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# Synthesis and Immunological Activity of Water-Soluble Thalidomide Prodrugs

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**Abstract**—A series of new water-soluble thalidomide prodrugs was prepared. All compounds were derivatized on the nitrogen of the glutarimide ring. Esters of natural amino acids and succinic acid derivatives have been introduced by reaction with the hydroxymethyl thalidomide **2**. Nicotinic acid derivatives were prepared from halomethyl derivatives. Additionally, a methoxymethyl derivative and a carboxymethyl derivative were prepared directly from thalidomide. Most compounds showed a very large increase in water solubility compared to thalidomide itself (0.012 mg/mL). The amorphous hydrochlorides of the *N*-methylalanine ester **8**, valine ester **9**, and glycylglycine ester **10**, respectively, were the most soluble compounds showing solubility greater than 300 mg/mL, which equals an increase greater than 15,000-fold. The lipophilicity of the prodrugs has been determined by their HPLC capacity factors $k'$. The stability of selected compounds was determined. The hydrolysis rates follow pseudo-first order kinetics. In order to assess the immunological activity, the prodrugs were tested using tumor necrosis factor-α and interleukin-2 inhibition assays. Selected compounds were additionally investigated on their abililty to inhibit the local Shwartzman reaction, an assay to determine the vascular permeability. The prodrugs retained high effectiveness in the inhibition of TNF-α release. Our results indicated that the more stable prodrugs exhibited higher activity in the immunological assays. Some compounds showed higher activity than thalidomide itself, suggesting a high affine binding to the pharmacophore. In conclusion, the prodrugs exhibited high water solubility and high activity and might therefore be used in therapeutic applications. 

## Introduction

Thalidomide (2-(2,6-dioxo-3-piperidyl-isoindoline-1,3-dione, Contergan®, **1**) was developed as a sedative by Grünenthal in the 1950's, but was shortly after its introduction removed from the market when its teratogenic side effects best described as heavy dysmelia (stunted limb growth) were discovered.[1–5] In recent years, thalidomide has regained scientific interest because of its effectiveness in various diseases including erythema nodosum leprosum, *graft-vs-host* disease, arthritis, tuberculosis and conditions associated with human immunodeficiency virus infections.[6] In 1998, thalidomide has been reintroduced to the market and is now under clinical investigation for a series of immunomodulatory and anti-inflammatory applications.[7,8]

Due to its bad solubility, the therapeutic use of thalidomide is limited. Attempts to solubilize thalidomide with cyclodextrines did not lead to therapeutic applications.[9,10] The development of water-soluble prodrugs would be a great advantage in order to administer the drug.

Furthermore, water-soluble prodrugs might be valuable tools to elucidate the mechanism of its beneficial and side effects. The immunological effects of thalidomide are partly mediated by modulation of tumor necrosis factor-α (TNF-α). For the rational development of thalidomide derivatives without teratogenic side effects, it is absolutely required to understand its molecular mechanism. Biological experiments with poor soluble drugs such as thalidomide are seldom reproducible and have often led to misinterpretation. Precipitation leads to unreliable biological results. The poor solubility of thalidomide presumably hampered the discovery of its teratogenicity earlier. Besides that, many of the investigated animal

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species did not respond to its teratogenic side effects.

Water-soluble prodrugs of thalidomide should be a valuable tool in the ongoing search for the mechanism of activity and teratogenicity.[11–14] Up to now, more than 30 hypotheses have been proposed. They include hydroxyl radical mediated inhibition of angiogenesis in embryoid bodies, inhibition of growth factors, intercalation with DNA synthesis as well as oxidative damage of DNA. Recently, with the prevention of homodimerization of *N*-cadherin monomers by interaction of thalidomide, instead of the dimerization promoting Trp2/Trp2′ residues, a new concept has been proposed.[13]

In the present study, we have prepared and investigated a series of new water-soluble prodrugs. All variations were introduced at the nitrogen of the glutarimide moiety, enabling most prodrugs to be metabolized to thalidomide. In general, Mannich bases (*N*-hydroxymethyl derivatives) of thalidomide were prepared. Furthermore, ether and carboxylic acid derivatives were synthesized. To enable the identification of the binding site of thalidomide to its orphan receptor(s), one compound for an affinity-labeling assay was additionally developed.

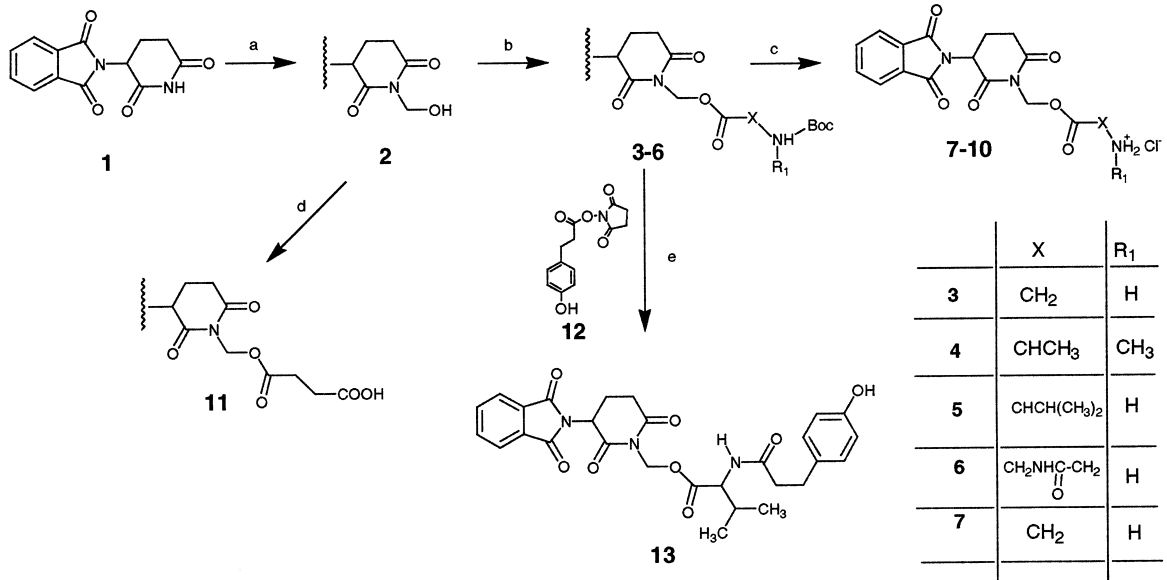
Using the appropriate derivatization, prodrugs improve the physicochemical properties of thalidomide and, thus, bioavailability and membrane transport.[15] This goal was achieved by increasing the water solubility of thalidomide by the introduction of both acidic and basic physiologically derived moieties such as amino acids or nicotinamide. Due to these ionizable groups, the water solubility should be greatly increased. The solubility of the prodrugs has been determined as well as the pH values of the aqueous solutions. In order to assess the lipophilicity and hydrolysis kinetics of the prodrugs, an HPLC method has been developed. Furthermore, we have investigated the immunological effects of the resulting prodrugs with respect to the introduced residues. Derivative **13** was synthesized in order to serve for affinity labeling of the potential biomolecule involved in the teratogenic mechanism.

## Results and Discussion

### Chemistry

Amino acid derived prodrugs **7–10** were synthesized starting from hydroxymethylthalidomide **2** and the appropriate Boc-protected amino acids (Scheme 1) using the dicyclohexylcarbodiimide method.[16–18] To improve yields, 4-pyrrolopyridine was added.[19] Compound **2** was prepared from racemic thalidomide reacted with formaldehyde. The Boc-protection group of **3**, **4**, **5** and **6**, respectively, was removed with trifluoroacetic acid in dichloromethane followed by ion exchange with Amberlite IR 45.[20–22]

Compound **13** was prepared from the Boc-protected valine ester **5** whereby the protection group was also removed with trifluoroacetic acid. The resulting residue was then reacted with the *N*-hydroxysuccinimidyl derivative **12** to afford **13**.[23,24] The succinic acid derivative **11** was synthesized from hydroxymethylthalidomide **2** and succinic anhydride.




| | X | $R_1$ |
|---|---|---|
| 3 | $CH_2$ | H |
| 4 | $CHCH_3$ | $CH_3$ |
| 5 | $CHCH(CH_3)_2$ | H |
| 6 | $CH_2NHC(=O)CH_2$ | H |
| 7 | $CH_2$ | H |
| 8 | $CHCH_3$ | $CH_3$ |
| 9 | $CHCH(CH_3)_2$ | H |
| 10 | $CH_2NHC(=O)CH_2$ | H |

**Scheme 1.** Synthetic route of the amino ester prodrugs **7–10**, the succinyl derivative **11** and the affinity-labeling compound **13**. Reaction conditions: (a) $CH_2O$, $\Delta t$; (b) *N*-Boc-aa, DCC, 4-PP, $CH_2Cl_2$, rt; (c) TFA (25%), $Cl^-$; (d) succinic anhydride, $NEt_3$, 4-PP, $CH_2Cl_2$, rt; (e) TFA, $NaHCO_3$, rt.

In order to prepare the azido derivative **16**, hydroxymethylthalidomide **2** was reacted with thionyl chloride under ice cooling to give the chloromethylthalidomide **14** in good yields (Scheme 2). To increase reactivity, the leaving group of chloromethylderivative **14** was exchanged. Therefore, **14** was reacted with potassium iodide and subsequently with sodium azide in acetone to afford **16.**

The nicotinamide derivative **17** was obtained from chloromethylthalidomide **14** reacted with nicotinamide.[25–27] For the preparation of dihydropyridine derivative **18**, **17** was reduced with sodium dithionite. Similarly, the nicotinic acid derivative **19** was prepared from nicotinic acid and the iodomethyl derivative **15**.

For the synthesis of the carboxymethyl derivative **21**, thalidomide was reacted with sodium hydride and *tert*-butyl iodoacetate in DMF (Scheme 3). Again the protection group was removed with trifluoroacetic acid. The methoxymethyl derivative **22** was prepared from thalidomide and formaldehyde dimethylacetal using phosphorus pentoxide in dichloromethane.[28]

Reagents, yields and analytical data are given in the Experimental section. All analytical data were in accordance with the proposed structures.

## Water solubility

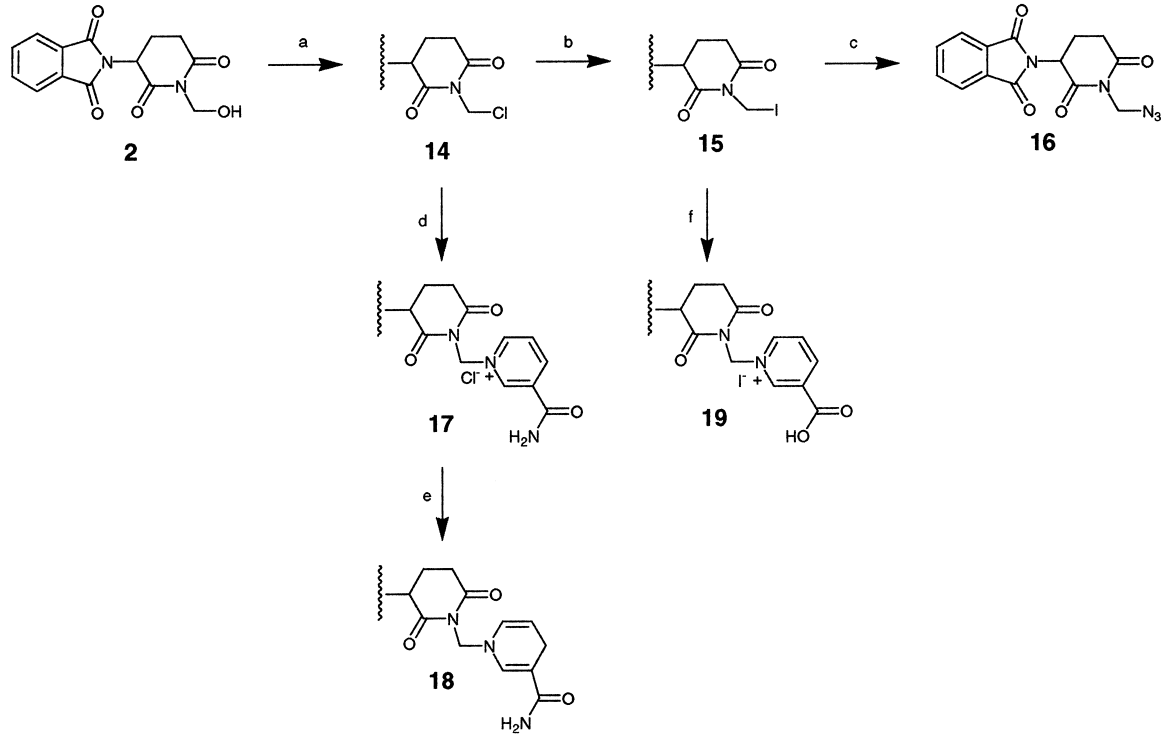
Thalidomide exhibits very low water solubility (0.012 mg/mL) which limits its therapeutic use. The aqueous solubility of selected prodrugs has been determined using UV. As presented in Table 1, the water solubility of the prodrugs has been greatly increased compared to thalidomide. The best soluble compounds were amorphous **8**, **9** and **10** with solubilities greater than 300 mg/mL, equivalent to more than 680 μmol/mL thalidomide. In these cases, the solubility was increased more than 15,000-fold. Solubility is not only dependent on the hydrophilicity of the compounds but also depends on the crystal structure. Due to their better accessibility to solvents, amorphous powders are generally more soluble than highly ordered crystals. The high increase in solubility of the amorphous amino acid ester prodrugs should enable therapeutic use, for example parenteral or topical application without addition of solubilizers like cyclodextrines. The solubility of the crystalline glycine ester **7** was increased 2780-fold, and the solubility of the succinic acid derivative **11** was increased 920-fold. The nicotinamide derivative **17** and the nicotinic acid derivative **19** showed lower increases of water solubility, namely 280 and 170-fold, respectively.

## Determination of the pH values of the aqueous solution

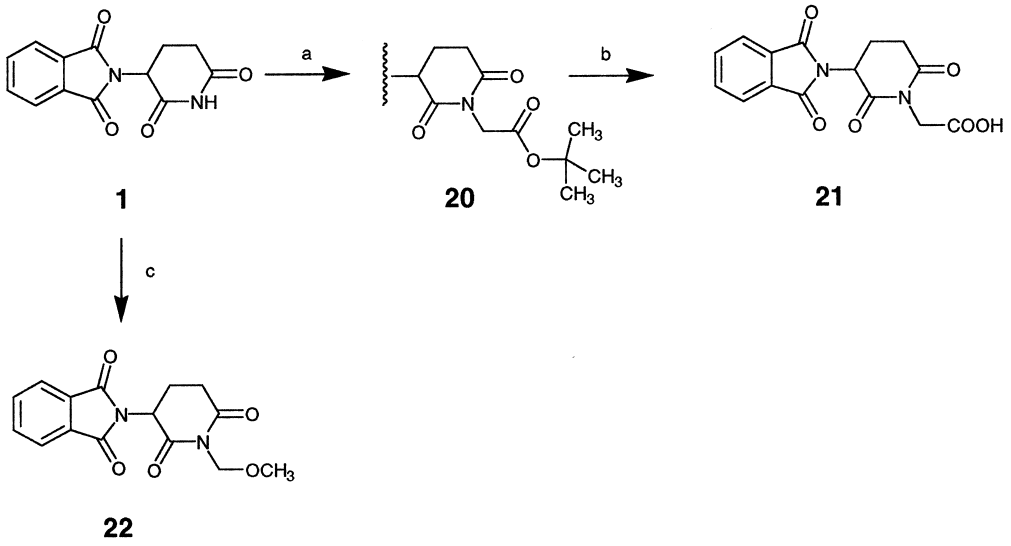
In addition to the water solubility, the pH values of the aqueous solutions were determined. As shown in Table 1, the pH values ranged from 2.3 for the nicotinic acid derivative **19** up to 5.7 for the nicotinamide derivative **17**. The pH values of the ester prodrugs were between 4.6 and 5.0. Since thalidomide has been proven to be very effective in skin lesions, some of the prepared prodrugs may be established for topical use.

## Lipophilicity parameters

Permeation through membranes is essential for the effectiveness of drugs. Therefore, not only aqueous




**Scheme 2.** Synthesis of the azido derivative **16** and the nicotinic acid derivative **17.** Reaction conditions: (a) $SOCl_2$, DMF; (b) KI, acetone; (c) $NaN_3$, acetone; (d) nicotinamide, DMF; (e) aq $NaHCO_3$, $Na_2S_2O_3$, 1 h, 0–5 °C; (f) nicotinic acid, acetonitrile, 2.5 h.




**Scheme 3.** Synthesis of the carboxymethyl derivative **21** and the methoxymethyl derivative **22**. Reaction conditions: (a) NaH, DMF, *tert*-butyl iodoacetate; (b) TFA, $CH_2Cl_2$, 1 h, rt; (c) $P_2O_5$, $CH_2Cl_2$, $HC(OCH_3)_3$, 72 h, rt.

**Table 1.** Physicochemical properties of selected compounds

| Compound | Solubility[a] (mg/ml) | Solubility (mmol/ml) | pH Value of the aqueous solution ($c = 5$ mg/mL) |
|---|---|---|---|
| **1** | 0.012[36] | 0.000047[36] | n.d. |
| **7** | 49.7 | 0.13 | 4.9 |
| **8** | > 300 | > 0.73 | 4.7 |
| **9** | > 300 | > 0.71 | 5.0 |
| **10** | > 300 | > 0.68 | 4.6 |
| **11** | 16.7[b] | 0.043[b] | n.d. |
| **17** | 5.7 | 0.013 | 5.7 |
| **19** | 4.2 | 0.008 | 2.3 (sat sol.) |

[a]Determined in water.
[b]Determined in phosphate buffer, pH 7.4.

solubility but also lipophilicity are important physicochemical properties. The lipophilicity of the thalidomide prodrugs was evaluated by means of reversed-phase HPLC capacity factors (Table 2). Additionally, some degradation compounds of thalidomide were investigated using the same method in order to compare stability (see the following section). The log k′ values of the prodrugs were correlated with log P values calculated by the Broto's method using the computer program ChemDraw Ultra (version 5).[29,30] No correlation was found for the log k′ and log P values for the investigated ester prodrugs (Table 2).

Lipophilicity parameters can be used as indicators for the permeability through membranes, suggesting the acid **11** as possibly the drug with the highest membrane permeability in this series. In order to achieve good absorption of orally administered drugs, a log P value greater than 2 is generally desired. The introduced peptide bonds, however, do rather lower than increase the log P values.[31] Nevertheless, the amino acid ester approach is successful in a variety of slightly soluble drugs.[17,32,33] This reported increase in effectiveness of amino acid prodrugs is not only due to the increased water solubility but also to the potential active transport into cells via oligopeptide permeases.[34,35]

Bodor reported a similar design with the successful introduction of the nicotinamide/dihydropyridine redox drug-delivery system to improve delivery through biological membranes.[25] The lipophilic dihydropyridine derivative **18** is supposed to penetrate easily through the skin and after enzymatic oxidation to the quarterny **17**, the ionized form is captured in the stratum corneum.

## Kinetics of hydrolysis and stability

The hydrolysis rates of the selected prodrugs at physiological pH = 7.4 were investigated in order to determine their chemical stability. As presented in Chart 1, most prodrugs were hydrolyzed into hydroxymethylthalidomide (**2**) and subsequently into thalidomide. The reactions were monitored by HPLC whereby the decrease of prodrug versus time was determined.

**Table 2.** Retention times, capacity factors $k'$ and log P values of investigated compounds

| Compound | Retention time (min) | Capacity factors k′ | Log P[a] |
|---|---|---|---|
| **1** | 17.9 | 12.77 | 0.49 |
| **2** | 17.2 | 12.23 | −0.42 |
| **7** | 9.2 | 6.08 | −1.08 |
| **8** | 13.3[c] | 9.23[c] | n.d.[b] |
| **9** | 22.8, 24.0[d] | 16.54, 17.46[d] | −0.51 |
| **10** | 11.1 | 7.54 | −2.42 |
| **11** | 48.7 | 36.46 | −0.24 |
| **19** | 8.0 | 5.15 | n.d.[b] |
| **23** | 6.2 | 3.77 | −0.06 |
| **24** | 4.1 | 2.15 | −0.06 |
| **25** | 2.8 | 1.15 | −0.94 |
| **26** | 2.7 | 1.08 | 0.58 |

[a]For the calculation of the log P values, the neutral compounds were used.
[b]n.d., not determined; the log P value of this compound was not calculable with the program.
[c]The diastereomers were not separated with this method.
[d]Two different retention times were observed for the diastereomers.

As shown in Chart 2, a variety of possible degradation compounds can occur during hydrolysis.[36] The main hydrolytic compound is *N*-(2,6-dioxo-piperidin-3-yl)-phthalamic acid **25** with a cleaved phthalimide ring, whereas the glutarimide ring is more stable and, therefore, the hydrolytic compounds **23** and **24** occur only in minor percentages.[36] The primary hydrolytic compounds **23, 24** and **25** are subjected to further hydrolysis.[36] The retention times of the investigated prodrugs and degradation compounds are given in Table 2, varying from 2.7 min for **26** to 48.7 min for **11**.

Kinetics of the chemical hydrolysis of the prodrugs followed a pseudo-first-order rate over several half-lives. The rates of prodrug hydrolysis were within a broad range (Table 3 and Fig. 1). On the one hand, the hydrolytic rate constant of the glycylglycine derivative **10** was so high that it could not be determined with the applied method. After 5 min, less than 10% of **10** was determined. The succinic acid ester **11**, on the other hand, was the most stable prodrug. The hydrolytic rate constant was less than 0.009 $min^{-1}$, corresponding to a half-life greater than 80 min. All other investigated prodrugs showed hydrolytic rate constants ranging from 0.013 to 0.060 $min^{-1}$ resulting in half-lives ($t_{1/2}$) ranging from 12 min for the *N*-methylalanine ester **8** up to 54 min for the relative stable valine ester **9**. In the series of the amino acid ester prodrugs **7**, **8** and **9**, the hydrolytic half-lives increased with increasing steric hindrance of the amino acid (Table 2). With the exception of **11** and **19**, all investigated prodrugs were converted into thalidomide, the parent compound. The hydrolytic products of **11** and **19** remain to be identified.

The determination of the hydrolytic products of the prodrugs is complicated by the fact that thalidomide undergoes hydrolytic cleavage itself. The half-life of thalidomide, however, is longer than that of most prodrugs (approx. 300 min).[36] Therefore, the formation of hydrolytic compounds did not hamper the determination of the hydrolysis rates of the prodrugs with the exception of the very stable **11**.

## Biological evaluation

The synthesized prodrugs were tested in TNF-α- and interleukin-2 assays. The inhibition of the TNF-α release was determined in the supernatant of human lipopolysaccharide-treated PBMCs.[37,38] All compounds were dissolved in DMSO and diluted in aqueous buffer solution.

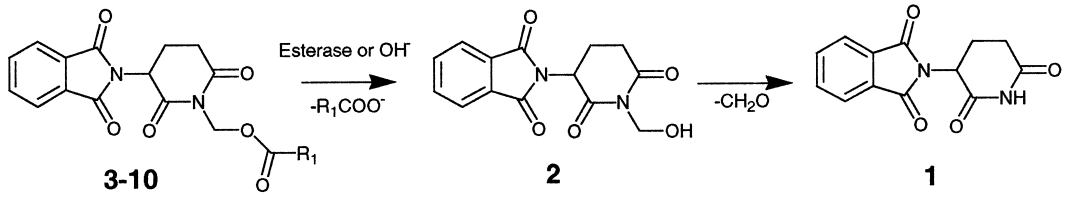



**Chart 1.** Hydrolysis of the ester prodrugs.

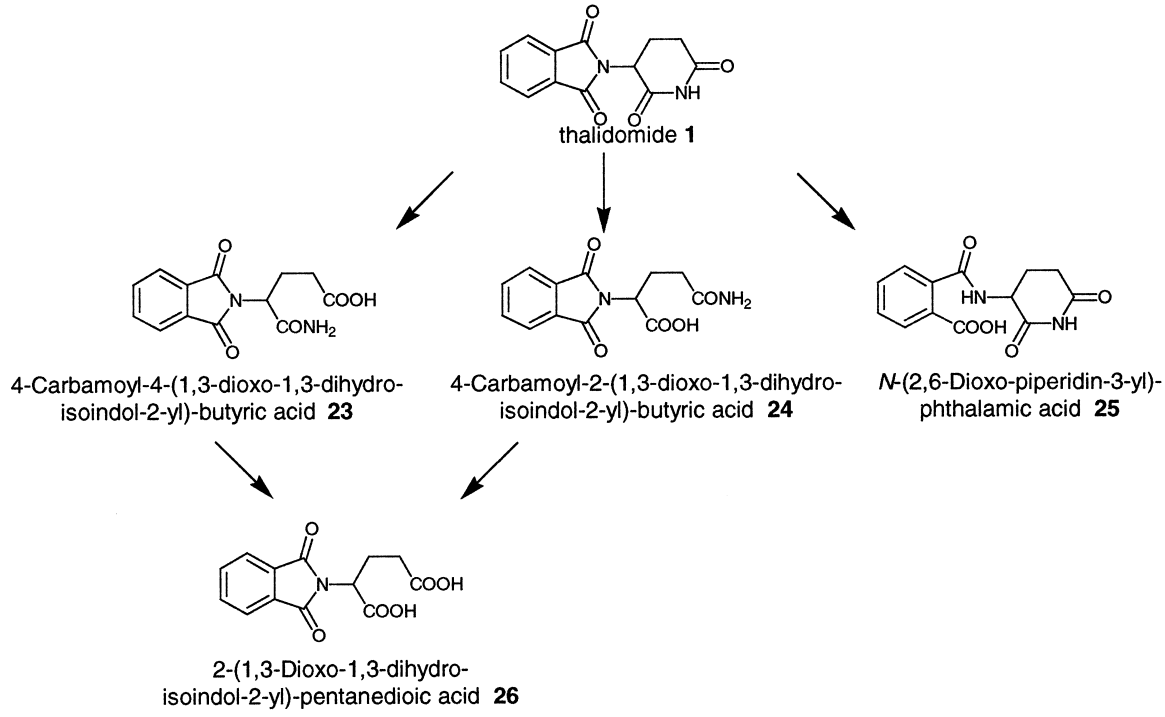



**Chart 2.** Hydrolytic degradation of thalidomide.

**Table 3.** Linearity, half-lives and hydrolytic rate constants of investigated prodrugs

| Compound | Linearity of the calibration curve (correlation coefficient) | Hydrolytic rate constant ($min^{-1}$) | Half-life (min) |
|---|---|---|---|
| **1** | $f(x) = 542.44x - 551.18$ ($r = 0.9999$) | 0.0023[a] | 300[a] |
| **7** | $f(x) = 332.21x + 3.3388$ ($r = 0.9998$) | 0.034 | 21 |
| **8** | $f(x) = 273.51x - 30.805$ ($r = 0.9998$) | 0.060 | 12 |
| **9** | $f(x) = 272.22x - 816.93$ ($r = 0.9999$) | 0.013 | 54 |
| **10** | n.d.[b] | > 0.23[c] | < 3[c] |
| **11** | n.d.[b] | ~0.009[c] | > 80[c] |
| **19** | $f(x) = 235.32x - 41.51$ ($r = 1.0$) | 0.034 | 21 |

[a]Data from ref 36
[b]n.d., not determined.
[c]Estimated from our own data.

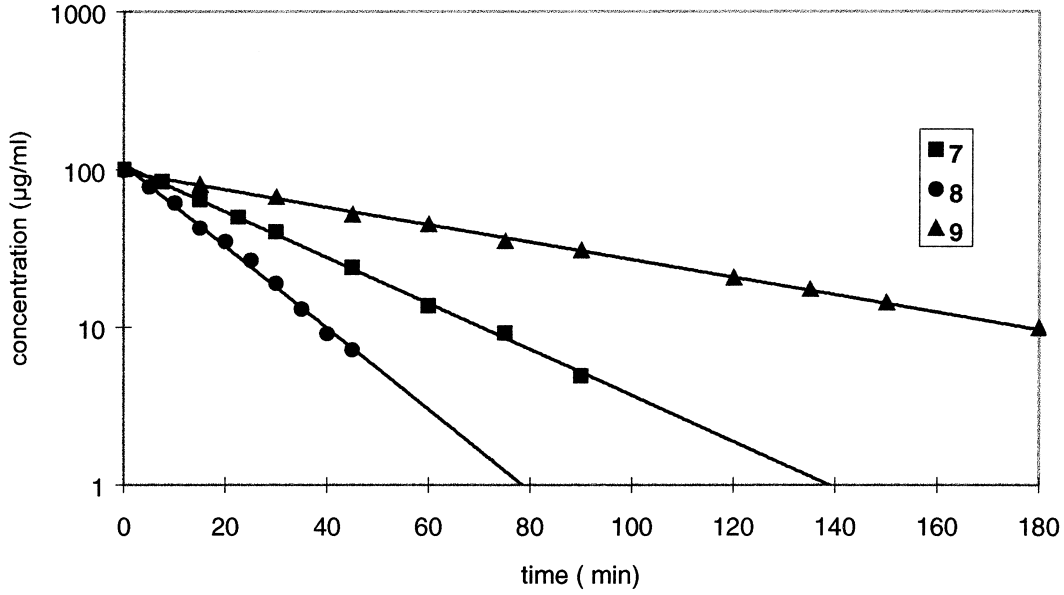



**Figure 1.** Pseudo-first-order kinetic plots for hydrolysis of prodrugs **7**–**9** in physiological phosphate buffer pH 7.4 at 37 °C.

Most compounds were additionally investigated for their ability to inhibit IL-2 in mice. The compounds were administered intraperitoneally in mice that were pretreated with staphylococcal super antigen to induce IL-2 production.[37,38] To investigate bioavailability, compounds **7**, **8** and **10** were additionally administered perorally. Inhibition of IL-2 was determined from heart-punctured blood using ELISA.

The selected compounds thalidomide, **2** and **7**, were also tested for their ability to inhibit the local Shwartzman reaction, an inflammatory lesion provoked by endotoxin exposition.[39] Therefore, the dorsal skin of male white mice was treated with lipopolysaccharide and TNF-α. The inhibition of the extravasation of the dye Evans Blue that was applied to the surrounding tissue was determined using UV.

### Inhibition of TNF-α release

The amino acid ester prodrugs **3** and **7**–**10** in which the Mannich base **2** is derivatized with a glycine, a valine, a *N*-methylalanine or a glycylglycine were investigated (Table 4). The valine ester **9** showed a greater inhibition (88%) than thalidomide itself. The $IC_{50}$ of **9** was 4.7 µM whereas the $IC_{50}$ of thalidomide has been reported to be 200 µM.[40] The glycine ester **7** showed 54% inhibition of TNF-α followed by the *N*-methylalanine ester **8** exhibiting only weak inhibition of 12%. Interestingly, a correlation between the stability of the prodrugs and the in vitro activity has been observed: the more stable the ester, the greater the inhibition of TNF-α. Additionally, the very unstable glycylglycine ester **10** did not inhibit but slightly increased the release of TNF-α. Furthermore, this observation was supported by the fact that the Boc-protected glycine ester **3** exhibited stronger inhibition of TNF-α than the unprotected **7**.

This finding was unexpected and showed that the prodrugs exhibited an effect of their own, rather than only the effect of the released thalidomide. It can be assumed that the ester prodrug **9** is binding itself with high affinity at the thalidomide recognition site of the pharmacophore.

The highly effective valine ester **9** was used to develop compound **13**. After radioactive labeling with $^{125}I$, **13** may be used for affinity labeling.[23,24] The ability of **13** to inhibit TNF-α is comparable to thalidomide. Therefore, **13** was developed as a pharmacological tool for the investigation of the binding sites of thalidomide.

**Table 4.** Inhibition of TNF-α release and interleukin-2 of thalidomide prodrugs

| Compound | TNF-α % inhibition[a,b] (concd (μM)) | Interleukin-2 i.p. administered $ED_{40}$[b] (μmol/kg) | Interleukin-2 p.o. administered % inhibition or $ED_{40}$[b] (μmol/kg) |
|---|---|---|---|
| **1** | 65 (194) | 663 | |
| **2** | 96 (174) | 1010 | |
| Ester derivatives | | | |
| **3** | 78 (112) | 898 | |
| **7** | 54 (131) | 602 | $ED_{40}$ 686 |
| **8** | 12 (122) | 976 | $ED_{40}$ 827 |
| **9** | 88 (118) | 944 | 55% (913) |
| **10** | −10 (114) | 912 | |
| **13** | 63 (93) | | |
| Nicotinic acid derivatives | | | |
| **17** | 26 (117) | | |
| **18** | 21 (127) | | |
| **19** | 53 (96) | 767 | |
| Other prodrugs | | | |
| **11** | 35 (129) | | |
| **16** | 14 (160) | | |
| **21** | 40 (150) | | |
| **22** | 24 (165) | 1323 | |

[a]Percent inhibition at the indicated concentration.
[b]Median of at least three experimental data; coefficient of variation was less than 20%.

The nicotinic acid derivative **19** exhibited 53% inhibition, whereas the nicotinamide derivative **17** and the dihydropyridine derivative **18** showed only weak inhibition of 26 and 21%, respectively. As presented in Table 3, the stability of **9** and **19** has been found to be very similar. Interestingly, their effectiveness to inhibit TNF-α was comparable too. Compound **19**, however, was not degraded into thalidomide, suggesting **19** itself inhibited the release of TNF-α. Again, this is in accordance with the above-mentioned finding that more stable prodrugs showed better activity.

Interestingly, the most effective compound in this series has been proven to be hydroxymethylthalidomide (**2**), inhibiting 96% of TNF-α-release, suggesting similar binding with high affinity.

Methoxymethylthalidomide **22** and the azido derivative **16** exerted only weak inhibition with 24 and 14%, respectively, whereas the succinic acid derivative **11** and the carboxymethyl derivative **21** have been determined to show reasonable inhibition of TNF-α. Similar to **19**, the carboxymethyl derivative **21** cannot be metabolized to thalidomide. This observation may also indicate that stable prodrugs or prodrugs that delay degradation of the intact glutarimide ring in thalidomide exhibited more effective inhibition of TNF-α release.

### Inhibition of interleukin-2

The inhibition of interleukin-2 was determined in vivo as described in the Experimental section. As reported in Table 4, thalidomide showed an inhibition of IL-2 of 56% at a concentration of 194 μM. The $ED_{40}$ was determined to be 663 μmol/kg body weight. The glycine ester **7** showed, with an $ED_{40}$ of 602 μmol/kg body weight a slightly better effect than thalidomide. Similarly, the nicotinic acid derivative **19** showed an $ED_{40}$ of 767 μmol/kg body weight. All other derivatives showed $ED_{40}$ values greater than 890 μmol/kg body weight. The test was performed under standard conditions within 2 h. Most prodrugs were not degraded completely at that time. In contrast to the in vitro TNF-α assay, no significant synergistic effects of the prodrugs compared to thalidomide were observed.

In order to determine oral bioavailability, compounds **7**, **8** and **10** have additionally been administered perorally. Compound **7** showed an $ED_{40}$ of 686 μmol/kg body weight and therefore similar effectiveness compared to intraperitoneal (ip) application. **8** showed an $ED_{40}$ of 827 μmol/kg body weight, that is a small increase compared to ip application. It is very likely that this effect is mainly mediated by thalidomide. In contrast to the i.p route, perorally administered drugs are subjected to biotransformation via first-pass effect in the liver. In the case of prodrugs, this can lead to fast bioactivation into thalidomide. The degradation of **10** was so fast that no difference between intravenous and peroral application could be observed (data not shown).

### Local Shwartzman reaction

The effects on the local Shwartzman reaction were recorded in order to determine vascular permeability. Thalidomide inhibited the extravasation of Evans Blue by 48% after three dosages of 40 mg/mL with an $ED_{50}$ of 178 mg/kg body weight. Hydroxymethyl derivative **2** and glycine ester **7** inhibited the extravasation in the same range, namely 40%.

## Conclusions

Using the prodrug approach, readily water-soluble derivatives were prepared. The water solubility not only depended on the introduced residue but also on the crystal form. Amorphous **8**, **9** and **10** showed 15,000-fold increase of water solubility compared to thalidomide. The pH values of the aqueous solution varied between 2.3 and 5.7, enabling therapeutic parenteral or topical use.

Furthermore, the synthesis of the affinity-labeling compound **13** exhibiting similar TNF-α inhibition compared to thalidomide provides a useful tool for further biological studies to explain the mechanism of activity and teratogenicity of thalidomide.

Most prodrugs retained activity in the immunological assays performed. Interestingly, a positive correlation between prodrug stability and activity was found, indicating a high affine binding of the prodrugs to the pharmacophore. Initially, the development of short-lived prodrugs was pursued in order to obtain prodrugs that release the active compound fast. Obviously, bulky residues increased stability by preventing degradation into thalidomide and subsequent degradation of thalidomide. In future studies, prodrugs with longer half-lives will be developed.

The majority of the new prodrugs might be useful water-soluble tools in the ongoing search for the mechanism of action of thalidomide. In particular, the stable valine ester **9** with a TNF-α inhibition value of 4.7 μM ($IC_{50}$) represents a new water-soluble lead for further optimization studies for therapeutic applications.

## Experimental

### Chemistry

All chemicals and reagents were of the highest possible purity. Thalidomide and *N*-phthaloylisoglutamine were a generous gift from Grünenthal GmbH. NMR spectra were measured on a Varian Gemini 300 spectrometer ($^1$H 300 MHz; $^{13}$C 75 MHz). The chemical shifts of the remaining protons of the deuterated solvents served as internal standard: δ ($^1$H, DMSO-$d_6$ = 2.50, $CDCl_3$ = 7.24, $^{13}$C, DMSO-$d_6$ = 39.7, $CDCl_3$ = 77.0). Unless otherwise noted, DMSO was used as solvent. Infra-red spectra were recorded on a Perkin-Elmer FT-IR PC 16 spectrophotometer. All compounds were checked for purity by TLC on 0.2 mm aluminium sheets with silica gel 60 $F_{254}$ (Merck). The following eluents were used: dichloromethane:acetone (9:1) is eluent A, dichloromethane/methanol (9:1) is eluent B, toluene/ethyl acetate (1:1) is eluent C, toluene/ethanol/acetic acid (5:4:1) is eluent D and toluene/ethylacetate/formic acid (10:9:1) is eluent E. Melting points were taken on a Büchi 535 melting point apparatus and are uncorrected. Thermospray (TSP, positive ionization), electron and chemical ionization mass spectra were recorded on a MS Engine HP 5989A mass spectrometer (Hewlett Packard). The GC mass spectrum was performed on HP5890/II-5971 mass spectrometer (Hewlett Packard). Elemental analyses, NMR spectra, and mass spectra were performed by the Institute of Organic Chemistry, University of Leipzig and University of Tübingen, respectively.

### Analytical methods

HPLC analyses were performed on a Sykam S 1000 pump, a 20 μL injection loop, a Linear UVis 205 detector using Axxiom 747 software for the registration and data evaluation. Chromatographic separation was achieved isocratically at ambient temperature in Nucleosil 120-5, $C_{18}$, 250×4.6 mm I.D. with a pore diameter of 10 nm (Macherey-Nagel, Düren, Germany) reversed-phase columns.

The mobile phase consisted of two volumes of acetonitrile and eight volumes of a 50 mM sodium dihydrogenphosphate buffer. The buffer was adjusted to pH 3.5 with concentrated phosphoric acid and was degassed with helium before and during use. Injection volume was 20 μL and flow rate was 1.0 mL/min. The system was equilibrated for approximately 30 min. Thalidomide, its prodrugs and hydrolysis compounds were monitored at 300 and 265 nm. The calibration curves of the prodrugs were linear over the range of 5–100 μg/mL. The method was validated with the following parameters: specifity, linearity, precision, limit of detection, limit of quantification, and stability. Acetonitrile was HPLC gradient grade quality and supplied by Merck (E. Merck, Darmstadt, Germany). All other reagents and salts were supplied by Aldrich (Steinheim, Germany). Ion-exchanged water (UO 100, Letzner GmbH, Hückeswagen, Germany) was further purified by bidistillation. Before use, the aqueous solution of sodium dihydrogen phosphate was filtered off through a cellulose acetate filter (0.45 μm).

The hydrolytic compounds **23**–**26** were synthesized according to Schumacher and Teubert.[37,41]

**2-(1-Hydroxymethyl-2,6-dioxo-piperidine-3-yl)-1,3-dihydro-2*H*-isoindole-1,3-dione (2).** A suspension of 12.9 g (50 mmol) of rac-**1** in 100 mL of a 35% aq formaldehyde solution was refluxed until dissolved and then allowed to cool down. After 24 h, the precipitate was collected by filtration and washed with 3% aq formaldehyde solution and dried. Yield: 10.1 g (70%); mp 165 °C; IR (KBR) ν 3200, 2970, 1783, 1680; $^1$H NMR δ 2.16–2.67 (m, 2H, H-4′), 2.87–3.11 (m, 2H, H-5′), 5.08 (d, $J_{ab}$ = 7.2 Hz, 2H, NC$H_2$OH), 5.52 (m, 1H, C$H$CH$_2$), 6.17 (t, $J_{ab}$ = 7.2 Hz, $J_{bc}$ = 7.2 Hz, 1H, OH), 7.92 (s, 4H, $H_{arom}$).

**General procedure for *N*-*tert*-butyloxycarbonyl-protected ester 3–6.** A solution of 4.32 g (15 mmol) **2**, 15 mmol of the appropriate *N*-Boc-protected amino acid, 3.09 g (15 mmol) of dicyclohexylcarbodiimide and 0.22 g of (1.5 mmol) 4-pyrrolidinopyridine in 75 mL of abs dichloromethane were reacted at rt for 24 h. Precipitated dicylcohexylurea was filtered off; the filtrate was washed 3× with 75 mL of 5% acetic acid and 3× with 75 mL of water. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was recrystallized two times from ethanol.

***N*-*tert*-butyloxycarbonyl-2-aminoacetic acid-[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindole-2-yl)-2,6-dioxo-piperidine-1-yl-methyl]ester (3).** N-Boc-glycine (2.63 g) was used. Yield: 3.88 g (58%); mp 108–111 °C; DC (eluent A) $R_f$-value = 0.51; IR (KBr) ν 3432, 3312, 2984, 2944, 1758, 1724, 1706, 1680, 1402, 1170, 722; $^1$H NMR δ 1.37 (s, 9H, C(C$H_3$)$_3$), 2.11–2.15 (m, 1H, H-4′), 2.56–2.71 (m, 1H, H-4′), 2.81–2.88 (m, 1H, H-5′), 3.01–3.15 (m, 1H, H-5′), 3.67 (d, $J_{(CH_2–NH)}$ = 6.2 Hz, 2H, NHC$H_2$CO$_2$), 5.35 (dd, $J_{3'a–4'e}$ = 5.3 Hz, $J_{3'a–4'a}$ = 13.1 Hz, 1H, H-3′), 5.66, 5.71 (AB, $J_{AB}$ = 9.6 Hz, 2H, NCH$_2$O), 7.23 (t, $J_{(NH–CH_2)}$ = 6.1 Hz, 1H, NH), 7.88–7.97 (m, 4H, $H_{arom.}$); $^{13}$C NMR δ 20.80 (C-4′), 28.14 (C(C$H_3$)$_3$), 31.07 (C-5′), 41.57 (NHC$H_2$COO), 49.47 (C-3′), 63.40 (NCH$_2$O), 78.33 (C(CH$_3$)$_3$), 123.52 (C-4, C-7), 131.20 (C-3a, C-7a), 134.98 (C-5, C-6), 155.78 (NHCOO), 167.04, 169.19, 169.48, 170.9 (C-1, C-3, C-2′, C-6′, CH$_2$COO); CIMS *m*/*z* 446 ($MH^+$). Anal. calcd for ($C_{21}H_{23}N_3O_8$): C, 56.63; H, 5.20; N, 9.43; found: C, 56.85; H, 5.19; N, 9.67%.

***N*-*tert*-butyloxycarbonyl-2-methylaminopropionic acid-[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindole-2-yl)-2,6-dioxo-piperidine-1-yl-methyl]ester (4).** *N*-Boc-L-*N*-methylalanine 4.32 g (15 mmol) was used. Crude **4** was chromatographed on silica using dichloromethane/acetone (9:1).

Yield: 4.44 g (63%); mp 123–125 °C; DC (eluent A) $R_f$-value = 0.63; IR (KBR) ν 2978, 1752, 1720, 1706, 1392, 720; $^1$H NMR δ 1.29–1.38 (m, 12H, C($CH_3$)$_3$, CHC$H_3$), 2.12–2.17 (m, 1H, H-4′), 2.58–2.64 (m, 1H, H-4′), 2.73 (s, 3H, $NCH_3$), 2.82–2.88 (m, 1H, H-5′), 3.02–3.08 (m, 1H, H-5′), 4.37, 4.60 (m, 1H, C*H*$CH_3$), 5.34 (dd, $J_{3'a-4'e}$ = 5.3 Hz, $J_{3'a-4'a}$ = 13.0 Hz, 1H, H-3′), 5.71 (s, 1H, $NCH_2O$), 7.88–7.96 (m, 4H, $H_{arom}$); $^{13}$C NMR δ ($CDCl_3$) 14.72, 15.09 (CH*C*$H_3$), 21.76 (C-4′), 28.33 (C(*C*$H_3$)$_3$), 30.49, 30.92 ($NCH_3$), 31.84 (C-5′), 49.91 (C-3′), 53.36, 54.79 (*C*HCH$_3$), 63.87 ($NCH_2O$), 80.40 (*C*($CH_3$)$_3$), 123.80 (C-4, C-7), 131.71 (C-3a, C-7a), 134.54 (C-5, C-6), 155.22, 155.83 (NCOO), 167.19, 167.93, 169.85, 169.93, 171.21 (C-1, C-3, C-2′, C-6′, CH*C*OO). Anal. calcd for ($C_{23}H_{27}N_3O_8$): C, 58.35; H, 5.75; N, 8.87; found: C, 58.31; H, 5.76; N, 8.80%.

***N*-*tert*-butyloxycarbonyl-2-amino-3-methylbutyric acid-[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindole-2-yl)-2,6-dioxo-piperidine-1-yl-methyl]ester (5).** *N*-Boc-L-valine 3.26 g (15 mmol) was used. Crude **5** was chromatographed on silica using dichloromethane/acetone (9/1). Yield: 3.85 g (53%); mp 82–85 °C; DC (eluent A) $R_f$-value = 0.68; IR (KBr) ν 3394, 2972, 2934, 1752, 1720, 1392, 720; $^1$H NMR δ 0.82–0.86 (m, 6H, CH(C$H_3$)$_2$), 1.37 (s, 9H, C($CH_3$)$_3$), 1.96–2.00 (m, 1H, C*H*($CH_3$)$_2$), 2.12–2.17 (m, 1H, H-4′), 2.60–2.66 (m, 1H, H-4′), 2.83–2.88 (m, 1H, H-5′), 3.03–3.09 (m, 1H, H-5′), 3.83 (t, $J_{(CH-NH)}$ = 6.1 Hz, 1H, CHNH), 5.30–5.38 (m, 1H, H-3′), 5.61 (dd, 1H, $NCH_{2(A)}O$), 5.75 (t, 1H, $NCH2_{(B)}O$), 7.13 (t, 1H, NH), 7.88–7.96 (m, 4H, $H_{arom}$); $^{13}$C NMR δ 18.04, 18.15, 18.75, 18.84 (CH(*C*$H_3$)$_2$), 20.80 (C-4′), 28.13 (C(*C*$H_3$)$_3$), 29.49, 29.54 (CH(*C*$H_3$)$_2$), 31.03 (C-5′), 49.46 (C-3′), 59.07, 59.12 (CHNH), 63.19, 63.27 ($NCH_2O$), 78.24 (*C*($CH_3$)$_3$), 123.45 (C-4, C-7), 131.17 (C-3a, C-7a), 134.93 (C-5, C-6), 155.68 (NHCOO), 166.97, 169.06, 170.86, 170.94, 171.07, 171.12 (C-1, C-3, C-2′, C-6′, CH*C*OO). Anal. calcd for ($C_{24}H_{29}N_3O_8$): C, 59.13; H, 6.00; N, 8.62; found: C, 58.74; H, 6.05; N, 8.64%.

**2-*N*-*tert*-butyloxycarbonyl-(aminoacetylamino)acetic acid-[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindole-2-yl)-2,6-dioxopiperidine-1-yl-methyl]ester (6).** *N*-Boc-glycylglycine (3.48 g) was used. Yield: 5.50 g (73%); mp 178–181 °C; IR (KBr) ν 3442, 3404, 2978, 2932, 1760, 1716, 1392, 1162, 996, 720; $^1$H NMR δ 1.38 (s, 9H, C(C$H_3$)$_3$), 2.12–2.17 (m, 1H, H-4′), 2.61–2.66 (m, 1H, H-4′), 2.83–2.89 (m, 1H, H-5′), 3.03–3.13 (m, 1H, H-5′), 3.59 (d, $J_{(CH_2-NH)}$ = 5.8 Hz, 2H, C$H_2$NHCOO), 3.86 (d, $J_{(CH_2-NH)}$ = 6.0 Hz, 2H, NHC$H_2$COO), 5.35 (dd, $J_{3'a-4'e}$ = 5.3 Hz, $J_{3'a-4'a}$ = 13.0 Hz, 1H, H-3′), 5.68, 5.73 (AB, $J_{AB}$ = 9.6 Hz, 2H, $NCH_2O$), 6.97 (t, $J_{(NH-CH_2)}$ = 5.8 Hz, 1H, NHCOO), 7.89–7.96 (m, 4H, $H_{arom}$), 8.20 (t, $J_{(NH-CH_2)}$ = 5.9 Hz, 1H, N*H*COCH$_2$); $^{13}$C NMR δ 20.80 (C-4′), 28.15 (C(*C*$H_3$)$_3$), 31.03 (C-5′), 40.17 (*C*$H_2$COO), 42.99 (*C*$H_2$NHCOO), 49.44 (C-3′), 63.31 ($NCH_2O$), 78.05 (*C*($CH_3$)$_3$), 123.48 (C-4, C-7), 131.16 (C-3a, C-7a), 134.93 (C-5, C-6), 155.72 (NHCOO), 167.0, 168.82, 169.15, 169.97, 170.95 (C-1, C-3, C-2′, C-6′, $CH_2$*C*ONH, $CH_2$*C*OO). Anal. calcd for ($C_{23}H_{26}N_4O_9$): C, 54.98; H, 5.22; N, 11.15; found: C, 55.18; H, 5.19; N, 10.89%.

**General procedure for prodrug esters 7–10.** A solution of 4 mmol of the appropriate *N*-Boc-protected ester **3**, **4**, **5** or **6**, respectively, in 25% of trifluoroacetic acid in dichloromethane was reacted at rt for 1 h. The solvent was removed in vacuo and the residue was coevaporated several times with dichloromethane until dry. The residue was dissolved in 160 mL of methanol and ion-exchange chromatographed using Amberlite IR 45, pretreated with 1 N hydrochloric acid. The solvent was concentrated in vacuo and the residue was suspended in boiling ethanol. Water was added dropwise until the solution was clear. After cooling, the precipitate was collected by filtration and dried.

**2-Aminoacetic acid-[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindole-2-yl)-2,6-dioxo-piperidine-1-yl-methyl]ester×HCl (7).** Compound **3** (1.78 g, 4 mmol) was used. Yield: 0.81 g (53 %); mp 227–232 °C; DC (eluent C) $R_f$-value = 0.6; IR (KBR) ν 2988, 2952, 2908, 2854, 2794, 2700, 2610, 1766, 1732, 1726, 1722, 1718, 1708, 1698, 1390, 1224, 720; $^1$H NMR δ 2.13–2.19 (m, 1H, H-4′), 2.54–2.69 (m, 1H, H-4′), 2.84–2.89 (m, 1H, H-5′), 3.04–3.16 (m, 1H, H-5′), 3.80 (s, 2H, $CH_2NH_3$), 5.35 (dd, $J_{3'a-4'e}$ = 5.4 Hz, $J_{3'a-4'a}$ = 13.1 Hz, 1H, H-3′), 5.77, 5.82 (AB, $J_{AB}$ = 9.6 Hz, 2H, $NCH_2O$), 7.89–7.97 (m, 4H, $H_{arom}$), 8.48 (s, 3H, $NH_3$); $^{13}$C NMR δ ($D_2O$): 21.10 (C-4′), 31.22 (C-5′), 40.12 (*C*$H_2NH_3$), 49.94 (C-3′), 64.55 (N*C*$H_2O$), 123.95 (C-4, C-7), 130.96 (C-3a, C-7a), 135.34 (C-5, C-6), 167.08, 169.02, 170.68, 173.45 (C-1, C-3, C-2′, C-6′, *C*OO); TSPMS *m*/*z* 346 ($M^+$). Anal. calcd for ($C_{16}H_{16}ClN_3O_6$): C, 50.34; H, 4.22; N, 11.01; found: C, 50.16; H, 4.30; N, 11.24%.

**2-Methylamino(propionic acid)-[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindole-2-yl)-2,6-di-oxo-piperidine-1-yl-methyl]ester×HCl (8).** Compound **4** (1.89 g, 4 mmol) was used. Deviating from the general procedure, the resulting residue was dissolved in a small amount of methanol and diethyl ether was added dropwise to precipitate **8**, which was collected by filtration.

Yield: 1.05 g (64 %); mp 147–151 °C; DC (eluent C) $R_f$-value 0.65; IR (KBR) ν 2966, 2704, 2434, 1756, 1718, 1394, 1106, 720; $^1$H NMR δ 1.42 (d, $J$ = 6.9 Hz, 3H, CHC$H_3$), 2.14–2.17 (m, 1H, H-4′), 2.54 (s, 3H, $NH_2CH_3$), 2.57–2.66 (m, 1H, H-4′), 2.84–2.89 (m, 1H, H-5′), 3.04–3.16 (m, 1H, H-5′), 4.10 (q, $J$ = 7.1 Hz, 1H, C*H*$CH_3$), 5.36 (dd, $J_{3'a-4'e}$ = 4.5 Hz, $J_{3'a-4'a}$ = 13.0 Hz, 1H, H-3′), 5.76 (d, $J_{AB}$ = 9.6 Hz, 1H, $NCH_{2(A)}O$), 5.89 (dd, $J_{AB}$ = 9.6 Hz, 1H, $NCH_{2(B)}O$), 7.89–7.96 (m, 4H, $H_{arom}$), 9.57 (bs, 2H, N$H_2CH_3$); $^{13}$C NMR δ 13.93 (CHC$H_3$), 20.83 (C-4′), 30.41, 30.46 (C$H_3NH_2$), 31.00 (C-5′), 49.41 (C-3′), 54.96 (C*H*$CH_3$), 63.81 ($NCH_2O$), 123.49 (C-4, C-7), 131.13 (C-3a, C-7a), 134.99 (C-5, C-6), 166.99, 168.34, 169.21, 170.97, 171.04 (C-1, C-3, C-2′, C-6′, $CH_2$*C*OO); TSPMS *m*/*z* 374 ($M^+$). Anal. calcd for ($C_{18}H_{20}ClN_3O_6$): C, 52.75; H, 4.92; N, 10.25; found: C, 52.42; H, 4.82; N, 10.17%.

**2-Amino-3-methylbutyric acid-[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindole-2-yl)-2,6-dioxo-piperidine-1-yl-methyl]ester×HCl (9).** Compound **5** (1.95 g, 4 mmol) was used. Deviating from the general procedure, the resulting

residue was dissolved in a small amount of ethanol and approximately a 10-fold excess of diethyl ether was added dropwise to precipitate **9**, which was collected by filtration. Yield: 1.01 g (60%); mp 145–150 °C; DC (eluent C) $R_f$-value = 0.73; IR (KBR) ν 2970, 2902, 1752, 1718, 1392, 720; $^1$H NMR δ 0.94 (t, 6H, CH(C$H_3$)$_2$), 2.14–2.18 (m, 2H, H-4′, C$H$(CH)$_2$), 2.59–2.63 (m, 1H, H-4′), 2.84–2.90 (m, 1H, H-5′), 3.07–3.16 (m, 1H, H-5′), 3.84 (bs, 1H, C$H$NH$_3$), 5.34–5.39 (m, 1H, H-3′), 5.69 (dd, $J_{AB}$ = 9.7 Hz, 1H, NCH$_{2(A)}$O), 5.88 (dd, $J_{AB}$ = 9.5 Hz, 1H, NCH$_{2(B)}$O), 7.88–7.95 (m, 4H, H$_{arom}$), 8.62 (bs, 3H, NH$_3$); $^{13}$C NMR δ 17.33, 17.44, 18.11, 18.25 (CH(C$H_3$)$_2$), 20.77 (C-4′), 29.33 (C$H$(CH$_3$)$_2$), 31.01 (C-5′), 49.43 (C-3′), 57.06 (CHNH$_3$), 63.79 (NCH$_2$O), 123.48 (C-4, C-7), 131.13 (C-3a, C-7a), 134.98 (C-5, C-6), 166.95, 168.01, 168.10, 169.12, 169.20, 170.92, 171.00 (C-1, C-3, C-2′, C-6′, C OO); CIMS $m/z$ 388 (M$^+$). Anal. calcd for ($C_{19}H_{22}ClN_3O_6$): C, 53.84; H, 5.23; N, 9.91; found: C, 54.06; H, 5.22; N, 9.62%.

**2-(Aminoacetylamino)acetic acid-[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindole-2-yl-2,6-dioxopiperidine-1-yl-methyl]ester× HCl (10).** A solution of 2.01 g (4 mmol) **6** was reacted as described for **7**. The compound was recrystallized from methanol:diethyl ether (1:1) to yield an amorphous foam. Yield 1.35 g (77 %); DC (eluent B): $R_f$-value = 0.15; IR (KBr) ν 3394, 3226, 3060, 1752, 1718, 1702, 1394, 720; $^1$H NMR δ 2.12–2.16 (m, 1H, H-4′), 2.59–2.68 (m, 1H, H-4′), 2.83–2.88 (m, 1H, H-5′), 3.03–3.17 (m, 1H, H-5′), 3.61 (s, 2H, C$H_2$NH$_3$), 3.96 (d, $J_{(CH_2–NH)}$ = 5.8 Hz, 2H, NHC$H_2$CO$_2$), 5.35 (dd, $J_{3'a–4'e}$ = 5.3 Hz, $J_{3'a–4'a}$ = 12.9 Hz, 1H, H-3), 5.68, 5.75 (AB, $J_{AB}$ = 9.6 Hz, 2H, NCH$_2$O), 7.90–7.97 (m, 4H, H$_{arom}$), 8.15 (s, 3H, NH$_3$), 8.87 (t, $J_{(NH–CH_2)}$ = 5.8 Hz, 1H, NH); $^{13}$C NMR δ (D$_2$O) 21.10 (C-4′), 31.24 (C-5′), 40.56, 42.12 (NH$C$H$_2$CO$_2$, CH$_2$NH$_3$), 49.96 (C-3′), 64.20 (NCH$_2$O), 123.95 (C-4, C-7), 131.03 (C-3a, C-7a), 135.33 (C-5, C-6), 167.79, 169.07, 169.99, 170.69, 173.50 (C-1, C-3, C-2′, C-6′, CONH, COO); TSPMS $m/z$ 403 (M$^+$). Anal. calcd for ($C_{18}H_{19}ClN_4O_7$): C, 49.27; H, 4.36; N, 12.77; found: C, 48.93; H, 4.26; N, 12.77%.

**Succinic acid mono[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindole-2-yl)-2,6-dioxo-piperidine-1-yl-methyl]ester (11).** A solution of 2.88 g (10 mmol) **2**, 2 g (20 mmol) succinic anhydride, 2.8 mL (20 mmol) of triethylamine and 0.15 g (1 mmol) 4-pyrrolidinopyridine in 50 mL of abs dichloromethane were stirred at rt for 24 h. The reaction mixture was extracted 3× with 50 mL of 5% hydrochloric acid and 3×times with 50 mL of water. The organic layer was dried over MgSO$_4$ and the solvent was evaporated in vacuo. The remaining residue was crystallized from ethyl acetate and recrystallized from ethanol. Yield 2.66 g (68%); DC (eluent E) $R_f$-value = 0.25; IR (KBr) ν 2990, 2960, 2904, 2784, 2684, 2574, 1740, 1716, 1704, 1392, 1208, 1162, 1100, 720; $^1$H NMR δ 2.14–2.17 (m, 1H, H-4′), 2.62–2.68 (m, 4H, C$H_2$C$H_2$COOH), 2.82–3.07 (m, 3H, 2×H-5′, H-4′), 5.04–5.10 (m, 1H, H-3′), 5.83, 5.88 (AB, $J_{AB}$ = 9.5 Hz, 2H, NCH$_2$O), 7.75–7.78 (m, 2H, H$_{arom}$), 7.87–7.89 (m, 2H, H$_{arom}$); $^{13}$C NMR δ 20.81 (C-4′), 31.03 (C-5′), 28.40 (C$H_2$C$H_2$COOH), 49.43 (C-3′), 63.11 (NCH$_2$O), 123.47 (C-4, C-7), 131.16 (C-3a, C-7a), 134.93 (C-5, C-6), 167.01, 169.15, 170.96, 171.23, 173.12 (C-1, C-3, C-2′, C-6′, C OOCH$_2$, C OOH); TSPMS $m/z$ 406 (M$^+$ + 18). Anal. calcd for ($C_{18}H_{16}N_2O_8$): C, 55.67; H, 4.15; N, 7.21; found: C, 55.73; H, 4.01; N, 7.33%.

**2-[3-(4-Hydroxyphenyl)propionylamino]-3-methylbutyric acid-[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindole-2-yl)-2,6-dioxo-piperidine-1-yl-methyl]ester (13).** A solution of 0.366 g (0.75 mmol) **5** in 10 mL of 25% trifluoroacetic acid in dichloromethane was reacted at rt for 1 h. The solvent was removed in vacuo and the residue was co-evaporated several times with dichloromethane until dry. The residue was taken up in 20 mL of aq phosphate buffer (0.067 M, pH 7.4) in an ice bath and treated with 0.395 g (1.5 mmol) **12** (3-(4-hydroxyphenyl)-propionic acid-2,5-dioxo-pyrrolidine-1-yl-ester) dissolved in dioxane. The pH of the mixture was brought up to 8–9 using an aq solution of sodium hydrogen carbonate and stirred at rt for 5 h. Subsequently, the pH of the mixture was adjusted to pH 2 using 1N hydrochloric acid. Dioxane was concentrated in vacuo; the remaining aq solution was 3× extracted with ethyl acetate. The combined organic layers were once washed with water and dried over Na$_2$SO$_4$. The solvent was removed in vacuo and the residue was chromatographed on silica (30 cm×2 cm) using eluent D to yield 0.218 g (54%) of a white foam.

DC (eluent D) $R_f$-value = 0.37; IR (KBR) ν 3388, 2966, 2932, 1750, 1720, 1654, 1614, 1516, 1392, 720; $^1$H NMR δ 0.80–0.82 (m, 6H, CH(C$H_3$)$_2$), 1.95–1.99 (m, 1H, C$H$(CH$_3$)$_2$), 2.12–3.08 (m, 8H, 2×H-4′, 2×H-5′, NHCOC$H_2$C$H_2$), 4.16 (t, 1H, C$H$NH), 5.32–5.37 (m, 1H, H-3′), 5.63 (dd, $J_{AB}$ = 9.7 Hz, 1H, NCH$_{2(A)}$O), 5.77 (dd, $J_{AB}$ = 9.6 Hz, 1H, NCH$_{2(B)}$O), 6.50 (d, $J_{3''–2'',5''–6''}$ = 8.3 Hz, 2H, H-3″, H-5″), 6.98 (d, $J_{2''–3'',6''–5''}$ = 8.3 Hz, 2H, H-2″, H-6″), 7.88–7.96 (m, 4H, H$_{arom}$), 8.06–8.10 (m, 1H, NH), 9.11 (s, 1H, OH); $^{13}$C NMR δ 17.96, 18.03, 18.74, 18.83 (C(C$H_3$)$_2$), 20.81 (C-4′), 29.72, 29.77 (C$H$(CH$_3$)$_2$), 30.27 (C$H_2$CONH), 30.91 (C-5′), 36.86 (CH$_2$-phenyl), 49.46 (C-3′), 57.02 (CHNH), 63.12 (NCH$_2$O), 114.96 (C-3″, C-5″), 123.47 (C-4, C-7), 128.96 (C-2″, C-6″), 131.17, 131.24 (C-3a, C-7a, C-1″), 134.93 (C-5, C-6), 155.37 (C-4″), 166.99, 169.07, 169.11, 170.71, 170.74, 170.89, 170.97, 172.04 (C-1, C-3, C-2′,C-6′, CONH, COO); TSPMS $m/z$ 553 (M$^+$ + 18), 536 (M$^+$ + 1). Anal. calcd for ($C_{28}H_{29}N_3O_8$): C, 62.79; H, 5.46; N, 7.85; found: C, 62.71; H, 5.61; N, 7.74%.

**2-(1-Chloromethyl-2,6-dioxo-piperidine-3-yl)-1,3-dihydro-2*H*-isoindole-1,3-dione (14).** To a solution of 11.52 g (40 mmol) of **2** in 100 mL of dimethylformamide, 8 mL of thionyl chloride were added slowly under ice cooling. After stirring for 1 h at 0 °C, the mixture was poured onto ice water. The precipitate was collected by filtration and recrystallized from ethanol. Yield: 8.52 g (69 %); mp 204–206 °C; DC (eluent A) $R_f$-value = 0.83; IR (KBR) ν 3074, 2908, 1786, 1772, 1716, 1702, 1392, 1156, 720; $^1$H NMR δ 2.11–2.16 (m, 1H, H-4′), 2.54–2.66 (m, 1H, H-4′), 2.84–2.90 (m, 1H, H-5′), 3.04–3.16 (m, 1H, H-5′), 5.38 (dd, $J_{3'a–4'e}$ = 5.3 Hz, $J_{3'a–4'a}$ = 13.0 Hz, 1H, H-3′), 5.52 (s, 2H, CH$_2$Cl), 7.89–7.96 (m, 4H, H$_{arom}$); $^{13}$C NMR δ (CDCl$_3$): 21.69 (C-4′), 31.85 (C-5′), 47.21 (CH$_2$Cl), 49.94 (C-3′), 123.88 (C-4, C-7), 131.70 (C-3a,

C-7a), 134.59 (C-5, C-6), 167.23, 167.40, 169.40 (C-1, C-3, C-2′, C-6′).

**2-(1-Iodomethyl-2,6-dioxo-piperidine-3-yl)-1,3-dihydro-2*H*-isoindole-1,3-dione (15).** To a solution of 6.13 g (20 mmol) **14** in 200 mL of abs acetone, 16.60 g (100 mmol) potassium iodide were added under argon atmosphere and refluxed for 6 h. The solvent was evaporated in vacuo. The residue was dissolved in 100 mL of a solution of 10% (m/V) sodium thiosulfate and 3× extracted with 50 mL of dichloromethane. Then, the combined organic layers were once extracted with 100 mL of a solution of 10% (m/V) sodium thiosulfate and dried over sodium sulfate. The solvent was evaporated in vacuo and the residue was crystallized from acetonitrile. Yield 5.58 g (70%); mp 207–209 °C; DC (eluent A): $R_f$-value = 0.86; IR (KBr) ν 3082, 2956, 2926, 2902, 1786, 1770, 1720, 1694, 1390, 1138, 718; $^1$H NMR δ ($CDCl_3$) 2.12–2.14 (m, 1H, H-4′), 2.76–2.78 (m, 1H, H-4′), 2.79–2.83 (m, 1H, H-5′), 3.02–3.07 (m, 1H, H-5′), 5.02–5.06 (m, 1H, H-3′), 5.52 (s, 2H, $CH_2I$), 7.77–7.79 (m, 2H, $H_{arom}$), 7.88–7.91 (m, 2H, $H_{arom}$); $^{13}$C NMR δ ($CDCl_3$) 5.22 ($CH_2I$), 21.57 (C-4′), 32.08 (C-5′), 50.17 (C-3′), 123.89 (C-4, C-7), 131.71 (C-3a, C-7a), 134.59 (C-5, C-6), 167.09, 167.21, 169.10 (C-1, C-3, C-2′, C-6′); TSPMS *m*/*z* 416 ($M^+ + 18$), 399 ($MH^+$). Anal. calcd for ($C_{14}H_{11}IN_2O_4$): C, 42.23; H, 2.78; N, 7.04; found: C, 42.56; H, 2.84; N, 6.70%.

**2-(1-Azidomethyl-2,6-dioxo-piperidine-3-yl]-1,3-dihydro-2*H*-isoindole-1,3-dione (16).** A solution of 1.59 g (4 mmol) **15** in 25 mL of abs acetone was reacted with 0.52 g (8 mmol) sodium azide at rt for 24 h. The reaction mixture was filtered and the filtrate was poured into ice water. After filtering off the precipitate, it was recrystallized from ethanol. Yield 0.85 g (68%); mp 148–151 °C; DC (eluent A) $R_f$-value = 0.79; IR (KBr) ν 3056, 2966, 2920, 2126, 2100, 1786, 1772, 1716, 1692, 1392, 718; $^1$H NMR δ 2.10–2.18 (m, 1H, H-4′), 2.55–2.66 (m, 1H, H-4′), 2.81–2.89 (m, 1H, H-5′), 3.01–3.13 (m, 1H, H-5′), 5.07, 5.12 (AB, $J_{AB}$ = 12.1, 2H, $CH_2N_3$), 5.36 (dd, $J_{3'a-4'e}$ = 5.3 Hz, $J_{3'a-4'a}$ = 13.0 Hz, 1H, H-3), 7.88–7.96 (m, 4H, $H_{arom}$); $^{13}$C NMR δ 20.95 (C-4′), 30.97 (C-5′), 49.38 (C-3′), 54.24 ($CH_2N_3$), 123.47 (C-4, C-7), 131.15 (C-3a, C-7a), 134.92 (C-5, C-6), 167.01, 169.69, 171.63 (C-1, C-3, C-2′, C-6′); TSPMS *m*/*z* 331 ($M^+ + 18$). Anal. calcd for ($C_{14}H_{11}N_5O_4$): C, 53.68; H, 3.54; N, 22.36; found: C, 53.77; H, 3.49; N, 22.19%.

**3-Carbamoyl-1-[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindoline-2-yl)-2,6-dioxo-piperidine-1-yl-methyl]pyridinium chloride (17).** A solution of 1.23 g (4 mmol) **14** in 30 mL of abs acetonitrile was refluxed with 0.98 g (8 mmol) nicotinamide for 40 h. After cooling to 70 °C, the precipitate was collected by filtration.

Yield 1.35 (79%); mp 277–280 °C; DC (eluent D) $R_f$-value = 0.05; IR (KBr) ν 3260, 3084, 1776, 1766, 1746, 1712, 1708, 1698, 1682, 1394, 1192, 726; $^1$H NMR δ 2.29–2.35 (m, 1H, H-4′), 2.63–2.73 (m, 1H, H-4′), 3.10–3.15 (m, 2H, 2×H-5′), 5.42 (dd, $J_{3'a-4'e}$ = 5.5 Hz, $J_{3'a-4'a}$ = 12.7 Hz, 1H, H-3′), 6.63, 6.69 (AB, $J_{AB}$ = 13.3 Hz, 2H, $NCH_2N$), 7.91 (s, 4H, $H_{arom}$), 8.30 (t, 1H, H-5″), 9.04 (d, $J_{4''-5''}$ = 7.9 Hz, 1H, H-4″), 9.22 (d, $J_{6''-5''}$ = 6.1 Hz, 1Hz, 1H, H-6″), 9.44 (s, 1H, H-2″); $^{13}$C NMR ($D_2O$) δ 20.98 (C-4′), 30.98 (C-5′), 49.81 (C-3′), 62.62 ($NCH_2N$), 124.00 (C-4, C-7), 128.64 (C-5″), 131.05 (C-3a, C-7a), 134.06 (C-3″), 135.40 (C-5, C-6), 145.21, 146.04, 147.45, (C-4″, C-2″, C-6″), 165.50, 169.08, 171.49, 173.61 ($CONH_2$, C-1, C-3, C-2′, C-6′). Anal. calcd for ($C_{20}H_{17}ClN_4O_5$): C, 56.02; H, 4.00; N, 13.07; found: C, 55.85; H, 4.04; N, 13.42%.

**1-[3-(1,3-Dihydro-1,3-dioxo-2*H*-isoindole-2-yl)-2,6-dioxo-piperidine-1-yl-methyl]-1,4-dihydropyridine-3-carboxylic acid amide (18).** A solution of 0.43 g (1 mmol) **17** in 70 mL of water was degassed with nitrogen and cooled to 0–5 °C. A mixture of 0.5 g sodium hydrogencarbonate and 0.7 g sodium dithionite were added and stirred for 1 h at 0–5 °C. Then, the mixture was extracted 5–6× with 50 mL of degassed dichloromethane until the aq phase was decolorized. The combined organic layers were once washed with water and dried over $Na_2SO_4$. The solvent was removed in vacuo and the yellow residue was recrystallized from ethanol. Yield 0.126 (32%); mp 207–210 °C; DC (eluent B) $R_f$-value = 0.60; IR (KBr) ν 3474, 3142, 1784, 1718, 1694, 1676, 1390, 716; $^1$H NMR δ 2.08–2.12 (m, 1H, H-4′), 2.56–2.61 (m, 1H, H-4′), 2.79–3.07 (m, 4H, 2×H-5′, 2×H-4″), 4.59–4.61 (m, 1H, H-5″) 4.94, 5.02 (AB, $J_{AB}$ = 13.9 Hz, 2H, $NHCH_2N$), 5.34 (dd, $J_{3'a-4'e}$ = 5.0 Hz, $J_{3'a-4'a}$ = 12.8 Hz, 1H, H-3′), 5.97 (d, $J_{6''-5''}$ = 7.7 Hz, 1H, H-6″), 6.58 (s, 2H, $CONH_2$), 7.02 (s, 1H, H-2″), 7.89–7.96 (m, 4H, $H_{arom}$); $^{13}$C NMR δ 20.97 (C-4′), 22.10 (C-4″), 31.15 (C-5′), 49.49 (C-3′), 56.43 ($NCH_2N$), 101.35 (C-3″), 101.81 (C-5″), 123.46 (C-4, C-7), 128.98 (C-6″), 131.17 (C-3a, C-7a), 134.91 (C-5, C-6), 137.63 (C-2″). 167.08, 168.77, 170.18, 172.18 (C-1, C-3, $CONH_2$, C-2′, C-6′); TSPMS *m*/*z* 395 ($M^+$). Anal. calcd for ($C_{20}H_{18}N_4O_5$): C, 60.91; H, 4.60; N, 14.21; found: C, 61.19; H, 4.67; N, 14.38%.

**3-Carboxy-1-[3-(1,3-dihydro-1,3-dioxo-2*H*-isoindoline-2-yl)-2,6-dioxo-piperidine-1-ylmetyl]pyridinium iodide (19).** A solution of 1.59 g (4 mmol) **15** in 20 mL of abs acetonitrile was refluxed with 0.49 g (4 mmol) nicotinic acid for 2.5 h. After cooling the precipitate was collected by filtration and washed with ethanol. Yield 1.11 (53%); mp 246–250 °C; DC (eluent D) $R_f$-value = 0.13; IR (KBr) ν 3058, 2904, 1784, 1772, 1712, 1392, 720; $^1$H NMR δ 2.15–2.18 (m, 1H, H-4′), 2.68–2.72 (m, 1H, H-4′), 2.95–3.01 (m, 2H, 2×H-5′), 5.32 (dd, $J_{3'a-4'e}$ = 4.8 Hz, $J_{3'a-4'a}$ = 12.9 Hz, 1H, H-3′), 6.53 (s, 2H, $NCH_2N$), 7.93 (s, 4H, $H_{arom}$), 8.28 (t, 1 H, H-5″), 9.02 (d, $J_{4''-5''}$ = 7.9 Hz, 1H, H-4″), 9.11 (d, $J_{6''-5''}$ = 5.8 Hz, 1H, H-6″), 9.40 (s, 1H, H-2″); $^{13}$C NMR δ 20.69 (C-4′), 31.33 (C-5′), 49.50 (C-3′), 61.77 ($NCH_2N$), 123.53 (C-4, C-7), 127.80 (C-5″), 131.09 (C-3a, C-7a), 132.20 (C-3″), 135.05 (C-5, C-6), 146.67 (C-2″, C-4″), 147.59 (C-6″), 162.84 (COOH), 166.95, 170.27, 172.04 (C-1, C-3, C-2′, C-6′); EIMS *m*/*z* 393 ($M^+$). Anal. calcd for ($C_{20}H_{16}IN_3O_6$): C, 46.08; H, 3.09; N, 8.06; found: C, 46.37; H, 3.14; N, 7.71%.

**[3-(1,3-Dihydro-1,3-dioxo-2*H*-isoindole-2-yl)-2,6-dioxo-piperidine-1-yl]-acetic acid *tert*-butyl ester (20).** A solution of 0.176 g (4.4 mmol) sodium hydride (60%) in 20 mL of

abs dimethylformamide was reacted with 1.03 g (4 mmol) thalidomide while cooling. After the formation of gas has ceased, 1.21 g (5 mmol) *tert*-butyl iodoacetate were added slowly and stirred for further 24 h. The mixture was poured into ice water; the pH was adjusted to 2.0 with 5% hydrochloric acid. The precipitate was filtered off and recrystallized from ethanol. Yield 0.92 (62%); mp 143–144 °C; DC (eluent A) $R_f$-value = 0.83; IR (KBr) ν 2978, 1786, 1772, 1722, 1688, 1392, 1170, 720; $^1$H NMR δ 1.40 (s, 9H, C(C$H_3$)$_3$), 2.12–2.17 (m, 1H, H-4′), 2.65–2.71 (m, 1H, H-4′), 2.83–2.89 (m, 1H, H-5′), 3.04–3.10 (m, 1H, H-5′) 4.26, 4.33 (AB, $J_{AB}$ = 16.7 Hz, 2H, C$H_2$COOH), 5.29 (dd, $J_{3'a-4'e}$ = 5.0 Hz, $J_{3'a-4'a}$ = 13.1 Hz, 1H, H-3′), 7.88–7.95 (m, 4H, $H_{arom}$); $^{13}$C NMR δ 20.99 (C-4′), 27.51(C(*C*H$_3$)$_3$), 30.95 (C-5′), 41.70 (*C*H$_2$COOH), 49.55 (C-3′), 81.42 (*C*(CH$_3$)$_3$), 123.43 (C-4, C-7), 131.17 (C-3a, C-7a), 134.90 (C-5, C-6), 166.32, 167.02, 169.07, 171.15 (C-1, C-3, C-2′, C-6′, COOH); TSPMS *m*/*z* 390 ($M^+$ + 18). Anal. calcd for ($C_{19}H_{20}N_2O_6$): C, 61.28; H, 5.41; N, 7.52; found: C, 61.56; H, 5.55; N, 7.84%.

**[3-(1,3-Dihydro-1,3-dioxo-2*H*-isoindole-2-yl)-2,6-dioxo-piperidine-1-yl]-acetic acid (21).** A solution of 2.98 g (8 mmol) **20** in a mixture of 40 mL of 25% trifluoroacetic acid in dichloromethane was reacted for 1 h at rt. The solvent was evaporated in vacuo. The remaining residue was coevaporated 3–4× until dry. The amorphous powder was recrystallized from water. Yield 2.27 g (85%); mp 111–113 °C; DC (eluent E) $R_f$-value = 0.24; IR (KBr) ν 3004, 2964, 2632, 2548, 1772, 1720, 1690, 1398, 1172, 722; $^1$H NMR δ 2.12–2.16 (m, 1H, H-4′), 2.66–2.71 (m, 1H, H-4′), 2.82–2.88 (m, 1H, H-5′), 3.04–3.10 (m, 1H, H-5′) 4.29, 4.34 (AB, $J_{AB}$ = 16.9 Hz, 2H, C$H_2$COOH), 5.29 (dd, $J_{3'a-4'e}$ = 5.0 Hz, $J_{3'a-4'a}$ = 13.0 Hz, 1H, H-3′), 7.91 (m, 4H, $H_{arom}$); $^{13}$C NMR δ 21.00 (C-4′), 30.94 (C-5′), 41.04 (*C*H$_2$COOH), 49.53 (C-3′), 123.42 (C-4, C-7), 131.16 (C-3a, C-7a), 134.90 (C-5, C-6), 167.04, 168.72, 169.12, 171.17 (C-1, C-3, C-2′, C-6′, COOH); TSPMS *m*/*z* 334 ($M^+$ + 18), 317 ($MH^+$). Anal. calcd for ($C_{15}H_{12}N_2O_6$ × $H_2O$): C, 53.90; H, 4.22; N, 8.38; found: C, 54.04; H, 4.28; N, 8.56%.

**2-(1-Methoxymethyl-2,6-dioxo-piperidine-3-yl)-1,3-dihydro-1,3-dioxo-2*H*-isoindole-1,3-dione (22).** A suspension of 1.03 g (4 mmol) **1** and 16 g phosphorus pentoxide in 30 mL of dichloromethane were reacted with 16 mL of formaldehyde 1,1-dimethoxymethane at rt for 72 h. Under cooling 20 mL of water was slowly added. The mixture was extracted 3× with 20 mL of diethyl ether. The combined organic layer was dried over $Na_2SO_4$ and the solvent was removed in vacuo. The residue was crystallized from ethanol. Yield 0.80 (66%); mp 153–155 °C; DC (eluent A) $R_f$-value = 0.64; IR (KBr) ν 2958, 2830, 1784, 1772, 1720, 1686, 1394, 1114, 720; $^1$H NMR δ 2.08–2.14 (m, 1H, H-4′), 2.54–2.67 (m, 1H, H-4′), 2.79–2.84 (m, 1H, H-5′), 2.99–3.11 (m, 1H, H-5′), 3.25 (s, 3H, $CH_3$), 5.04, 5.09 (AB, $J_{AB}$ = 9.9 Hz, 2H, $NCH_2O$), 5.32 (dd, $J_{3'a-4'e}$ = 5.4 Hz, $J_{3'a-4'a}$ = 13.1 Hz, 1H, H-3′), 7.88–7.95 (m, 4H, $H_{arom}$); $^{13}$C NMR δ 20.98 (C-4′), 31.15 (C-5′), 49.52 (C-3′), 56.53 ($CH_3O$), 70.17 ($NCH_2O$), 123.44 (C-4, C-7), 131.18 (C-3a, C-7a), 134.89 (C-5, C-6), 167.08, 169.80, 171.68 (C-1, C-3, C-2′, C-6′); GCMS *m*/*z* 302 ($M^+$). Anal. calcd for ($C_{15}H_{14}N_2O_5$): C, 59.60; H, 4.67; N, 9.27; found: C, 59.54; H, 4.72; N, 9.38%.

### Solubility determination

The solubility of selected compounds was determined using UV. In general, a saturated solution (200 mg/mL) of the compounds in water was prepared and allowed to reach equilibrium while stirring at rt for 1 h. Compound **11** was dissolved in buffer, pH 7.4. After filtration through cotton, the saturated stock solutions were diluted in order to enable UV determination. The solubility of the compounds was then calculated using calibration curves, previously carried out. Due to the good water solubility of the compounds **8**, **9** and **10**, it was possible to dissolve them in concentrations as high as 300 mg/mL. No attempt was made to prepare saturated solutions in these cases.

### Determination of the pH of the aqueous solution

Five mg/mL of the compounds were dissolved in water and the pH was measured at rt using a Metrohm 691 pH Meter (Metrohm, Herisau, Switzerland).

### Lipophilicity parameters

In this method, the capacity factor ($k'$) of the solute was taken as a measure of the relative lipophilicity and was calculated as $k' = (t_R - t_0)/t_0$, where $t_R$ is the retention time of the solute and $t_0$ is the elution time of the solvent. For analysis, a reversed phase Nucleosil column equipped with a guard column was used. The $k'$ values were determined using acetonitrile/phosphate buffer (50 mM, pH 3.5) (20/80). The flow rate was 1.0 mL/min, the UV detector was set at 300 and 265 nm, respectively.

### Hydrolysis kinetics

The hydrolysis of selected compounds was determined using HPLC. Physiological phosphate buffer pH 7.4 at 37 °C was used. Aqueous solutions of the prodrugs were added to the buffered solution to yield an initial concentration of 100 μg/mL. At selected time intervals, samples were withdrawn and analyzed by HPLC.

### Assays for inhibition of TNF-α synthesis by human peripheral blood mononuclear cells (PBMCs)

PBMCs isolated from three healthy human blood donors were cultured in RPMI 1640 (containing 10% fetal calf serum, 100 μmol β-mercaptoethanol, 50 μg/mL penicillin, 2 mM glutamine and 50 μg/mL streptomycin) at a density of 1–3×10$^6$ cells/mL at 37 °C, 5% $CO_2$ in presence of test compounds in 24-well plates (Sigma, Deisenhofen, Germany). Test compounds were routinely dissolved in DMSO (dimethylsulfoxide, Merck, Darmstadt, Germany) and applied 1 h before release of TNF-α was stimulated by addition of lipopolysaccharide from *Escherichia coli* serotype 0127: B8 (Sigma) at a

final concentration of 0.1 μg/mL. The final concentration of DMSO was adjusted to 0.1%, the final compound concentration was 50 μg/mL, corresponding to 93–194 μM, for each culture sample. After incubation for 20 h at 37 °C and 5% $CO_2$, the TNF-α concentration of all culture supernatants was determined using a commercial TNF-α-ELISA (Boehringer Mannheim, Mannheim, Germany). The percent inhibition of release of TNF-α was calculated relative to cultures treated with DMSO alone. The $IC_{50}$ values were calculated by linear regression.

### Assay for inhibition of IL-2 synthesis in mice

Male white mice (20–30 g body weight) were used. The animals were kept under standardized housing conditions. Test compounds were applied ip in a concentration of 400 mg/kg using carboxymethylcellulose as vehicle and as control. After 30 min, staphylococcal-superantigen was injected iv in a concentration of 200 μg/animal. After 2 h, the inhibition of the IL-2 concentration was determined collecting blood samples by puncturing the heart. The IL-2 concentration of all blood samples was determined using a commercial ELISA (Boehringer Mannheim). The percent inhibition of IL-2 synthesis was calculated relative to the blood samples treated with carboxymethylcellulose alone. The $ED_{40}$ of compounds **7** and **8** was additionally determined after peroral application.

### Effect on local Shwartzman reaction in mice

The assay was carried out as previously described.[39] Briefly, lipopolysacharide (LPS) from *Salmonella typhimurium* was injected intradermally at a dose of 100 μg in a volume of 30 μL on one side of the shaved dorsum. On the other side, the same volume of a sterile salt solution was applied as a control. After 24 h, the Evans Blue solution was injected in the ear vene. Immediately after this injection, TNF-α (133 ng/0.4 mL) was injected subcutaneously on the site of the intradermal injection. Thalidomide derivatives were administered ip three times in a concentration of 400 mg/kg, 10 min before and 8 h after LPS injection as well as 10 min before the TNF-α challenge injection. The dorsal skin was excised. Evans Blue was extracted using formamide and its extinction was determined at 623 nm.

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