inhibition of microvessel outgrowth by the indicated compounds).

^[c] 94 $\pm$ 8 %.

^[d] Data from ref. [14].

^[e] 83 $\pm$ 13 %.

^[f] 84 $\pm$ 9 %.

^[g] Data from ref. [10].

^[h] 82 $\pm$ 16 %.