Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 5249

www.rsc.org/obc

PAPER

Prodrug design for the potent cardiovascular agent N^{ω}-hydroxy-L-arginine (NOHA): Synthetic approaches and physicochemical characterization†

Dennis Schade,*‡ Jürke Kotthaus, Nikola Klein, Joscha Kotthaus and Bernd Clement*

Received 6th December 2010, Accepted 20th April 2011
DOI: 10.1039/c0ob01117g

N^{ω}-Hydroxy-L-arginine (NOHA)—the physiological nitric oxide precursor—is the intermediate of NO synthase (NOS) catalysis. Besides the important fact of releasing NO mainly at the NOS-side of action, NOHA also represents a potent inhibitor of arginases, making it an ideal therapeutic tool to treat cardiovascular diseases that are associated with endothelial dysfunction. Here, we describe an approach to impart NOHA drug-like properties, particularly by wrapping up the chemically and metabolically instable *N*-hydroxyguanidine moiety with different prodrug groups. We present synthetic routes that deliver several more or less highly substituted NOHA derivatives in excellent yields. Versatile prodrug strategies were realized, including novel concepts of bioactivation. Prodrug candidates were primarily investigated regarding their hydrolytic and oxidative stabilities. Within the scope of this work, we essentially present the first prodrug approaches for an interesting pharmacophoric moiety, *i.e.*, *N*-hydroxyguanidine.

Introduction

Nitric oxide (NO) is involved in a myriad of (patho)physiological processes.[1,2] An impaired NO bioavailability is well-recognized in the pathology of endothelial dysfunction and atherosclerosis, and therefore is relevant for many cardiovascular diseases.[3] To date, multiple up- and downstream targets have been identified that are available for pharmacologic intervention within the NO/cGMP system. Among these, new developments suggest a paradigm shift from purely symptomatic to more causative therapeutic approaches. Interference with the NO generating/regulating system opens up opportunities to not only treat ischemic heart disease but many more diseases that are characterized by endothelial dysfunction.[4] It is highly desirable to more selectively affect pathologically altered processes of NO generation and availability, thereby providing a safe and effective therapy. As a consequence, a great deal of research has focused on modulation of the predominant enzymes involved in the regulation of endogenous NO levels, with the nitric oxide synthase (NOS) representing the central enzyme of NO formation.

NOSs catalyze the 5-electron oxidation of L-arginine to L-citrulline and NO *via* intermediate formation of N^{ω}-hydroxy-L--arginine (NOHA).[5] Although NOS is a desirable target, the NOS active site has demonstrated very little promiscuity, and to date few alternative substrates or modulators have been identified.[4] We consider the physiological *N*-hydroxyguanidine NOHA the ideal NOS substrate for therapeutic applications. Besides the important fact of releasing NO mainly at the NOS-site of action, NOHA also represents a potent inhibitor of arginases, with the latter being well recognized as contributing to the development of endothelial dysfunction.[6]

Although NOHA can serve as a substrate for NOS and thereby stimulate NO generation, direct therapeutic application is prohibited by its little drug-like character. The major problem to be solved is NOHA's deleterious chemical and metabolic properties (see Fig. 1).

N-Hydroxyguanidines in general are thermolabile as they are already unstable at room temperature and should be stored at –20 °C. Usually, they are most stable in the form of salts of strong acids and are labile towards bases.[7,8] *N*-Hydroxyguanidines undergo two and three electron oxidative degradation reactions, with different oxidants leading to distinct products, such as cyanamides or urea derivatives.[9–14] Furthermore, NOHA and other *N*-hydroxyguanidines are known to be readily metabolized by the *N*-reductive biotransformation pathway.[15–19]

Although the formed metabolite L-arginine represents the actual substrate for NOSs and may be considered an active metabolite from this *N*-reduction, it would be favorable to "smuggle" the

Department of Pharmaceutical Chemistry, Pharmaceutical Institute, Christian-Albrechts-University of Kiel, Gutenbergstraße 76-78, D-24118 Kiel, Germany. E-mail: dschade@sanfordburnham.org, bclement@pharmazie.uni-kiel.de; Fax: +49-880-1352; Tel: +49-880-1126

† Electronic supplementary information (ESI) available: Complete synthetic protocols and spectral data of all synthesized compounds (including intermediates); synthetic details for attempts towards *O*-acylated NOHA prodrugs; determination of enantiomeric purity by HPLC; *in vitro* bioactivation of **13**; detailed hydrolytic stability graphs of selected prodrugs. See DOI: 10.1039/c0ob01117g

‡ Current address: Sanford-Burnham Medical Research Institute and Human BioMolecular Research Institute, 5310 Eastgate Mall, San Diego, CA 92121, USA. Fax: +1-858-459-9311; Tel: +1-858-459-9305

This journal is © The Royal Society of Chemistry 2011

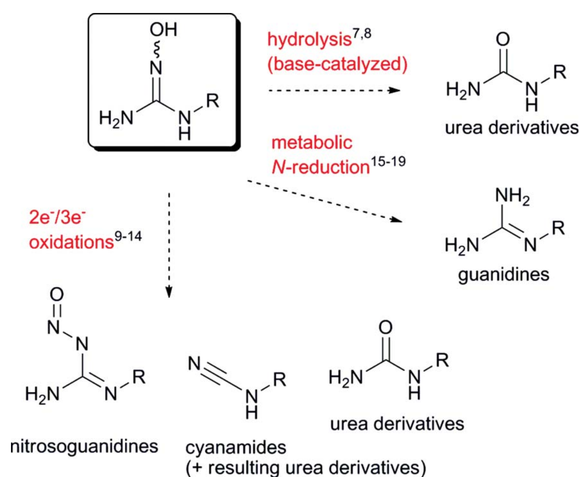


Fig. 1 Chemical and metabolic degradation of *N*-hydroxyguanidines.

direct NO-precursor NOHA through the first-pass effect passage without having significant amounts of L-arginine formed. The background is that L-arginine underlies a plethora of metabolic pathways,[20] whereas NOHA physiologically only serves NO production, as is so far known.

To circumvent the above stability issues associated with NOHA, we decided to synthetically pursue chemically stabilized NOHA analogues. *O*- and *N*-substitutions were designed which may suppress radical formation, abstraction of protons, or nucleophilic attack at the hydroxyguanidine-carbon. Concurrently, such modifications were expected to increase membrane permeation processes, either active or passive ones. Synthetic feasibility for novel NOHA prodrug candidates and model compounds was explored, along with their physicochemical stabilities.

Results and discussion

General synthetic concept

Within the course of our ongoing studies with various N^{δ}- and N^{ω}-substituted L-arginines,[21,22] we have already reported on a synthetic strategy that allowed access to N^{ω}-substituted L-arginines.[23] Among these, one *O*-methylated NOHA derivative could be prepared in excellent yields. The underlying reaction employs a concept that has been first described by Linton *et al.* for the synthesis of guanidines.[24] Martin *et al.* could successfully apply this strategy to the preparation of NOHA and N^{ω}-amino-L-arginines.[25,26] Essentially, a highly substituted guanidine (or *N*-hydroxyguanidine) is built up by reacting a carbamoylated thiourea with the desired amine or hydroxylamine in the presence of a desulfurizing agent, such as EDCI. This strategy provided an attractive basis for the preparation of the herein described NOHA derivatives as it avoids direct *O*-substitution of the *N*-hydroxyguanidine group. Direct substitution was expected to be hampered by the unstable character of the *N*-hydroxyguanidine as rather harsh reaction conditions are required.

Our general synthetic approach to a diverse set of protected NOHA derivatives is outlined in Scheme 1. Thioureas **2** could be conveniently obtained by reaction of the protected L-ornithine **1** with the respective isothiocyanate. That way, either a simple protecting group (Cbz) could be inserted or a prodrug group (Eoc) that was supposed to be retained for some of the final molecules.

Synthesis of *O*-alkylated prodrugs

One approach to increase the stability of *N*-hydroxyguanidines was by suitable *O*-alkylations. A similar approach has already been described for amidoximes, such as pafuramidine (DB289), an *O*-methylated amidoxime with antiprotozoal activity.[27] The primary aim of this prodrug concept was to increase oral bioavailability and permeation of the blood-brain barrier rather than improving its overall stability.

Our first attempts to prepare such *O*-methylated NOHA prodrugs used the corresponding cyanamide and methoxylamine as starting materials in accordance with a literature protocol.[28] But even after optimization of this reaction (80% yield), the general applicability to other derivatives remained limited.

Using the outlined synthetic scheme (see Scheme 1), we were not only able to further increase the overall yields but also gained access to quite diversely substituted derivatives (Fig. 2). Thus, we could realize free amino acid prodrug candidates **8a** and **8c** as well as the corresponding ethylesters **8b** and **8d**. For complete deprotection, **6a** was treated with TFA/thioanisole. For esterification, dry HCl gas was carefully bubbled through an ethanolic solution of **8a** (or **8c**) at −10 °C, which was then left at 4 °C for

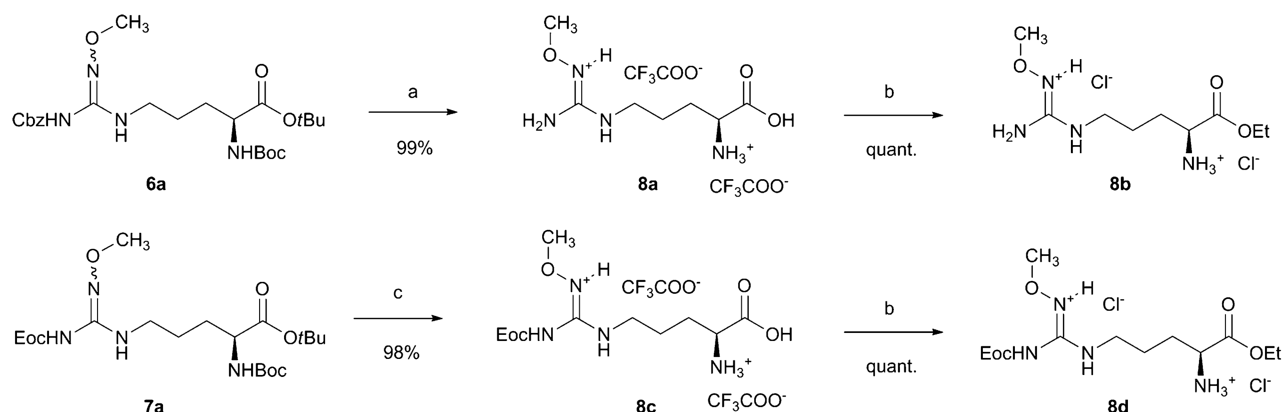
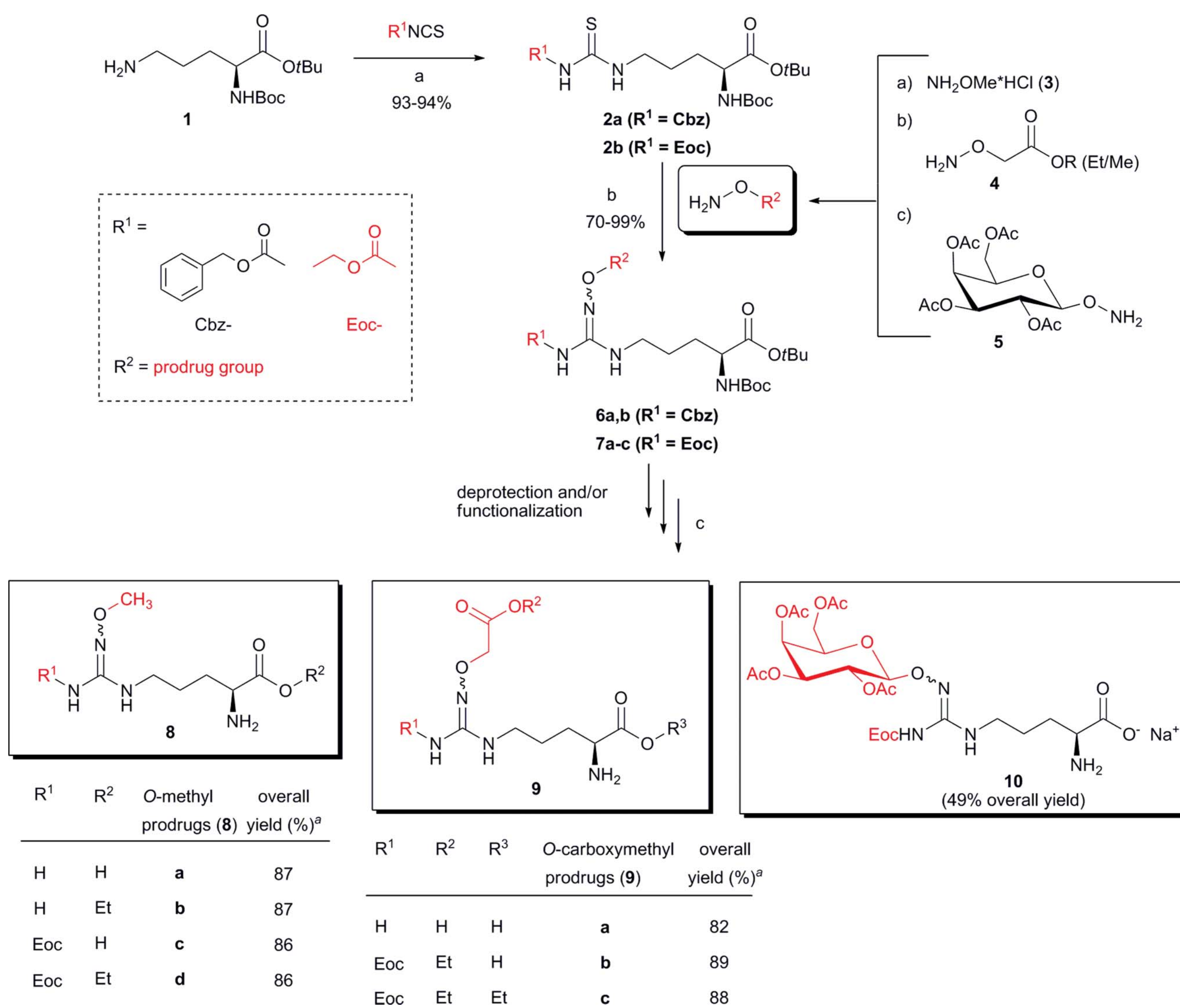


Fig. 2 **Synthesis of *O*-alkylated prodrugs of NOHA**. *Reagents and conditions*: (a) TFA/thioanisole, 30 min, r.t.; (b) $HCl_{(g)}$ in EtOH, 1 h at −10 °C, 36 h at 4–8 °C; (c) TFA, 30 min at 0 °C, 3 h at r.t.

 This journal is © The Royal Society of Chemistry 2011



R^1	R^2	*O*-methyl prodrugs (**8**)	overall yield (%)[a]
H	H	**a**	87
H	Et	**b**	87
Eoc	H	**c**	86
Eoc	Et	**d**	86

R^1	R^2	R^3	*O*-carboxymethyl prodrugs (**9**)	overall yield (%)[a]
H	H	H	**a**	82
Eoc	Et	H	**b**	89
Eoc	Et	Et	**c**	88

Scheme 1 General synthetic approach to highly substituted NOHA prodrugs. *Reagents and conditions*: (a) in CH_2Cl_2, r.t., 2 h; (b) DIPEA, EDCI, r.t., 24–48 h, in CH_2Cl_2; (c) for details see Fig. 2 and 3. [a] Overall isolated yield, starting from **1**.

1.5 days until the reaction was complete. The desired ethylesters **8b/d** were obtained in high yield and purity after lyophilization. TFA was simultaneously esterified by these procedures, and the ester was subsequently removed *in vacuo*. In addition, with **8c** and **8d**, Eoc could be introduced in the molecules as a carbamate-based prodrug moiety. For chemoselective deprotection of the α-amino acid group in **7a**, thioanisole was omitted and dry conditions guaranteed to prevent loss of the Eoc moiety. The product **8c** could be conveniently purified by flash chromatography on RP-18 silica gel. With **8c** the *N*-hydroxyguanidine group was already highly substituted, but a further esterification of the carboxy function to **8d** was expected to increase the lipophilicity of the molecule and thereby its general tendency to pass biological membranes by passive diffusion processes.

One possible disadvantage of simple *O*-methylation may be seen in the involvement of cytochrome P450 enzymes in the bioactivation cascade as has been shown for DB289.[29] The consequence might be an interaction potential with other substances metabolized by the respective enzyme system.

Therefore, we envisioned a similar *O*-alkyl prodrug approach for NOHA, utilizing a novel mechanism of bioactivation *via* action of the peptidylglycine α-amidating monooxygenase (PAM).[30] PAM exhibits a broad substrate specificity and recognizes many glycine-like structures, including *N*-, *O*- and *S*-carboxymethylated compounds.[31] We previously demonstrated that *O*-carboxymethylated amidoximes and simple *N*-hydroxyguanidines are accepted as substrates by PAM.[30] For the synthesis of the respective *O*-carboxymethylated NOHA prodrugs **9a–c**, we essentially applied the same chemistry as described for the *O*-methyl prodrugs **8a–d**, but using aminooxyacetic acid derivatives **4** as the hydroxylamine component (see Scheme 1). As an exception, the synthesis of the fully deprotected N^{ω}-carboxymethoxy-L-arginine (**9a**) (as a reference compound for stability and pharmacokinetic studies) had to be slightly altered. The *O*-carboxymethylester was unexpectedly stable towards TFA/thioanisole treatment, and complete deprotection required 5 h incubation at 50–60 °C with 6 N aqueous HCl. The optical purity of **9a** was assessed by chromatography on

a Crownpak Cr(+) column and was preserved in this synthetic sequence (>99%*ee*). Several other *O*-alkylated NOHA prodrugs were also analyzed on chiral stationary phase and shown to be pure by >99%*ee* (see the ESI†).

Synthesis of *O*-acetalic prodrugs

Considering that *O*-alkylations should contribute most to the overall stability of the *N*-hydroxyguanidine moiety, another modification of this kind was realized. Inclusion of the *N*-hydroxy group into an acetal was expected to afford stable hydroxyguanidine prodrugs that can be bioactivated by monooxygenase-independent hydrolytic mechanisms.

Especially, the incorporation into a carbohydrate-based acetal was deemed attractive as (1) they could be bioactivated by glycosidases in addition to slow chemical hydrolysis, (2) the released monosaccharides are rather uncritical from a toxicological point of view, and (3) the conjugate might be taken up into cells by facilitated transport.

This approach was particularly inspired by the work of Melisi *et al.* who used galactosyl-conjugates of D- and L-arginine (attached at the α-amino acid group) as a prodrug principle to increase permeation in, and thereby NO release within, cancer cells.[32] Other similar examples in the field of NO-donors are carbohydrate conjugates of the diazeniumdiolates (NONOates) and *S*-nitrosothiols, that were successfully optimized in terms of stability and pharmacokinetics.[33,34]

Because such strategies were not previously applied to *N*-hydroxyguanidines, we also synthesized a simple model compound for *in vitro* characterization purposes. The only described preparation of an *O*-glycosylic hydroxyguanidine was reported by our group for the *N*-hydroxylated metabolite of the antihypertonic agent debrisoquine.[16] However, the actual glycoside formation was attempted by direct substitution of the *N*-hydroxy function using the respective sugar-1-bromide, with resulting yields of <1%. Following the synthetic strategy described here, we used the 1-aminooxyl derivative of peracetylated β-D-galactose (**5**, prepared according to a modified literature procedure)[35] in order to build up the glycosylated *N*-hydroxyguanidine under mild conditions. Importantly, the β-configuration is required for the targeted galactosidases.[36] Using this strategy, we could prepare the desired galactose conjugates **7c** and **12** in very good yields (70% and 90%, see Fig. 3).

Further deprotection of **12** was achieved with a strong anion exchange resin to afford the deacetylated β-D-galactose conjugate **13** as the model compound (see B in Fig. 3). On the basis of NOHA derivative **7c** we achieved chemoselective deprotection of the α-amino acid moiety without affecting the acid-labile glycosidic group by careful bubbling of HCl gas through an absolute diethyl ether solution, after which the amino acid **10** precipitated as a hydrochloride salt (see A in Fig. 3). To avoid an autocatalyzed hydrolysis of this hydrochloride upon contact with water, we attempted isolation of the respective sodium salt by dissolving the powder in 0.5 M sodium bicarbonate and subsequent purification on reversed-phase (RP-18) silica gel. Using this method, the desired *O*-galactosyl NOHA prodrug **10** could be prepared in good yields in its peracetylated form. Compound **10** represented the preferred form as a prodrug candidate due to its higher stability towards presystemic inactivation by chemical and/or enzymatic degradation.

Synthesis of *O*-acylated prodrugs

A final set of NOHA prodrugs was envisioned that was solely comprised of carboxylic ester- and carbamate-based prodrug groups. In terms of bioactivation, this group of compounds was most appealing as esterases are ubiquitously present and reveal high catalytic activities as well as broad substrate specificities.[37] However, we faced substantial problems with the preparation of these NOHA derivatives (see Scheme 2).

Initial attempts to apply the above described synthetic strategy (see Scheme 1) failed since, in our hands, the required *O*-acylated hydroxylamines could not be prepared according to a literature procedure.[38] We were unable to deprotect several *O*-acylated ethyl imidates using HCl in different solvents, such as nitromethane, diethyl ether, or 1,4-dioxane. They were not commercially available, and thus, other approaches were pursued.

A second strategy was to acylate the protected NOHA derivative **14** (see A in Scheme 2). Compound **14** was prepared in two steps according to a literature protocol.[39] However, despite efforts exploring several different solvent systems under various conditions with acid chlorides and with anhydrides, none of the

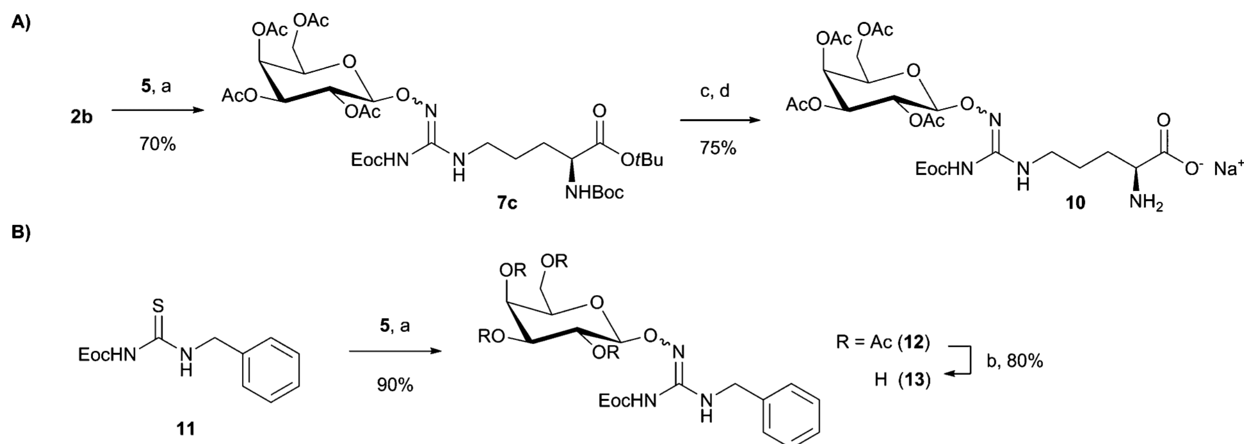
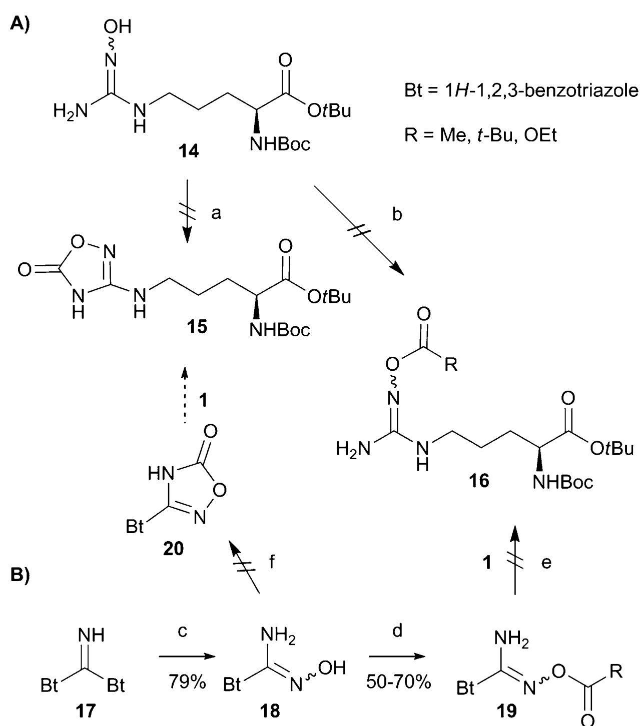


Fig. 3 Synthesis of *O*-galactosylated *N*-hydroxyguanidines. *Reagents and conditions*: (a) DIPEA, EDCI, r.t., 24 h, in CH_2Cl_2; (b) basic anion exchanger, MeOH, r.t., 24 h; (c) Et_2O, −15 °C, argon, $HCl_{(g)}$ for 5 min, 4–8 °C overnight; (d) 0.5 M $NaHCO_3$, RP-18 flash chromatography.

 This journal is © The Royal Society of Chemistry 2011



Scheme 2 Attempts to prepare *O*-acyl type prodrugs of NOHA. *Reagents and conditions*: (a) carbonyldiimidazole (CDI) or ethylchloroformate, in THF, DMF or EtOH; or *via O*-THP protected Eoc-substituted **14** (see the ESI†); (b) acid chlorides or anhydrides, in dry DCM, Et_2O, pyridine, temperatures from −30 °C to r.t.; (c) NH_2OH (free base), in dioxane, 60 °C, 2 h; (d) acid chloride, TEA, CH_2Cl_2; (e) conventional or microwave conditions, from r.t. up to 100 °C, neat or in CH_2Cl_2, THF or EtOH; (f) carbonyldiimidazole (CDI) or ethylchloroformate in THF, DMF or EtOH, or phosgene in toluene. Experimental data for these reactions are found in the ESI.†

compound **16** *O*-acyl derivatives could be prepared. We usually obtained complex product mixtures instead. One persistent side reaction was the incidental *N*-acylation of the *N*-hydroxyguanidine moiety, even when using the less reactive pivalic acid chloride. *N*-Acetylation occurred after formation of a mixed anhydride with acetic acid, which was present in the starting material **14** after chromatographic workup. Furthermore, no significant amounts of the desired *O*-acyl derivatives could be obtained from the free base form of **14**, which was found to be unstable. This is consistent with a study by Belzecki *et al.* concerning peracetylations of similar *N*-hydroxyguanidines, which states that successful selective *O*-acetylation was only achieved for very few highly substituted *N*-hydroxyguanidines.[40–42]

The cyclic carbamate (1,2,4-oxadiazol-5-one) of NOHA (**15**) represents an extension of the "*O*-acyl" prodrug principle for NOHA and was based on a similar compound described by Rehse *et al.*[43] However, their NOHA-based oxadiazolone turned out to be inactive as a NO donor, a characteristic likely attributable to the chemically and metabolically highly stable N^{α}-acetyl group. In addition, the racemic nature of their compound eventually lowers efficacy 2-fold. We therefore wanted to circumvent a synthetic protocol that delivers racemic material or N^{α}-substituted derivatives. To date, our attempts to cyclize **14** to afford the respective cyclic carbamate **15** failed, using CDI or ethylchloroformate under different conditions. Other attempts included the preparation of *O*-THP-protected, N^{ω}-Eoc-substituted NOHA (and simple model compound analogs) in order to induce cyclization (elimination of EtOH) after *in situ* chemoselective deprotection of the THP group (see the ESI†).

The third strategy was to prepare an agent that should transfer an *O*-acylamidoxime moiety (**19**) or a 1,2,4-oxadiazol-5-one group (**20**) to the side-chain amine of protected L-ornithine **1** to afford the respective *O*-acylhydroxyguanidine **16** or cyclic carbamate **15**, respectively (see B in Scheme 2). According to the various pioneering applications described by the Katritzky group, we deemed benzotriazole to be a suitable leaving group for this purpose. In fact, Katritzky *et al.* have described similar applications to the synthesis of amidoximes and *N*-hydroxyguanidines.[44,45] After slight modifications of the literature protocols we could prepare *N′*-hydroxy-1*H*-1,2,3-benzotriazole-1-carboximidamide (**18**) and from there, different *O*-acyl derivatives (**19**; acetyl, pivaloyl and ethoxycarbonyl) in good yields. However, neither under conventional nor microwave conditions did these reagents react with **1** to give the desired products **16**. The only detected reaction was elimination of the hydroxylamine with replacement by the amine (*i.e.*, the protected L-ornithine **1**), as demonstrated by TLC and LC/MS analysis of crude products. Moreover, reagent **20** could not be prepared using CDI, ethylchloroformate, phosgene or phosgene-like reagents.

In vitro characterization

Hydrolytic stability. As indicated in the introduction, *N*-hydroxyguanidines are particularly susceptible to base-catalyzed hydrolysis. Therefore, the pH stability of different NOHA prodrugs was assessed at 37 °C for up to 24 h, and the mixtures were analyzed by HPLC (see Fig. 4, A). A more detailed analysis including the analysis of samples at several time points over the 24 h incubation is included in the ESI.†

When comparing the most simple (*i.e.*, least substituted) prodrugs **8a** and **9a** with NOHA, it is apparent that *O*-alkylations improve the stability at pH 9. The *O*-methylated N^{ω}-carbamate prodrug **8c** also exhibits increased stability at this pH suggesting that N^{ω}-substitution does not have deleterious effects on stability but rather improves it. Surprisingly, NOHA underwent only about 10% degradation after 24 h at 37 °C at physiological pH (*i.e.*, 7.4).

NOHA prodrugs **8b** and **9c** revealed an expected hydrolytic degradation due to hydrolysis of the α-amino acid ester. Interestingly, this ester appears to be more sensitive towards hydrolysis than the ethyloxycarbonylmethoxy group in **9b**.

NOHA prodrug **10** was not included in this stability study, as the presence of four acetyl groups was expected to overly complicate interpretation of the data. Instead, the deacetylated β-D-galactose-conjugate **13** was used, allowing for focused analysis of the stability of the sugar-acetal function. Unfortunately, we were not yet able to isolate the corresponding deacetylated NOHA prodrug in sufficient purity for the herein presented stability studies. Strikingly, **13** proved very stable at all three pHs. Considering the actual lability of acetals towards acidic media, the stability of **13** at pH 2 was quite surprising and might be due to steric as well as electronic effects.

Oxidative stability. We selected a set of distinct oxidants that have been reported in the literature to be capable of oxidizing

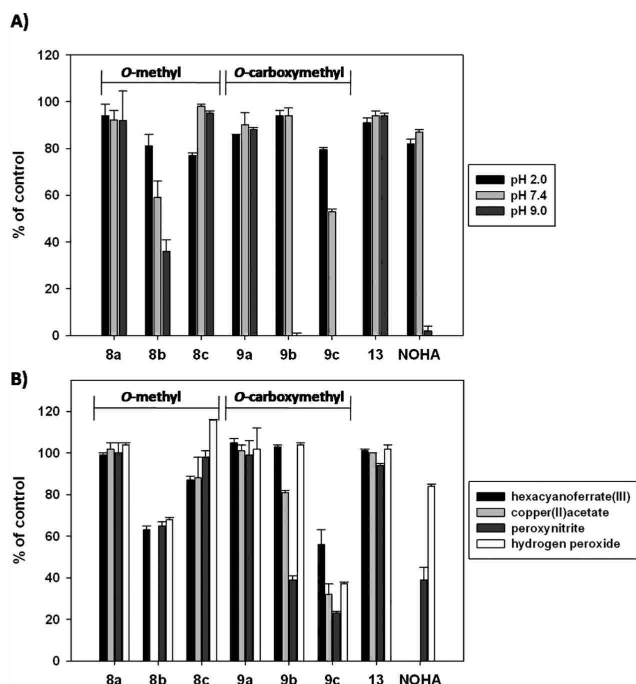


Fig. 4 ***In vitro*** **stability studies**. (**A**) pH stability: 250 μM compounds in 10 mM potassium phosphate buffer pH 2, pH 7.4 and pH 9 after incubation at 37 °C for 24 h (mean value of at least three independent experiments ± SD); (**B**) oxidative stability: 250 μM compounds in 10 mM phosphate buffer pH 7.4 and 500 μM potassium hexacyanoferrate(III), 500 μM copper(II) acetate, 500 μM peroxynitrite or 29 mM hydrogen peroxide at 37 °C for 24 h (mean value of at least three independent experiments ± SD); control = concentration at $t = 0$ h.

N-hydroxyguanidines, with a special focus on chemicals that are of significance in NO-related biochemistry. Because oxidation of NOHA by these compounds has been proposed to lead to a variety of products, we opted to monitor the stability of the parent compound, rather than formation of products. Hydrogen peroxide (H_2O_2) and peroxynitrite (ONO_2^-) were used to mimic oxidative stress conditions. Elevated levels of such reactive oxygen species (ROS) are especially of interest in the targeted tissues/cells (*e.g.*, endothelium) under pathophysiological conditions, and are known to affect the bioavailability of NO.[46,47] Copper and iron were also used as oxidants, as two- and three-valent metal ions that are considered to contribute to the metabolic fate of NOHA and of *N*-hydroxyguanidines in general. Normally, metal metabolism is highly regulated. However, NO has been shown to elicit metal ions from metalloproteins and to disrupt metal metabolism (see Cho *et al.* and references therein).[12] The Fukuto lab pointed at the physiological relevance of interactions between *N*-hydroxyguanidines and cupric ions, providing comprehensive insights into the underlying reactions and mechanisms.[12] As an example of three-valent ions, iron(III) was used in the form of its ferricyanide complex ($K_3[Fe(CN)_6]$). This oxidant has also been reported in the literature to oxidize *N*-hydroxyguanidines.[13]

The oxidative stability of NOHA and its different prodrugs was monitored over 24 h at 37 °C by HPLC (Fig. 4). Under the tested conditions, NOHA exhibited large sensitivity towards copper(II) acetate and ferricyanide, but was moderately insensitive towards ONO_2^-. In fact, *N*-hydroxyguanidines have been demonstrated to inhibit peroxynitrite-induced oxidation.[48] NOHA showed little sensitivity to H_2O_2.

Simple *O*-alkylation resulted in a remarkable increase of stability as can be seen for prodrugs **8a** and **9a**. An additional N^ω-carbamate group (see data for **8c**) provides a similarly stable prodrug. This is also observed for **9b** but with an increased lability towards ONO_2^-. Data for **8b** and **9c** should be carefully interpreted considering their general stability profile without addition of oxidants, *i.e.*, at pH 7–9 for 24 h at 37 °C (see A in Fig. 4). Only **8b** turned out exceptionally labile in incubations with cupric ions, a phenomenon that does not parallel with other results of this study (*e.g.*, **8a** as an *O*-methyl analog, or **9c** as a N^ω-carbamate analog). The apparent stability of galactose-conjugate **13** towards all examined oxidants is remarkable. This result again underscores the utility of this prodrug concept for *N*-hydroxyguanidines.

It is apparent that *O*-substitution (alkyl-, acetal-type) efficiently protects from oxidation. Several groups have suggested that initial formation of an iminoxy radical (**IIIa/b**, and the less favored *C*-centered radical **IIIc**) is key to oxidative degradation reactions of *N*-hydroxyguanidines (see Fig. 5).[9–11]

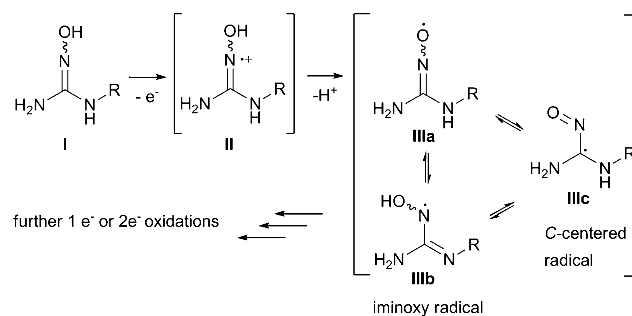


Fig. 5 First step of *N*-hydroxyguanidine oxidation according to literature-proposed mechanisms.

Our data therefore suggest formation of this iminoxy radical is prohibited by *O*-substitution. Cleavage of the O–H bond in NOHA or *N*-hydroxyguanidines is an expected requisite for formation of the stabilized radical (**III**), and proton abstraction is greatly limited by *O*-substitutions. Although **IIIb** does represent a possible radical intermediate for an *O*-alkyl/acetalic NOHA prodrug, it lacks the possibility of tautomeric stabilization. Stabilization of this radical would require dimerization, or reaction with other radicals, such as NO.[12] Since cleavage of the O–C bond is thermodynamically much less favorable, it effectively protects from this oxidation. Moreover, prodrug candidates with an electron-withdrawing substituent (such as Eoc) exhibit a lower electron density rendering them less prone to initial 1e⁻ oxidation (preferably at the double-bonded nitrogen).

Another consequence of *O*-substitution is that the *N*-hydroxyguanidine group has no chance any more to chelate Cu(II) and Fe(III). Especially for cupric ions, this has been discussed as the mechanistic basis for its oxidative potency since the calculated redox potentials for *N*-hydroxyguanidines present in solution make Cu(II)-mediated reduction thermodynamically prohibitive.[12]

Conclusions

Our ultimate goal is to establish a prodrug concept for NOHA that ensures its proper bioavailability, and thereby, sufficient NO

 This journal is © The Royal Society of Chemistry 2011

levels in pathophysiologically altered situations that are associated with endothelial dysfunction. We postulate that physiological conditions could be ideally mimicked with a suitable prodrug candidate that ensures a prolonged and sustained release of NOHA.

Here, we have demonstrated synthetic routes to diversely substituted NOHA derivatives. The overall yields are very good for *O*-alkylated compounds **8** and **9** in this 3–4 step sequence (82–89%), and reasonable for galactose conjugate **10** (49%). The selected prodrug groups addressed different pharmaceutical aspects, particularly with regards to NOHA's chemical and metabolic stability profiles. Our difficulties with the preparation of *O*-acylated prodrug candidates indicates that this type of modification may not lead to compounds with improved pharmaceutical properties.

The performed hydrolytic and oxidative stability studies clearly demonstrated that *O*- and *N*-substitution of the *N*-hydroxyguanidine moiety benefits the drug-likeness of these NOHA derivatives. In summary, especially compared to unsubstituted NOHA , its lability towards bases, two- and three-valent ions as well as the ROS peroxynitrite was solved primarily by *O*-substitution (*O*-alkyl, *O*-acetal). However, it needs to be clarified whether NOHA's stability at pH 2–7 and its moderate stability towards hydrogen peroxide and peroxynitrite is sufficient for *in vivo* (peroral) applications. It might still be challenged by distinct oxidative situations, particularly under conditions of temporarily high local concentrations of oxidatively relevant species.

Notably, we not only wanted to address stability issues but also the general pharmacokinetic profile with respect to passive diffusion processes and targeted active transport mechanisms (such as amino acid and carbohydrate transporters) for a good oral bioavailability. Therefore, novel prodrug concepts were pursued as there are none reported for the class of *N*-hydroxyguanidines so far.

Our future efforts are dedicated to investigate the different bioactivation pathways and general metabolic profiles, respectively. Using model compound **13** we were already able to show very efficient bioactivation by β-galactosidases *in vitro* (see the ESI†). However, without a comprehensive metabolic study it is difficult to name the best prodrug candidate at this stage. Each implicated concept has its assets and drawbacks depending on the desired route of application and formulation (pharmaceutical), velocity of drug release (retardation principles), clinical application (disease), *et cetera*.

Experimental section

Syntheses

General remarks. Melting points are uncorrected. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were obtained on a Bruker ARX 300 spectrometer at 300 K. Chemical shifts (δ values) are reported in ppm relative to TMS, 3-(trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (TPS) as an internal standard, or alternatively, relative to the residual solvent signal. All coupling constants (J values) were obtained by first order analysis of the multiplets. Low resolution mass spectra were recorded on a Bruker–Esquire–LC with an electrospray ionization (ESI). Recordings of high mass resolution spectra were conducted on a Bruker 7.4 Tesla FTICR mass spectrometer BioApex II equipped with an ESI-ion source (Agilent); substances were dissolved in 60% acetonitrile in water and introduced by flow injection analysis. Elemental analyses were performed on a CHNS analysator (HEKAtech GmbH). Reactions were monitored by TLC on precoated silica gel plates (SiO_2 60, F_{254}). All compounds could be visualized by either UV detection, ninhydrine spray or Ce(IV)-sulfate staining and heating at 120 °C. Purification of synthesized compounds was performed by column chromatography using silica gel (particle size 40–63 µm). Flash chromatography on a RP-18 Redi*Sep*® column (43 g) was performed with a CombiFlash®RETRIEVE system. Chromatography on chiral stationary phase was carried out using a Crownpak Cr(+) column (see the ESI for details†). All starting materials were commercially available and used without further purification. N^{α}-Boc-L-ornithine *tert*-butylester hydrochloride (**1**) was purchased from Bachem. CbzNCS was prepared according to literature procedures.[23–25] All solvents were distilled and dried according to standard procedures.

N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-benzyloxycarbonyl-L-thiocitrulline *t*-Butylester (2a)[23,25]. Has been prepared in analogy to the thiourea **2b** described below. Yield: 93%.

N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-ethoxycarbonyl-L-thiocitrulline *t*-butylester (2b). N^{α}-(*t*-Butyloxycarbonyl)-L-ornithine-*t*-butylester (2.02 g, 7.0 mmol) is dissolved in 250 mL of dry CH_2Cl_2. The solution is chilled on ice and ethoxycarbonylisothiocyanate (917 mg, 7.0 mmol), dissolved in 15 mL of dry CH_2Cl_2, is added dropwise for about 30 min. The reaction mixture is stirred for 2 h at room temperature, concentrated to one third of the original volume and washed with 25 mL 1% HCl, water and brine. The organic phase is dried with Na_2SO_4 and concentrated *in vacuo*. Thiourea **2b** is at this point already pure by >95% (TLC). The product was dissolved in a small amount of eluent (Cy/EtOAc, 4 : 1), charcoal added and purified on a short silica gel column (*ca.* 20 g SiO_2). Yield: 2.76 g of a colorless oil (94%) that solidifies upon standing in the refrigerator; mp. 81 °C; TLC: R_f 0.31 (Cy/EtOAc, 4 : 1); ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.31 (t, 3H, ^3J = 7.1 Hz, CH_2–CH_3), 1.45, 1.47 (2 × s, 9H, $C(CH_3)_3$), 1.61–1.90 (m, 4H, β,γ-CH_2), 3.66 (pseudo q, 2H, N–CH_2), 4.22 (q, 2H, ^3J = 7.1 Hz, CH_2–CH_3), 5.08 (m, 1H, α-CH), 8.06, 9.70 (2 × br s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ (ppm) = 14.9 (CH_2–CH_3), 24.9 (γ-CH2), 28.7, 29.0 (2 × $C(CH_3)_3$), 31.0 (β-CH_2), 45.8 (N–CH_2), 54.3 (α-CH), 63.4 (O–CH_2), 82.8 ($C(CH_3)_3$), 153.4 (CO-Eoc), 156.2 (CO-Boc), 172.5 (COO^{t}Bu), 180.1 (C═S); MS (ESI): m/z = 442 $[M + Na]^+$, 420 $[M + H]^+$, 308 $[M - 2 \times C_4H_8 + H]^+$, 264 $[M - 2 \times C_4H_8 - CO_2 + H]^+$; Anal. calcd for $C_{18}H_{33}N_3O_6S$ (419.54): C 51.53, H 7.93, N 10.02, S 7.64; Found: C 51.68, H 7.97, N 10.06, S 7.62.

General procedure for the synthesis of fully protected NOHA derivatives 6 and 7. A literature protocol for the preparation of carbamoylguanidines **6** and **7** was used for the herein presented protected NOHA derivatives.[23–25] 1.5 Equivalents of DIPEA, *O*-substituted hydroxylamine (**3–5**) and EDCI were reacted with 0.5 mmol thiourea (**2a,b**) in 10 mL of dry CH_2Cl_2. For hydroxylamines that were applied as hydrochlorides (= methoxylamine hydrochloride, **3**) 3 equivalents of DIPEA were used. Unless noted otherwise, reactions were complete after stirring overnight. The organic phase was diluted with 10 mL of CH_2Cl_2

This journal is © The Royal Society of Chemistry 2011

and washed with small amounts of 1% aqueous HCl, water and brine. The resulting oils were purified by column chromatography on silica gel.

N^{ω}-Benzyloxycarbonyl-N^{α}-*t*-butyloxycarbonyl-$N^{\omega'}$-methoxy-L-arginine *t*-butylester (6a)[23]. Yield: 95%.

N^{ω}-Benzyloxycarbonyl-N^{α}-(*t*-butyloxycarbonyl)-$N^{\omega'}$-(methoxycarbonyl)methoxy-L-arginine *t*-butylester (6b). Eluent: Cy/EtOAc (3 : 2), R_f 0.48; yield: 254 mg (92%) of a colorless oil; ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.44, 1.46 (2 × s, 9H, $C(CH_3)_3$), 1.50–1.87 (m, 4H, β,γ-CH_2), 3.07 (m, 2H, N–CH_2), 3.73 (s, 3H, O–CH_3), 4.16 (m, 1H, α-CH), 4.41 (s, 2H, O–CH_2), 5.08 (m, 1H, NH), 5.15 (s, 2H, CH_2-Cbz), 6.37 (br t, ^3J = 5.3 Hz, 1H, NH), 7.30–7.39 (m, 5H, ArH), 8.23 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ (ppm) = 25.5 (γ-CH_2), 28.7 (β-CH_2), 29.0, 30.9 (2 × $C(\underline{C}H_3)_3$), 41.2 (N–CH_2), 52.4 (O–CH_3), 54.5 (α-CH), 68.3 (CH_2-Cbz), 71.2 (O–CH_2), 80.3, 82.5 (2 × $\underline{C}(CH_3)_3$), 129.0, 129.28, 129.34 (ArCH), 135.9 (ArC), 150.8 (C═N), 153.7 (CO-Cbz), 156.0 (CO-Boc), 171.7, 172.4 (COO^{t}Bu, COOMe); MS (ESI): m/z = 575 [M + Na]$^+$, 553 [M + H]$^+$, 497 [M – C_4H_8 + H]$^+$.

N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-ethoxycarbonyl-$N^{\omega'}$-methoxy-L-arginine *t*-butylester (7a). Eluent: CH_2Cl_2/MeOH (98 : 2), R_f 0.26; yield: 203 mg (94%) of a colorless oil; ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.27 (t, ^3J = 7.1 Hz, 3H, CH_2–$C\underline{H}_3$), 1.43, 1.45 (2 × s, 9H, $C(CH_3)_3$), 1.56–1.89 (m, 4H, β,γ-CH_2), 3.09 (m, 2H, N–CH_2), 3.66 (s, 3H, O–CH_3), 4.16 (br q, ^3J = 7.1 Hz, 3H, $C\underline{H}_2$–CH_3, α-CH), 5.10, 6.26 (2 × br m, 1H, NH), 7.80 (br s, 1H, NH); ^{13}C-NMR ($CDCl_3$): δ (ppm) = 14.9 (CH_2–$\underline{C}H_3$), 25.6 (γ-CH_2), 29.7 (β-CH_2), 29.0, 30.9 (2 × $C(\underline{C}H_3)_3$), 41.2 (N–CH_2), 54.5 (α-CH), 62.0 ($\underline{C}H_2$–CH_3), 62.6 (O–CH_3), 80.2, 82.5, (2 × $\underline{C}(CH_3)_3$), 149.0 (C═N), 153.8 (CO-Eoc), 156.0 (CO-Boc), 172.4 (COO^{t}Bu); MS (ESI): m/z = 455 [M + Na]$^+$, 433 [M + H]$^+$, 377 [M – C_4H_8 + H]$^+$; Anal. calcd for $C_{19}H_{36}N_4O_7$ (432.52): C 52.76, H 8.39, N 12.95; Found: C 53.65, H 8.57, N 13.24.

N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-ethoxycarbonyl-$N^{\omega'}$-(ethoxycarbonyl)methoxy-L-arginine *t*-butylester (7b). Eluent: Cy/EtOAc (3 : 2), R_f 0.51; yield: 250 mg (99%) of a colorless oil; ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.26 (t, ^3J = 7.1 Hz, 3H, CH_2–$C\underline{H}_3$), 1.27 (t, ^3J = 7.1 Hz, 3H, CH_2–$C\underline{H}_3$), 1.42, 1.44 (2 × s, 9H, $C(CH_3)_3$), 1.51–1.85 (m, 4H, β,γ-CH_2), 3.06 (m, 2H, N–CH_2), 4.16 (q, ^3J = 7.11 Hz, 2H, $C\underline{H}_2$–CH_3), 4.19 (q, ^3J = 7.17 Hz, 2H, $C\underline{H}_2$–CH_3), 4.39 (s, 2H, O–CH_2), 5.08 (m, 1H, NH), 6.40 (br t, 1H, NH), 8.19 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ (ppm) = 14.1, 14.2 (2 × CH_2–$\underline{C}H_3$), 24.8 (γ-CH_2), 27.9, 28.3 (2 × $C(\underline{C}H_3)_3$), 30.2 (β-CH_2), 40.5 (N–CH_2), 53.7 (α-CH), 60.8, 61.9 (2 × $\underline{C}H_2$–CH_3), 70.7 (O–CH_2), 79.5, 81.8 (2 × $\underline{C}(CH_3)_3$), 150.4 (C═N), 153.2 (CO-Eoc), 155.3 (CO-Boc), 170.7, 171.7 (COOEt, COO^{t}Bu); MS (ESI): m/z = 527 [M + Na]$^+$, 505 [M + H]$^+$, 449 [M – C_4H_8]$^+$; Anal. calcd for $C_{22}H_{40}N_4O_9$ (504.59): C 52.37, H 7.99, N 11.10; Found: C 53.09, H 7.90, N 11.44.

N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-ethoxycarbonyl-$N^{\omega'}$-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranos-1-yl)oxy-L-arginine *t*-butylester (7c). Eluent: CH_2Cl_2/MeOH (97 : 3), R_f 0.35; yield: 262 mg (70%) of a colorless oil; ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.29 (t, ^3J = 7.1 Hz, 3H, CH_2–$C\underline{H}_3$), 1.43, 1.45 (2 × s, 9H, $C(CH_3)_3$), 1.52–1.86 (m, 4H, β,γ-CH_2), 1.98, 2.02, 2.05, 2.13 (4 × $COCH_3$), 3.08 (m, 2H, N–CH_2), 3.96 (br t, ^3J = 6.8 Hz, 1H, 5′-CH), 4.11–4.21 (m, 5H, α-CH, $C\underline{H}_2$–CH_3, 3′-CH, NH), 4.85 (d, ^3J = 8.3 Hz, 1H, 1′-CH), 5.06 (m, 2H, 6′-CH_2), 5.25 (dd, ^3J = 10.4, 8.3 Hz, 1H, 2′-CH), 5.39 (dd, ^3J = 3.5, 1.0 Hz, 1H, 4′-CH), 6.47 (br t, 1H, NH), 7.65 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ (ppm) = 14.3 (CH_2–$\underline{C}H_3$), 20.55, 20.6, 20.7, 20.8 (4 × $CO\underline{C}H_3$), 24.9 (γ-CH_2), 28.0, 28.3 (2 × $C(\underline{C}H_3)_3$), 30.3 (β-CH_2), 40.5 (N–CH_2), 53.7 (α-CH), 61.0 (6′-CH_2), 62.1 ($\underline{C}H_2$–CH_3), 66.9, 68.0, 70.5, 71.0 (2′,3′,4′,5′-CH), 79.6, 81.9 (2 × $\underline{C}(CH_3)_3$), 102.5 (1′-CH), 150.5 (C═N), 153.1 (COEoc), 158.9 (COBoc), 170.01, 170.03, 170.2, 170.3 (4 × $\underline{C}OCH_3$), 171.7 (COO^{t}Bu); MS (ESI): m/z = 772 [M + Na]$^+$, 750 [M + H]$^+$, 707 [M – C_2H_2O + H]$^+$.

N^{ω}-Methoxy-L-arginine bis(trifluoroacetate) (8a)[23]. Described in the literature. Yield: 99%.

N^{ω}-Methoxy-L-arginine ethylester dihydrochloride (8b). For esterification 238 mg of the free amino acid precursor **8a** (0.55 mmol) were dissolved in 5 mL of absolute ethanol under an argon atmosphere. This solution was stirred for 30 min at −10 °C before gently bubbling HCl gas through for 5–10 min. For completion of the reaction, it is stirred for one hour at 0 °C and left in the refrigerator for 36 h. The mixture is carefully concentrated under reduced pressure and lyophilized to obtain a hygroscopic amorphous solid, that liquifies upon contact with air. Yield: 168 mg (99%) of a colorless oil; R_f 0.18 (*i*-propanol/H_2O/AcOH, 8 : 1 : 1);%*ee* = 99.6 ± 0.1; ^{1}H NMR (DMSO-d_6): δ (ppm) = 1.24 (t, ^3J = 7.2 Hz, 3H, CH_2–$C\underline{H}_3$), 1.47–1.90 (m, 4H, β,γ-CH_2), 3.22 (m, 2H, N–CH_2), 3.64 (s, 3H, O–CH_3), 3.99 (m, 1H, α-CH), 4.21 (q, ^3J = 7.2 Hz, 2H, $C\underline{H}_2$–CH_3), 8.04 (br s, 2H, NH_2), 8.31 (br t, 1H, NH), 8.72 (br s, 3H, NH_3^+), 11.33 (br s, 1H, NH^+); ^{13}C NMR (DMSO-d_6): δ (ppm) = 13.9 (CH_2–$\underline{C}H_3$), 24.0 (γ-CH_2), 27.0 (β-CH_2), 40.0 (N–CH_2), 51.4 (α-CH), 61.7 ($\underline{C}H_2$–CH_3), 64.4 (O–CH_3), 157.2 (C═N), 169.2 (CO); HRMS (m/z): Calculated for $C_9H_{21}N_4O_3$ [M + H]$^+$ = 233.16082, found: 233.16064; Anal. calcd for $C_9H_{20}N_4O_3 \cdot 2.0\ HCl \cdot 0.7\ H_2O$ (317.82): C 34.01, H 7.42, N 17.63; Found: C 33.65, H 7.66, N 18.20.

N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-methoxy-L-arginine bis(trifluoroacetate) (8c). 200 mg of the fully protected precursor **7a** (0.46 mmol) are dissolved in 5 mL of TFA at 0 °C, stirred at that temperature for 30 min and then 3 h at room temperature. TFA is removed in a vacuum (not exceeding 20 °C) and the residue is taken up in a minimum amount of water. The crude product is purified by flash chromatography on a RP-18 column (0.1% TFA in water). Ninhydrin-positive fractions are combined and concentrated on a rotary evaporator (not exceeding 30 °C) to a volume of *ca.* 10 mL, and were finally lyophilized. Yield: 225 mg (97%) of a colorless oil; R_f 0.44 (*i*-propanol/H_2O/AcOH, 8 : 1 : 1); %*ee* = 99.5 ± 0.1; ^{1}H NMR (DMSO-d_6): δ (ppm) = 1.22 (t, ^3J = 7.1 Hz, 3H, CH_2–$C\underline{H}_3$), 1.50–1.87 (m, 4H, β,γ-CH_2), 3.12 (br t, 2H, N–CH_2), 3.62 (s, 3H, O–CH_3), 3.90 (m, 1H, α-CH), 4.13 (q, ^3J = 7.1 Hz, 2H, $C\underline{H}_2$–CH_3), 7.35 (br s, 1H, NH), 8.28 (br s, 3H, NH_3^+); ^{13}C NMR (DMSO-d_6): δ (ppm) = 14.1 (CH_2–$\underline{C}H_3$), 24.6 (γ-CH_2), 27.3 (β-CH_2), 40.6 (N–CH_2), 51.7 (α-CH), 61.6 ($\underline{C}H_2$–CH_3), 62.1 (O–CH_3), 149.0 (C═N), 153.5 (CO-Eoc), 170.9 (CO); HRMS (m/z): Calculated for $C_{10}H_{21}N_4O_5$ [M + H]$^+$ = 277.15065, found: 277.15049; Anal. calcd for $C_{10}H_{20}N_4O_5 \cdot 2.0\ CF_3COOH \cdot 0.4\ H_2O$ (513.56): C 32.74, H 4.87, N 10.91; Found: C 32.44, H 4.69, N 10.53.

N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-methoxy-L-arginine ethylester dihydrochloride (8d). The esterification of the free amino acid **8c** was

This journal is © The Royal Society of Chemistry 2011

carried out as described for **8b**. Yield: 150 mg (99%) of a colorless oil, starting from 200 mg (0.397 mmol) of **8c**; ^{1}H NMR (DMSO-d_6): δ (ppm) = 1.23, 1.25 (2 × t, ^3J = 7.0 Hz, 3H, CH_2–C$\underline{H}_3$), 1.55–1.88 (m, 4H, β,γ-CH_2), 3.32 (br t, 2H, N–CH_2), 3.71 (s, 3H, O–CH_3), 3.95 (m, 1H, α-CH), 4.19 (m, 4H, 2 × C$\underline{H}_2$–CH_3), 8.80 (br s, 4H, NH_3^+, NH); ^{13}C NMR (DMSO-d_6): δ (ppm) = 13.9, 14.0 (2 × CH_2–$\underline{C}H_3$), 24.0 (γ-CH_2), 26.9 (β-CH_2), 41.1 (N–CH_2), 51.4 (α-CH_2), 61.7, 62.5 (2 × $\underline{C}H_2$–CH_3), 63.9 (O–CH_3), 150.7 (C═N), 152.6 (CO-Eoc), 169.2 (COOEt); HRMS (*m*/*z*): Calculated for $C_{12}H_{25}N_4O_5$ [M + H]$^+$ = 305.18195; found: 305.18176; Anal. calcd for $C_{12}H_{24}N_4O_5$·2.0 HCl·0.6 H_2O (388.08): C 37.14, H 7.06, N 14.44; Found: C 36.67, H 7.59, N 15.00.

N^{ω}-Carboxymethoxy-L-arginine dihydrochloride (9a). The fully protected precursor **6b** (270 mg, 0.489 mmol) is stirred for 4 h at 50–60 °C in 5 mL aqueous 6 N HCl. The mixture is concentrated to dryness, taken up with *ca.* 1–2 mL water and subjected to flash chromatography on a RP-18 column (eluent = 0.1% TFA in water). Ninhydrin-positive fractions are combined, concentrated to a few residual millilitres and lyophilized. Yield: 150 mg of a fine, amorphous solid (96%); R_f 0.53 (*i*-propanol/H_2O/AcOH, 6 : 3 : 1); %*ee* = 99.1 ± 0.1; ^{1}H NMR (D_2O): δ (ppm) = 1.76–2.18 (m, 4H, β,γ-CH_2), 3.41 (br t, ^3J = 6.7 Hz, 2H, N–CH_2), 4.17 (br t, ^3J = 6.2 Hz, α-CH), 4.63 (s, 2H, O–CH_2); ^{13}C NMR (D_2O, TPS): δ (ppm) = 26.3 (γ-CH_2), 29.6 (β-CH_2), 43.2 (N–CH_2), 55.3 (α-CH), 75.5 (O–CH_2), 161.0 (C═N), 174.6, 175.3 (2 × CO); MS (ESI): *m*/*z* = 249 [M + H]$^+$; Anal. calcd for $C_8H_{16}N_4O_5$·2.0 HCl·0.5 H_2O (330.17): C 29.10, H 5.80, N 16.97; Found: C 29.00, H 5.99, N 17.16.

N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-(ethoxycarbonyl)methoxy-L-arginine bis(trifluoroacetate) (9b). Deprotection of **7b** is carried out in analogy to the protocol for **8c**, but starting from 0.5 mmol (252 mg) starting material **7b**. The purification was performed by flash chromatography on a RP-18 column using a stepwise gradient (0.1% TFA in water with MeOH from 5–30%). Ninhydrin-positive fractions were combined, concentrated (not exceeding 30 °C) and lyophilized. Yield: 277 mg of a colorless oil (96%); R_f 0.62 (i-propanol/H_2O/AcOH, 8 : 1 : 1); %*ee* = 99.0 ± 0.1; ^{1}H NMR (DMSO-d_6): With DMSO-d_6 as the solvent **9b** appears as isomers in the ratio of *ca.* 8.6 : 1.4 (at 300 K, referred to the CH_2 singlet of the ethoxycarbonylmethoxy group). The stated ppm values refer to the main isomer; δ (ppm) = 1.19 (t, ^3J = 7.10 Hz, 3H, CH_2–C$\underline{H}_3$), 1.21 (t, ^3J = 7.08 Hz, 3H, CH_2–C$\underline{H}_3$), 1.48–1.87 (m, 4H, β,γ-CH_2), 3.02 (m, 2H, N–CH_2), 3.89 (m, 1H, α-CH), 4.10 (q, ^3J = 7.04 Hz, 2H, C$\underline{H}_2$–CH_3), 4.12 (q, ^3J = 7.13 Hz, 2H, C$\underline{H}_2$–CH_3), 4.37 (s, 2H, O–CH_2), 6.54 (br s, 1H, NH), 8.24 (br s, 3H, NH_3^+), 10.64 (br s, COOH); ^{13}C NMR (DMSO-d_6): δ (ppm) = 14.0, 14.2 (2 × CH_2–$\underline{C}H_3$), 27.4 (CH_2), 40.3 (N–CH_2), 51.8 (α-CH), 60.2, 61.3 (2 × $\underline{C}H_2$–CH_3), 70.3 (O–CH_2), 169.9, 171.0 (3 × CO); HRMS (*m*/*z*): Calculated for $C_{13}H_{25}N_4O_7$ [M + H]$^+$ = 349.17178, found: 349.17155; Anal. calcd for $C_{13}H_{24}N_4O_7$·3.2 CF_3COOH·1.5 H_2O (740.26): C 31.48, H 4.11, N 7.57; Found: C 31.52, H 4.24, N 7.40.

N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-(ethoxycarbonyl)methoxy-L-arginine ethylester dihydrochloride (9c). The esterification of the free amino acid **9b** was carried out as described for **8b**. Yield: 222 mg (99%) of a colorless oil, starting from 288 mg (0.5 mmol) of **9b**; R_f 0,57 (i-propanol/H_2O/AcOH, 8 : 1 : 1); ^{1}H NMR (DMSO-d_6): δ (ppm) = 1.20 (t, ^3J = 7.16 Hz, 3H, CH_2–C$\underline{H}_3$), 1.23 (t, ^3J = 7.13 Hz, 3H, CH_2–C$\underline{H}_3$), 1.26 (t, ^3J = 7.10 Hz, 3H, CH_2–C$\underline{H}_3$), 1.51–1.87 (m, 4H, β,γ-CH_2), 3.17 (m, 2H, N–CH_2), 3.95 (m, 1H, α-CH), 4.14 (q, ^3J = 7.12 Hz, 2H, C$\underline{H}_2$–CH_3), 4.15 (q, ^3J = 7.10 Hz, 2H, C$\underline{H}_2$–CH_3), 4.17–4.25 (m, 2H, C$\underline{H}_2$–CH_3), 4.51 (s, 2H, O–CH_2), 7.80 (br s, 1H, NH), 8.55 (br s, 1H, NH), 8.72 (br s, 3H, NH_3^+); ^{13}C NMR (DMSO-d_6): δ (ppm) = 13.9, 14.0, 14.1 (3 × CH_2–$\underline{C}H_3$), 24.2 (γ-CH_2), 27.2 (β-CH_2), 40.8 (N–CH_2), 51.5 (α-CH), 60.5, 61.7, 61.9 (3 × $\underline{C}H_2$–CH_3), 71.1 (O–CH_2), 150.3 (C═N), 153.0 (CO-Eoc), 169.1, 169.3 (2 × CO); HRMS (*m*/*z*): Calculated for $C_{15}H_{29}N_4O_7$ [M + H]$^+$ = 377.20308, found: 377.20287.

Sodium N^{ω}-ethoxycarbonyl-$N^{\omega'}$-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranos-1-yl)oxy-L-argininate (10). In a Schlenk flask 120 mg (0.16 mmol) of the fully protected, thoroughly dried precursor **7c** is dissolved in absolute Et_2O under an argon atmosphere. The solution is stirred for *ca.* 30 min at −15 °C, and HCl gas is carefully bubbled through the solution. The reaction mixture is left in the refrigerator overnight and then concentrated in a vacuum. The white-yellow solid residue is taken up with *ca.* 1–2 mL of 0.5 M aqueous $NaHCO_3$ and purified by flash chromatography on a RP-18 column (eluent = water, with a stepwise MeOH gradient of 10%, 25%, 50%). The product containing fractions were combined, concentrated on a rotary evaporator (not exceeding 30 °C) and lyophilized. Yield: 74 mg of a fine, white powder (75%). ^{1}H NMR (D_2O, TPS): **10** appears as isomers in the ratio of *ca.* 6 : 4 (at 300 K, referred to the CH_3 triplet of the ethoxycarbonyl group). The stated ppm values refer to the main isomer; δ (ppm) = 1.28 (t, ^3J = 7.1 Hz, 3H, CH_2–C$\underline{H}_3$), 1.52–1.97 (m, 4H, β,γ-CH_2), 2.02, 2.08, 2.12, 2.23 (4 × s, 3H, $COCH_3$), 3.16 (br t, ^3J = 6.8 Hz, 2H, 6′-CH_2), 3.75 (t, ^3J = 6.3 Hz, 1H, 5′-CH), 4.15–4.30 (m, 5H, C$\underline{H}_2$–CH_3, α-CH, N–CH_2), 5.07 (d, ^3J = 8.0 Hz, 1H, 1′-CH), 5.21–5.33 (m, 2H, 2′,3′-CH), 5.47 (m, 1H, 4′-CH); ^{13}C NMR (D_2O, TPS): δ (ppm) = 16.2 (CH_2–$\underline{C}H_3$), 22.7, 22.8, 22.9, (4 × CO$\underline{C}H_3$), 26.6 (γ-CH_2), 30.6 (β-CH_2), 42.7 (N–CH_2), 57.2 (α-CH), 64.6 (6′-CH_2), 65.8 ($\underline{C}H_2$–CH_3), 70.6, 70.7, 73.4, 74.1 (2′,3′,4′,5′-CH), 104.1 (1′-CH), 155.6 (C═N), 156.8 (CO-Eoc), 175.4, 175.8, 176.2, 177.1 (4 × $\underline{C}OCH_3$); MS (ESI): *m*/*z* = 615 [M + Na]$^+$, 593 [M + H]$^+$, 551 [M − C_2H_2O + H]$^+$, 331 [$C_{14}H_{19}O_9$]$^+$; HRMS (*m*/*z*): Calculated for $C_{23}H_{33}N_4O_{14}Na$ [M + Na]$^+$ = 615.21202, found: 615.21164; anal. calcd for $C_{23}H_{35}N_4NaO_{14}$ (614.53): C 44.95, H 5.74, N 9.12; Found: C 45.08, H 6.21, N 8.77.

***N*-Benzyl-*N*′-ethoxycarbonyl-*N*″-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranos-1-yl)oxy]guanidine (12).** 119 mg of thiourea **11**[24] (see also ESI for analytical data†) (0.5 mmol), 218 mg of the protected 1-aminooxygalactose **5** (0.6 mmol) and 104.5 µL DIPEA (0.6 mmol) are dissolved in 5 mL of dry CH_2Cl_2. The mixture is cooled to 0 °C, after which 115 mg of EDCI (0.6 mmol) is added. This mixture is stirred for 48 h at room temperature. For work up, 10 mL of CH_2Cl_2 are added and the organic phase is washed with 1% HCl, water and brine (5 mL each). The organic phase is dried with Na_2SO_4 and concentrated in a vacuum. The crude product is purified by column chromatography on SiO_2 using Cy/EtOAc (1 : 1) as the eluent. Yield: 255 mg of a colorless oil (90%); TLC: R_f 0.54 (Cy/EtOAc, 1 : 1); ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.30 (t, ^3J = 7.1 Hz, 3H, CH_2–C$\underline{H}_3$), 1.99, 2.02, 2.06, 2.15 (4 × s, 3H, $COCH_3$), 3.98 (br t, ^3J = 6.7 Hz, 1H, 5′-CH), 4.10–4.30 (m, 6H, 6′-CH_2, N–CH_2, C$\underline{H}_2$–CH_3), 4.90 (d, ^3J = 8.3 Hz, 1H, 1′-CH), 5.08 (m, 1H, 3′-CH), 5.29 (br t, ^3J = 9.6 Hz, 1H, 2′-CH), 5.41 (m, 1H,

4′-CH), 6.79 (br t, 1H, NH), 7.22–7.35 (m, 5H, ArH), 7.72 (br s, 1H, NH); ^{13}C NMR (CDCl$_3$): δ (ppm) = 14.3 (CH$_2$–$\underline{C}$H$_3$), 20.6, 20.7, 20.9 (4 × CO$\underline{C}$H$_3$), 45.1 (N–CH$_2$), 61.1 (6′-CH$_2$), 62.2 ($\underline{C}$H$_2$–CH$_3$), 67.0, 68.1, 70.7, 71.0 (2′,3′,4′,5′-CH), 102.7 (1′-CH), 127.4, 127.8, 128.6 (ArCH), 138.3 (ArC), 150.4 (C═N), 153.2 (CO-Eoc), 170.0, 170.2, 170.3 (4 × $\underline{C}$OCH$_3$); MS (ESI): m/z = 590 [M + Na]$^+$, 568 [M + H]$^+$, 526 [M − C$_2$H$_2$O + H]$^+$, 331 [C$_{14}$H$_{19}$O$_9$]$^+$.

***N*-Benzyl-*N*′-ethoxycarbonyl-*N*″-[(β-D-galactopyranos-1-yl)-oxy]guanidine (13).** 150 mg of the protected precursor **12** (0.26 mmol) are dissolved in 5 mL MeOH and gently stirred with *ca.* 30 mg of strong anion exchanger resin (Merck Ionenaustauscher III, OH$^-$) over night. The solution is filtered, washed with small amounts of MeOH and concentrated on a rotary evaporator. The product is further purified by flash chromatography on SiO$_2$ using a step-wise gradient of EtOAc/MeOH (9 : 1 to 8 : 2). Yield: 84 mg of a fine white powder (80%); TLC: R_f 0.66 (EtOAc/MeOH, 8 : 2); ^{1}H NMR (MeOH-d_4): In MeOH-d_4 as the NMR solvent the substance occurs as isomers at a ratio of *ca.* 8 : 2 (300 K, referred to the triplet of the ethyloxycarbonyl methyl-group). In DMSO-d_6 this ratio is *ca.* 6 : 4. The stated ppm values refer to the main isomer; δ (ppm) = 1.38 (t, ^3J = 7.1 Hz, 3H, CH$_2$–C$\underline{H}_3$), 3.58–3.67 (m, 2H, 6′-CH$_2$), 3.71–3.96 (m, 4H, 2′,3′,4′,5′-CH), 4.28 (q, ^3J = 7.1 Hz, 2H, C$\underline{H}_2$–CH$_3$), 4.36 (br s, 2H, N–CH$_2$), 4.61 (d, ^3J = 8.1 Hz, 1H, 1′-CH), 7.31–7.44 (m, 5H, ArH); ^{13}C NMR (MeOH-d_4): δ (ppm) = 14.6 (CH$_2$–$\underline{C}$H$_3$), 45.8 (N–CH$_2$), 62.5 (6′-CH$_2$), 63.2 ($\underline{C}$H$_2$–CH$_3$), 70.2, 71.2, 75.0, 76.8 (2′,3′,4′,5′-CH), 106.6 (1′-CH), 128.3, 128.6, 129.6 (ArCH), 139.8 (ArC), 152.8 (C═N), 155.1 (CO); HRMS (m/z): Calculated for C$_{17}$H$_{26}$N$_3$O$_8$ [M + H]$^+$ = 400.17144, found: 400.17147; Anal. calcd for C$_{17}$H$_{33}$N$_4$O$_{14}$Na [M + Na]$^+$ = 422.15339, found: 422.15327.

In vitro assays

Materials and methods. Commercially available materials were purchased from either Sigma Aldrich, Fluka, Roth or Merck. HPLC-grade methanol was obtained from Mallinckrodt Baker. D-Galactose concentration was analysed using the testkit "Lactose/Galactose" of Boehringer Mannheim.

Analytical reversed phase HPLC. Amino acids were derivatized with *o*-Pa and separated at 37 °C on a Nova–Pak RP$_{18}$ 4 µm (4 × 150 mm, VDS Optilab) with a Phenomenex C18, 4 × 3 mm guard column. HPLC system: Waters autosampler 717plus, Waters 600 Controller and Waters 470 scanning fluorescence detector, set at λ_{ex}: 338 nm, λ_{em}: 425 nm. For derivatization, the autosampler was set to mix 14 µL of *o*-Pa reagent with 10 µL of sample and allowed to react for 2 min at room temperature before injection. *o*-Pa reagent was prepared by dissolving 50 mg *o*-Pa in 9 mL of 0.2 M potassium borate buffer pH 9.4 and 53 µL of 2-mercaptoethanol. Eluent A: 10 mM potassium phosphate buffer (pH 4.7)/MeOH (78%/22%); eluent B: methanol/aqua bidest. (80%/20%). The following elution conditions were used: flow-rate was kept at 1 mL min^{-1}; 0–12 min isocratic with 90% eluent A and 10% eluent B; 12–25 min linear gradient to 100% eluent B, 25–27 min, linear gradient to 90% eluent A, 27–35 min reequilibration with 90% eluent A and 10% eluent B.

pH stability. Compounds were dissolved in 10 mM potassium phosphate buffer pH 2, pH 7.4 and pH 9 to a final concentration of 250 µM. Samples were incubated at 37 °C and reactions analyzed immediately at the desired time points. Subsequent samples were analyzed by HPLC as described above.

Oxidative stability. Stability was determined at 37 °C in 10 mM phosphate buffer pH 7.4 containing either 500 µM potassium hexacyanoferrate(III), 500 µM copper(II)-acetate, 500 µM peroxynitrite or 29 mM hydrogen peroxide, respectively. Prodrugs were applied in a concentration of 250 µM. After 24 h samples were taken and immediately analyzed *via* HPLC as described above.

Acknowledgements

We thank Dr Ulrich Girreser for performing NMR and MS experiments and gratefully acknowledge the excellent technical assistance of Ms. Melissa Zietz, Ms. Johanna Blauth and Mr. Sven Wichmann. We are also indebted to Dr. Erik Ralph for his meticulous and valuable proofreading.

Notes and references

1 K. Bian, M. F. Doursout and F. Murad, *J. Clin. Hypertens.*, 2008, **10**, 304–310.
2 S. Moncada and E. A. Higgs, *Br. J. Pharmacol.*, 2006, **147**(Suppl 1), S193–201.
3 C. Napoli, F. de Nigris, S. Williams-Ignarro, O. Pignalosa, V. Sica and L. J. Ignarro, *Nitric Oxide*, 2006, **15**, 265–279.
4 D. Schade, J. Kotthaus and B. Clement, *Pharmacol. Ther.*, 2010, **126**, 279–300.
5 D. J. Stuehr, N. S. Kwon, C. F. Nathan, O. W. Griffith, P. L. Feldman and J. Wiseman, *J Biol Chem*, 1991, **266**, 6259–6263.
6 W. Durante, F. K. Johnson and R. A. Johnson, *Clin. Exp. Pharmacol. Physiol.*, 2007, **34**, 906–911.
7 J. B. Walker and M. S. Walker, *J. Biol. Chem.*, 1959, **234**, 1481–1484.
8 J. M. Fukuto, *Methods Enzymol.*, 1996, **268**, 365–375.
9 T. Cai, M. Xian and P. G. Wang, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 1507–1510.
10 A. Jousserandot, J. L. Boucher, Y. Henry, B. Niklaus, B. Clement and D. Mansuy, *Biochemistry*, 1998, **37**, 17179–17191.
11 H. G. Korth, R. Sustmann, C. Thater, A. R. Butler and K. U. Ingold, *J. Biol. Chem.*, 1994, **269**, 17776–17779.
12 J. Y. Cho, A. Dutton, T. Miller, K. N. Houk and J. M. Fukuto, *Arch. Biochem. Biophys.*, 2003, **417**, 65–76.
13 J. M. Fukuto, G. C. Wallace, R. Hszieh and G. Chaudhuri, *Biochem. Pharmacol.*, 1992, **43**, 607–613.
14 J. M. Fukuto, D. J. Stuehr, P. L. Feldman, M. P. Bova and P. Wong, *J. Med. Chem.*, 1993, **36**, 2666–2670.
15 B. Clement, M. Demesmaeker and S. Linne, *Chem. Res. Toxicol.*, 1996, **9**, 682–688.
16 A. K. Froehlich, U. Girreser and B. Clement, *Drug Metab. Dispos.*, 2005, **33**, 1532–1537.
17 B. Clement, M. H. Schultze-Mosgau and H. Wohlers, *Biochem. Pharmacol.*, 1993, **46**, 2249–2267.
18 B. Clement, T. Kunze and S. Heberling, *Biochem. Biophys. Res. Commun.*, 2006, **349**, 869–873.
19 J. Kotthaus, B. Wahl, A. Havemeyer, J. Kotthaus, D. Schade, D. Garbe-Schönberg, R. Mendel, F. Bittner and B. Clement, *Biochem. J.*, 2011, **433**, 383–391.
20 S. M. Morris Jr., *Am. J. Clin. Nutr.*, 2006, **83**, 508S–512S.
21 J. Kotthaus, D. Schade, K. Topker-Lehmann, E. Beitz and B. Clement, *Bioorg. Med. Chem.*, 2008, **16**, 2305–2312.
22 J. Kotthaus, D. Schade, N. Muschick, E. Beitz and B. Clement, *Bioorg. Med. Chem.*, 2008, **16**, 10205–10209.
23 D. Schade, J. Kotthaus and B. Clement, *Synthesis*, 2008, **15**, 2391–2397.
24 B. R. Linton, A. J. Carr, B. P. Orner and A. D. Hamilton, *J. Org. Chem.*, 2000, **65**, 1566–1568.
25 N. I. Martin, J. J. Woodward and M. A. Marletta, *Org. Lett.*, 2006, **8**, 4035–4038.
26 N. I. Martin, W. T. Beeson, J. J. Woodward and M. A. Marletta, *J. Med. Chem.*, 2008, **51**, 924–931.

 This journal is © The Royal Society of Chemistry 2011

27 T. Wenzler, D. W. Boykin, M. A. Ismail, J. E. Hall, R. R. Tidwell and R. Brun, *Antimicrob. Agents Chemother.*, 2009, **53**, 4185–4192.
28 F. L. Wagenaar and J. F. Kerwin, *J. Org. Chem.*, 1993, **58**, 4331–4338.
29 M. Z. Wang, J. Q. Wu, A. S. Bridges, D. C. Zeldin, S. Kornbluth, R. R. Tidwell, J. E. Hall and M. F. Paine, *Drug Metab. Dispos.*, 2007, **35**, 2067–2075.
30 D. Schade, J. Kotthaus, H. Hungeling and B. Clement, *ChemMedChem*, 2009, **4**, 1595–1599.
31 A. G. Katopodis and S. W. May, *Biochemistry*, 1990, **29**, 4541–4548.
32 D. Melisi, A. Secondo, P. Montoro, S. Piacente, M. G. Rimoli, M. Minale, P. de Caprariis and L. Annunziato, *J. Med. Chem.*, 2006, **49**, 4826–4833.
33 P. G. Wang, M. Xian, X. Tang, X. Wu, Z. Wen, T. Cai and A. J. Janczuk, *Chem. Rev.*, 2002, **102**, 1091–1134.
34 X. X. T. Wu, M. Xian and P. G. Wang, *Tetrahedron Lett.*, 2001, **42**, 3779–3782.
35 O. Renaudet and P. Dumy, *Tetrahedron Lett.*, 2001, **42**, 7575–7578.
36 B. Testa and J. M. Mayer, in *Hydrolysis in Drug and Prodrug Metabolism. Chemistry, Biochemistry and Enzymology*, Helvetica Chimica Acta, Zürich, 2003, pp. 664–672.
37 B. Testa and J. M. Mayer, in *Hydrolysis in Drug and Prodrug Metabolism. Chemistry, Biochemistry and Enzymology*, Helvetica Chimica Acta, Zürich, 2003, pp. 365–387.
38 G. Zinner, *Arch. Pharm.*, 1960, **293**, 657–661.
39 B. Clement, E. Schnorwangen, T. Kampchen, P. Mordvintcev and A. Mulsch, *Arch. Pharm.*, 1994, **327**, 793–798.
40 C. Belzecki, B. Hintze and S. Kwiatkowska, *Bull. Acad. Pol. Sci. Chem.*, 1970, **18**, 569–572.
41 C. Belzecki and J. Trojnar, *Bull. Acad. Pol. Sci. Chem.*, 1970, **18**, 379–383.
42 C. Belzecki, K. Piotrowska and B. Hintze, *Bull. Acad. Pol. Sci. Chem.*, 1971, **19**, 367–370.
43 K. Rehse and S. Bade, *Arch. Pharm.*, 1996, **329**, 535–540.
44 A. R. Katritzky, N. M. Khashab, N. Kirichenko and A. Singh, *J. Org. Chem.*, 2006, **71**, 9051–9056.
45 A. R. Katritzky, N. M. Khashab, S. Bobrov and M. Yoshioka, *J. Org. Chem.*, 2006, **71**, 6753–6758.
46 Y. Higashi, K. Noma, M. Yoshizumi and Y. Kihara, *Circ. J.*, 2009, **73**, 411–418.
47 C. Szabo, H. Ischiropoulos and R. Radi, *Nat. Rev. Drug Discovery*, 2007, **6**, 662–680.
48 G. J. Southan, A. L. Salzman and C. Szabo, *Free Radical Biol. Med.*, 1998, **25**, 914–925.

This journal is © The Royal Society of Chemistry 2011