Synthesis and Antiapoptotic Activity of a Novel Analogue of the Neutral Sphingomyelinase Inhibitor Scyphostatin

Ralf A. Claus,[b] Annette Wüstholz,[c] Stefan Müller,[c] Clemens L. Bockmeyer,[b] Norman H. Riedel,[c] Ralf Kinscherf,[d] and Hans-Peter Deigner*[a]

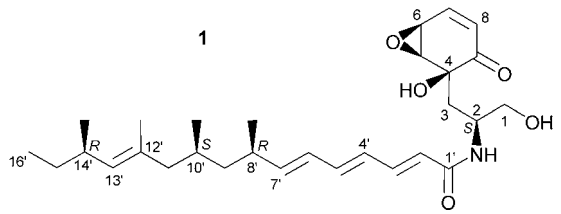
*The enantioselective synthesis of an analogue of scyphostatin, a potent inhibitor of the neutral sphingomyelinase, is described. The synthesis starts with cyclohexanone and a protected D-serine derivative. The key step is an asymmetric hydroxylation to access a hydroxycyclohexanone, which is transformed into a substituted hydroxycyclohexenone. This is converted into the scyphostatin analogue **14**, a chemically and metabolically stabilised compound lacking the epoxy function of the natural congener and carrying a palmitic acid group instead of the native trienoyl residue. An evaluation of the biological activity of **14** revealed neutral sphingomyelinase inhibition in several in vivo test systems (monocytes, macrophages, hepatocytes) monitoring antiapoptotic effects and the inversion of phorbolester-induced translocation of green fluorescent protein labelled kinase (protein kinase C-α).*

Introduction

Sphingolipids—integral constituents of eukaryotic membranes—have emerged as a particularly rich source of bioactive molecules such as ceramides. The generation of products serving as signalling molecules and bioeffectors is initiated by degradation of membrane sphingolipids. The hydrolysis of sphingomyelin by specific enzymes, sphingomyelinases (SMases), which is also observed in response to various extracellular stimuli including vitamin D3, interferon-γ (IFN-γ), tumour necrosis factor alpha (TNF-α) and interleukin-1, results in transient and sustained elevations in the levels of ceramide (Cer) with variable kinetics.[1] Due to the plethora of biological effects modulated by ceramides, including cellular differentiation, proliferation, cell-cycle control and induction of apoptosis, the neutral isoforms of these enzymes (nSMases)—which display maximal activities at neutral pH values[1]—emerge as highly interesting targets for the development of novel antiapoptotic,[2,3] antineurodegenerative[4] and anti-inflammatory drugs.[5]

Scyphostatin (**1**), the first specific potent low-molecular-weight inhibitor of the nSMases isolated from the mycelial extract of *Trichopeziza mollissima*, was described several years ago (Scheme 1).[5,6] Due to the unique and stimulating structure of this compound as well as its important pharmacological activity, the synthesis and structural elucidation[5–7] of scyphostatin and its analogues have recently been the subject of several synthetic studies.[8–11] Several approaches to the core structure have appeared lately.[12] In the course of on-going investigations towards an efficient synthesis, Izuhara and Katoh first reported the synthesis of the protected cyclohexenone moiety.[8] Recently, a (4*R*,5*R*)-2,3-dimethoxybutanediyldioxy protected cyclohexenone was prepared as a building block for scyphostatin synthesis.[13] Kenworthy et al. used a tethered aminohydroxylation of 1-allyl-cyclohexanol to yield a β-amino alcohol intermediate with the required stereochemistry.[14] The asymmetric synthesis of the side-chain unit was also successfully achieved and its stereochemistry unambiguously confirmed, first by Hoye and Tennakoon, and again recently by Japanese groups using asymmetric carboalumination.[7] It is of note that during the review process of this paper, Inoue et al. described the total synthesis by starting from a D-arabinose derivative and Garner aldehyde for preparation of the cyclohexene moiety and from a vinyl iodide derivative for the fatty acid.[15]

The key feature of the structure of scyphostatin is a highly functionalised cyclohexenone moiety with a quaternary carbon



Scheme 1. *Structure of the neutral sphingomyelinase inhibitor Scyphostatin (**1**).*

[a] *Prof. Dr. H.-P. Deigner*
Biomedicinal Chemistry, School of Chemical Sciences and Pharmacy
University of East Anglia, Norwich NR4 7TJ (UK)
Fax: (+44) 1603-592003
E-mail: h-p.deigner@uea.ac.uk

[b] *Dr. R. A. Claus, C. L. Bockmeyer*
Department for Anaesthesiology and Intensive Care Therapy
Division for Experimental Anaesthesiology
Friedrich-Schiller-University Jena, Research Centre Lobeda
Erlanger Allee 101, 07747 Jena (Germany)

[c] *Dr. A. Wüstholz, Dr. S. Müller, Dr. N. H. Riedel*
Institute for Pharmacy and Molecular Biotechnology
University of Heidelberg
Im Neuenheimer Feld 364, 69120 Heidelberg (Germany)

[d] *Priv.-Doz. Dr. R. Kinscherf*
Department of Anatomy and Cell Biology III
University of Heidelberg
Im Neuenheimer Feld 307, 69120 Heidelberg (Germany)

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centre and an α,β-unsaturated ketone, as well as a lipophilic branched trienoyl side chain. Our intention was to gain straightforward synthetic access to scyphostatin analogues with simplified structures and with retention of the inhibitory potency of the natural congener. We report here the stereocontrolled preparation of an analogue lacking the epoxy function and with highly interesting biological activities.

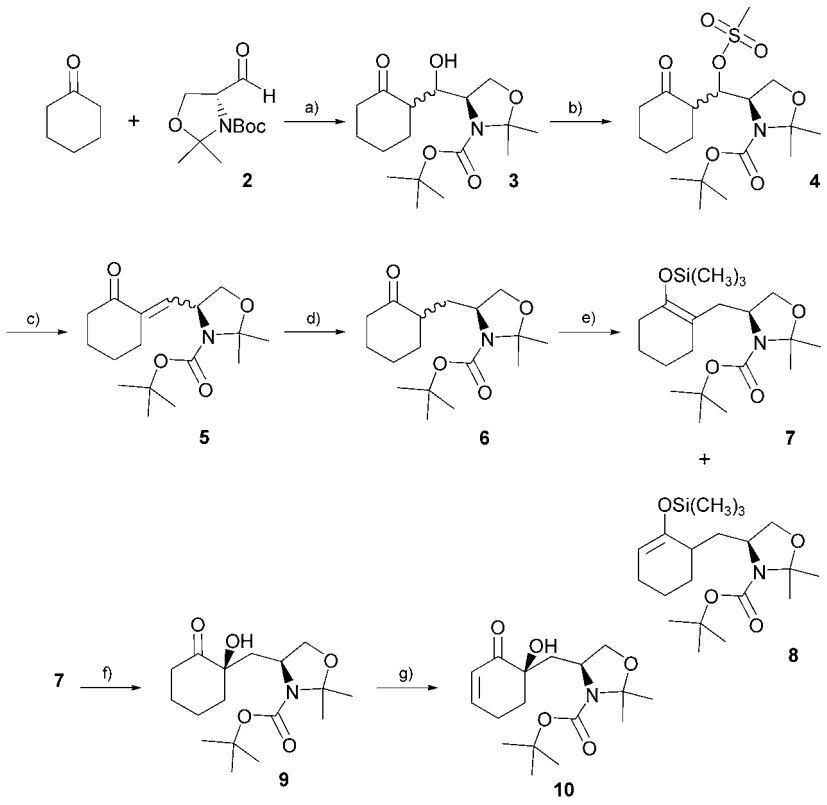
Chemistry—Synthesis of the Scyphostatin Analogue

The general synthetic strategy is based on the functionalisation of cyclohexanone by the protected D-serine derivative serinal (Scheme 2). The aldehyde **2** is accessible by diisobutylaluminium hydride (DIBALH) reduction of D-serine methyl ester hydrochloride.[16] The alcohol is transformed into the α,β-unsaturated ketone **5** by mesitylation followed by an elimination with DBU.[17] Compound **5** is hydrogenated to give the saturated ketone **6**. It is important to check the conditions for hydrogenation meticulously: overdosing with the utilised catalyst or an improper extension of the reaction time gives high amounts of the corresponding cyclohexanol, due to hydrogenation of the enol structure of the enone **5**.[18]

Upon coupling of the aldehyde **2** to cyclohexanone, the desired *S* stereochemistry is obtained at the asymmetric amino-substituted carbon atom. In all other early reaction steps, the new asymmetric carbon atoms generated lead to diastereomers; separation of the resulting diastereomers, however, is not required since only the stereochemistry of the carbon atom carrying the methyleneoxazolidine is essential for the final product.

Transformation of the substituted cyclohexanone **6** into the corresponding silyl enol ether **7** followed by an asymmetric bishydroxylation according to the Sharpless method affords the second asymmetric carbon atom of the cyclohexenone moiety with the correct *S* stereochemistry (86% diastereomeric excess, determined by HPLC). By use of hexamethyldisilazane and trimethylsilyliodide, the thermodynamically stable enol ether **7** is preferentially formed over the kinetic product **8**, and the structures were confirmed by ^{1}H NMR analysis to be in a ratio of 85:15.[19] The enol ethers **7** and **8** can be separated chromatographically.

Asymmetric bishydroxylation of the silyl enol ether **7** to form the α-hydroxyketone **9** under conditions reported by Sharpless and co-workers with AD mix β in basic milieu enriched by osmium(VIII) oxide was found to be the most efficient method for simultaneous generation of the stereogenic centres.[20] The correct stereochemistry was confirmed by NOE experiments and comparison with the respective NMR data of the natural product.[5,6] Remarkably, there were no rotamers from the BOC protecting group present in the spectra of substances **9**–**11** and **15**–**18**, possibly due to conformational rigidisation by hydrogen bonds between the hydroxy groups and the nitrogen atom.



Scheme 2. *Synthesis of the cyclohexenone moiety* ***10****. a) LDA, THF, −78 °C for 2 h, RT for 1 h, 84%; b) Et_3N, MsCl, CH_2Cl_2, 0 °C, 2 h, 98%; c) DBU, CH_2Cl_2, 0 °C for 1 h, RT for 12 h, 73%; d) Pd/C, H_2 (1 atm), EtOH (96%), 30 min, 90%; e) HMDS, TMSI, THF, 0–25 °C, 2 h, 89%; f) AD mix β, $NaHCO_3$, OsO_4, t$BuOH/H_2O$ (1:1), 0–25 °C, 36 h, 58%; g) 1. LDA, Br_2, THF, CH_2Cl_2, −78 °C, 2 min, 98%; 2. DBU, 115 °C, 5 h, 62%. BOC = tert-butoxycarbonyl, LDA = lithiumdiisopropylamide, THF = tetrahydrofuran, Ms = methanesulfonyl, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, HMDS = 1,1,1,3,3,3-hexamethyldisilazane, TMS = trimethylsilyl.*

Bromination of the α-hydroxyketone **9** results in a bromo derivative,[21] which is transformed into the cyclohexenone **10** by dehydrobromination with DBU.[22] Attempts to introduce an additional C–C double bond by allylic bromination,[23] by introduction of a phenylselenyl substituent in the allyl position[24] followed by oxidation of **11** (Scheme 3) or by direct oxidation of **10** with DDQ,[25] however, were unsuccessful. The subsequent dehydrobromination gave complex reaction mixtures, and efforts to isolate the desired oxidation product remained unsuccessful.

The amino hydroxyketone **13** is accessible by deprotection of the cyclohexenone moiety **10** and can be directly converted into the scyphostatin analogue **14** by stirring with palmitic acid chloride at −78 °C (Scheme 3).

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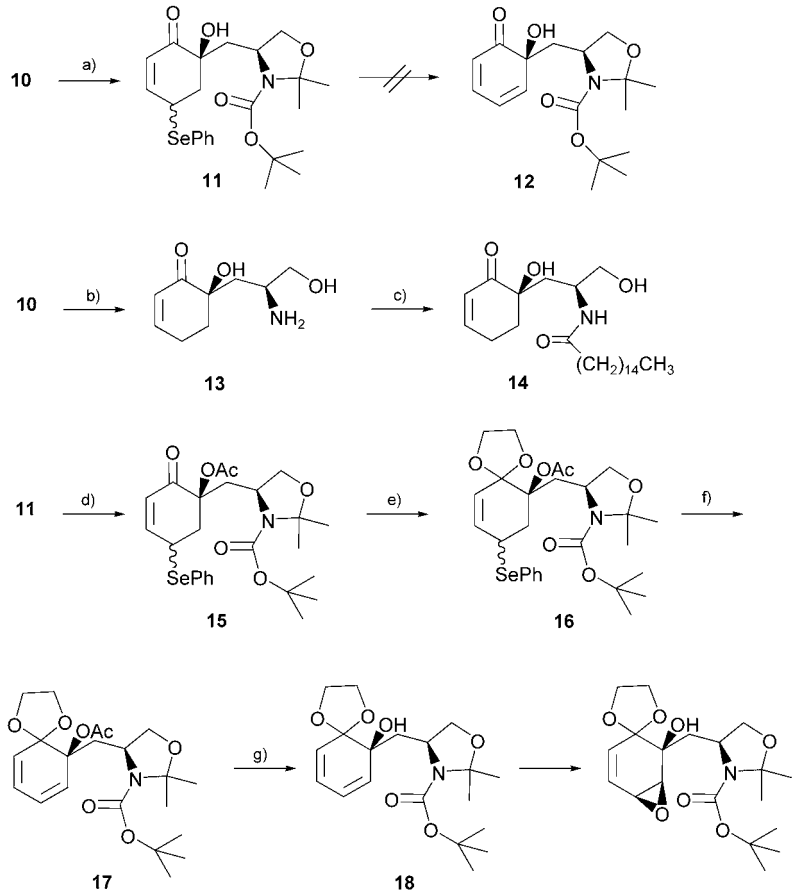

Scheme 3. *Synthesis of the scyphostatin analogue **14**. a) LDA, PhSeCl, THF, −78 °C, 15 min, 80 %; b) TFA, CH_2Cl_2, H_2O, RT, 4 h, 89 %; c) $CH_3(CH_2)_{14}COCl$, Et_3N, THF, −78 °C, 91 %; d) H_3CCOCl, DMAP, CH_2Cl_2, 4 h, 93 %; e) $Me_3SiOCH_2CH_2OSiMe_3$, $Me_3SiOSO_2CF_3$, CH_2Cl_2, 14 h, 63 %; f) H_2O_2, CH_2Cl_2, RT, 2 h, 46 %; g) NaOH, THF, RT, 30 min, quant. TFA = trifluoroacetic acid, DMAP = 4-dimethylaminopyridine.*

It is known that elimination by oxidation of selenenyl ketones results in complex product mixtures.[26] Elimination of the corresponding ethylene ketals, in contrast, affords good yields of the desired products. Therefore, the synthetic strategy was altered by the introduction of additional protecting groups. The protection of the hydroxy group proved to be necessary in order to obtain ketalysation of compound **11**.[27] Hydrolysis of the acetyl ester under basic conditions after ketalysation leads to allylic alcohol **18**. Subsequent epoxidation of **18**, however, was unsuccessful, possibly due to steric hindrance by the spiro ketal group.

Biological Evaluation of the Scyphostatin Analogue

Compound **14** was first examined for antiapoptotic properties in a minimally modified low-density lipoprotein (mmLDL) or TNF-α induced model of programmed cell death (PCD) with human monocytes or macrophages.[3] By using fluorescence microscopy, with untreated cells as controls (spontaneous apoptosis rate 4.3 %), mmLDL-exposed macrophages revealed a degree of 19.4 % of stained (apoptotic) cells, a value which was set as 100 %. The analogue significantly inhibited mmLDL-induced apoptosis in a concentration-dependent manner at concentrations up to 5 μM (Figure 1). As depicted, the EC_{50} value calculated on the basis of a regression analysis ($r^2 = 0.98$) is about 1.5 μM. In order to analyse potential effects on TNF-α-induced apoptosis, freshly isolated monocytes were treated with the cytokine (10 ng mL^{-1}, 4 h) to result in an apoptosis rate of 18.4 % (untreated sample: 9.4 %). As depicted in Table 1 A, pretreatment with the analogue **14** diminished the relative amount of fluorescent (apoptotic) cells to 9.7 % at a concentration of 0.1 μM. In all experiments, no apoptogenic effect of the plain analogue **14** was observed. Moreover, by flow cytometric analysis, the inhibition of PCD induction was determined by annexin staining in comparison to propidium iodide. In this experimental setting, TNF-α stimulation also results in a doubling of the apoptosis rate (0.57 versus 1.08 % annexin(V)-positive cells), whereas preincubation with **14** abolished the cytokine-induced effect (0.58 %, $p < 0.05$, ANOVA

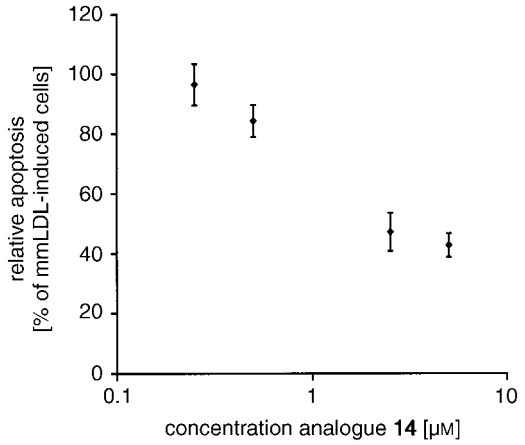

Figure 1. *Concentration-dependent decrease of mmLDL-induced apoptosis by preincubation with **14**. Apoptosis of human MΦ was determined at day 7 after isolation of monocytes: MΦ were incubated with **14** at concentrations as indicated for 30 minutes prior to mmLDL addition (27 μg mL^{-1} for 4 h). Apoptosis is expressed as the percentage of YOPRO-1 staining (2.5 μg mL^{-1}) relative to Hoechst staining.[3] The data represent the mean values ± the standard deviation of three experiments. The mmLDL-treated cells were set as 100 % (absolute: 19.4 %). Regression analysis results in a correlation coefficient of $r^2 = 0.98$.*

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Table 1. *Biological properties of compound* **14**.

	A[a]	B[b]	C[c]	D[d]	E[e]
	rate of apoptotic cells [%]			SM metabolism	nSMase
	YOPRO-1	annexin(V)	propidium iodide	[Cer/SM ratio]	[Cer/μg]
stimulus	TNF-α	TNF-α	TNF-α	mmLDL	mmLDL
untreated control	9.4 ± 0.58	0.57 ± 0.39[f]	1.17 ± 0.13	18.49 ± 1.59[g]	6.40 ± 1.72[g]
+ stimulus	18.9 ± 1.05	1.08 ± 0.14	1.07 ± 0.89	25.52 ± 2.46	14.42 ± 1.56
14 (10.0 μM) + stimulus	n.d.	n.d.	n.d.	17.96 ± 1.59[g]	n.d.
14 (1.0 μM) + stimulus	11.8 ± 1.90	0.58 ± 0.27[f]	0.75 ± 0.34	21.10 ± 0.89[g]	6.62 ± 1.75[g]
14 (0.1 μM) + stimulus	9.7 ± 1.65	n.d.	n.d.	22.84 ± 1.08	10.64 ± 1.01[g]
14 (0.01 μM) + stimulus	n.d.	n.d.	n.d.	n.d.	14.03 ± 0.77
14	11.9 ± 2.60	0.64 ± 0.30[f]	0.54 ± 0.18	19.21 ± 1.10[g]	7.98 ± 1.26[g]

[a] Analogue **14** inhibits TNF-α-induced apoptosis in monocytes. Human monocytes were plated in four-well plates, incubated overnight and preincubated with **14** as indicated for 30 min. Apoptogenic processes were then induced by addition of hr TNF-α (10 ng mL^{-1}, 4 h). Apoptotic cells were determined as described in the legend of Figure 4. **14** (1 μM) was used as a control. Mean values of two independent experiments are given. [b] Analogue **14** inhibits TNF-α-induced apoptosis in CD14$^+$ monocytes. Human monocytes were stimulated as described in [a]. Annexin(V)-positive, apoptotic monocytic cells were determined by flow cytometry as described in the Experimental Section. **14** (1 μM) was used as a control. Mean values of six independent experiments are given. [c] Analogue **14** was without effect, as determined by counter-staining for dead cells. Human monocytes were stimulated as described in [a]. Propidium iodide stained, dead monocytic cells were determined by flow cytometry as described in the Experimental Section. **14** (1 μM) was used as a control. Mean values of six independent experiments performed in parallel with [b] are given. [d] Inhibition of intracellular SM metabolism. Freshly isolated human monocytes were labelled overnight with a BODIPY-SM/BSA complex according to Martin and co-workers.[28] Monolayers were preincubated with **14** and exposed to mmLDL (27 μg mL^{-1}) for 4 hours. Cellular lipids were extracted by the method of Bligh and Dyer,[44] and separated by HPLC. See the Experimental Section for further details. The turnover rate was determined by calculation of the SM/Cer ratio. **14** (5 μM) was used as a control. Mean values of three independent experiments are given. [e] Inhibition of nSMase activity by **14**. Human monocytes (day 7 after isolation) were preincubated with **14** as indicated and exposed to mmLDL (80 μg mL^{-1}) for 2 hours. The cells were collected, lysed in nSMase reaction buffer[42] and normalised for protein content, then the sphingolytic activity was determined. Sample lipids were separated by HPLC and the ceramide content was determined by 505/515 Em/Ex analysis. See the Experimental Section for further details. Under these conditions, nSMase activity is increased approximately twofold (R. A. Claus, A. Wuestholz, H. P. Deigner, unpublished results). **14** (1 μM) was used as a control. Mean values of two independent experiments, performed in duplicate, are given. [f] $p < 0.05$ versus TNF-α-stimulated sample, with ANOVA-testing. [g] $p < 0.05$ versus mmLDL-stimulated sample, with ANOVA-testing.

testing, Table 1 B). For verification of apoptosis, a counterstaining for dead cells with propidium iodide was performed in parallel. Neither the stimulus nor the inhibitor revealed a significant effect.

To analyse the effect of **14** on the metabolism of endogenous sphingomyelin (SM), we used monocytes labelled with a fluorescent SM analogue.[28] The intracellular hydrolysis of SM to Cer was found to be 18.5% in untreated controls (Table 1 D); the turnover rate, however, rose to 25.5% after exposure to mmLDL (27 μg mL^{-1} for 4 h) as determined by HPLC analysis, according to previous results.[3] In this experimental setting, preincubation with the synthetic analogue significantly reduced the relative content of Cer (at 1.0 and 0.1 μM inhibitor **14**, respectively) and completely inhibited a change of the Cer/SM ratio, so the values of the respective controls were retained at 10 μM inhibitor concentration. We then examined the effect on nSMase activity in cell extracts of (stimulated) cells: in fact, **14** inhibited the stimulus-dependent increase in sphingolytic activity in a concentration-dependent manner (Table 1 E).

In addition, in order to analyse potential cytotoxic effects, freshly isolated human monocytes were incubated with **14** (0.01–10 μM) for 16 h. No cytotoxic effects were observed in human monocytes as determined by the release of lactate dehydrogenase (LDH). The percentage cytotoxicity was calculated according to the manufacturer's instructions on the basis of a background control, with a high control value representing the maximum release of LDH (100%) due to stimulation with Triton X-100 (0.1%) and a low control value representing the spontaneous LDH release from untreated cells (0%). Treatment with **14** (10 μM) gave a value of −13.6% (three independent experiments), a result indicating that the compound displayed no cytotoxic effects but may rather increase viability.

In order to determine the inhibitory effect towards a neutral SMase of bacterial origin (*Bacillus cereus*), we measured enzymatic activities over five orders of magnitude of inhibitor concentration up to 10 μM. We found that **14** had no effect on the isolated bacterial nSMase (bSMase): no significant change of enzyme activity was observed after pretreatment of the bSMase with **14** prior to addition of substrate (two independent experiments, data not shown).

A crude cellular preparation containing Mg^{2+}-dependent nSMase from a human cell line without previous stimulation was used to study the mechanism of compound **14** as an inhibitor of neutral sphingomyelinase. Enzyme activity was determined with and without preincubation of cell lysate for 60 min with the compound tested (**14**, 100 μM) in the absence of substrate. Basal sphingolytic activity in the presence of Mg^{2+} amounted to 168 pM/(min×μg) ± 12.3 and was diminished by preincubation with compound **14** for 60 min, which resulted in an activity of 59 pM/(min×μg) ± 4.8. Without preincubation, a residual activity of 67 pM/(min×μg) ± 8.2 was analysed. The Mg^{2+}-independent hydrolytic activity of the preparation was assayed at 32 pM/(min×μg) ± 1.5. (Data are given as the mean ± the standard deviation of three independent experiments.)

To monitor directly the intracellular effects of the prepared compound, we used a method originally described by Signorelli et al.[29] and modified by Kajimoto et al.[30] Preincubation of hepatocytes with phorbol-12-myristate-13-acetate (PMA) and subsequent stimulation of intracellular generation of Cer by IFN-γ resulted in an inverse translocation of an enhanced green fluorescent protein (EGFP) tagged protein kinase C-α (PKC-α), as observed by fluorescence microscopy. The effect of

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IFN-γ on the translocation of PKC-α was monitored in HepG2 cells, since these cells are known to be responsive to IFN-γ stimulation with subsequent intracellular Cer generation mediated by the nSMase.[31] To examine whether IFN-γ induced inverse translocation of the tagged PKC isoform is mediated by the activation of the Mg^{2+}-dependent nSMase, we performed PKC-α-shift assays, as shown in Figure 2, in the presence of extracellular Mg^{2+}. The presence of magnesium eliminates the effects of other sphingo- or phospholipid-hydrolysing enzymes.[30] In fact, the inverse translocation was abolished by preincubation with **14** in appropriate concentrations (10 μM), but it could be induced by subsequent application of exogenous Cer (30 μM). See the legend of Figure 2 for details. The

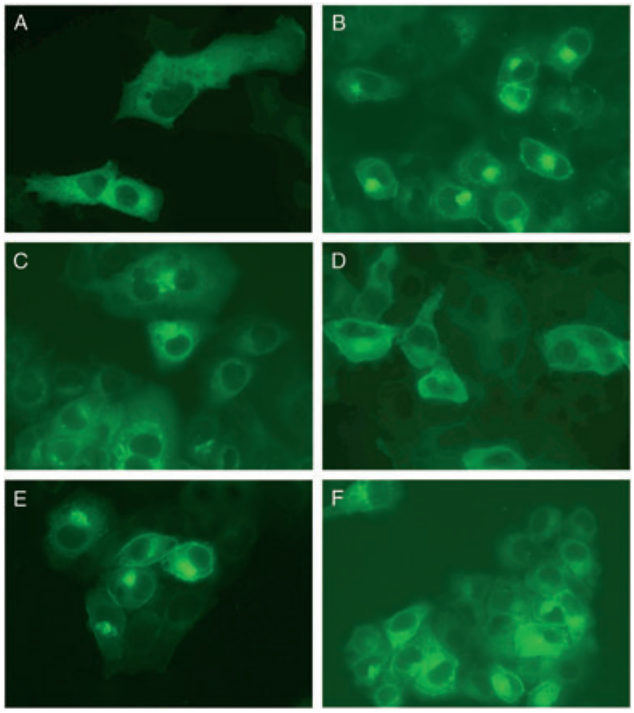

Figure 2. *SMase-dependent reverse translocation of PKC-α in HepG2 cells. HepG2 cells were stable transfected with EGFP-tagged PKC-α and protein distribution was visualised by fluorescence microscopy. Cells were left unstimulated (A) or were treated as follows by using cell-culture RPMI medium supplemented with 0.1% bovine serum albumin (BSA) and 1 mM $MgCl_2$: B) PMA (750 nM, 75 min); C) PMA (750 nM, 30 min) then treatment with IFN-γ (400 U mL^{-1}, 45 min); D) PMA (750 nM, 30 min) followed by treatment with Cer (30 μM, 45 min); E)* ***14*** *(10 μM, 30 min) followed by PMA (750 nM, 30 min) and subsequently IFN-γ (400 U mL^{-1}, 45 min); F)* ***14*** *(10 μM, 30 min) followed by PMA (750 nM, 30 min) and subsequently Cer (30 μM, 45 min). In unstimulated cells, PKC-α was detected throughout the cytoplasm (A), whereby the stimulation with PMA induced its translocation to the cellular membrane (B). The addition of exogenous ceramide (C) or the stimulation of endogenous Cer generation by IFN-γ (D) result in an nSMase-dependent reverse translocation of the tagged protein. This effect is abolished by pretreatment with the nSMase inhibitor* ***14*** *(E). The application of exogenous Cer after pretreatment with analogue* ***14*** *bypassed the inhibition of the nSMase and resulted in a diffuse distribution of the tagged protein in the cytoplasm (F). Plain Cer (30 μM, 45 min), IFN-γ (400 U mL^{-1}, 45 min) and analogue* ***14*** *(10 μM, 1 h 45 min) did not affect PKC-α translocation (not shown). The omission of Mg^{2+} supplementation in this experimental setting abrogated PKC-α translocation as a result of IFN-γ-induced intracellular Cer generation (not shown). These images are representative fields from at least three independent experiments, all of which showed similar results.*

treatment of cells with media without Mg^{2+} supplemention entirely blocked IFN-γ-induced inverse translocation in PMA-stimulated hepatocytes (not shown), thereby confirming isoform specificity. The results obtained by fluorescence microscopy were further confirmed by independent methods such as Western blotting experiments after subcellular fractionation, as shown in Figure 3. ANOVA testing revealed a significant effect after densitometric analysis ($p < 0.05$). In order to verify that these morphological changes were part of an ongoing apoptotic process, biological evaluation of **14** was further elucidated by analysis of poly(adenosine diphosphate–ribose) polymerase (PARP) protein fragmentation as an independent method for inhibition of apoptosis. As shown in Figure 4, Western blotting experiments indicated a significant increase in TNF-α- (6.17 ± 0.70) and IFN-γ- (14.87 ± 2.93) treated cells of PARP fragmentation (untreated control: 2.90 ± 0.45). The presence of the nSMase inhibitor **14** (10 μM) partially blocked TNF-α- (5.73 ± 0.75) or IFN-γ- (6.39 ± 0.18) induced appearance of the 85 kDa PARP fragment (median ± standard deviation of three experiments).

Discussion

We describe here an efficient and modular strategy for synthesising a biologically active analogue of the natural compound scyphostatin with improved chemical and metabolic stability and report the antiapoptotic activity of this analogue by applying current PCD models. Scyphostatin is described as a selective, noncovalent inhibitor of nSMase. The question of whether or to what extent the membrane-bound, Mg^{2+}-dependent nSMase or the lysosomal acid isoform (aSMase) is involved in Cer production in response to various stimuli is controversial.[32] Specific inhibitors capable of differentiating between distinct isoforms represent valuable tools for the characterisation of specific biological roles of SMase isoforms, to clarify the involvement of these enzymes in the induction of apoptosis, as well as for the characterisation of the role of Cer. We found that the synthetic analogue **14** prevents activation of nSMase in whole cells and that the compound displays antiapoptogenic activity in PCD models, as shown by staining of nuclei and cellular membranes of apoptotic cells. The fragmentation of the PARP protein is known to function as a reliable marker of PCD. In fact, both TNF-α- and IFN-γ-mediated activation of neutral sphingomyelinase result in the induction of cell death and can be monitored by PARP cleavage.[33] Our results obtained by using independent methodologies clearly indicate an inhibitory effect of compound **14** on cytokine-triggered cell death. The use of compound **14** as an inhibitor of neutral sphingomyelinase is in agreement with previous reports demonstrating that at least two major routes for the induction of apoptosis are affected by compounds inhibiting neutral SMases, such as GW4869 or **14**.[33]

The inhibitory activity of compound **14** was also confirmed at a more cellular level. Hepatocyte responses to IFN-γ, including cell-cycle arrest, apoptosis,[34] activation of nSMase and stimulation of Cer generation and metabolism, are mediated by IFN regulatory factor 1.[31] In this context, we have studied

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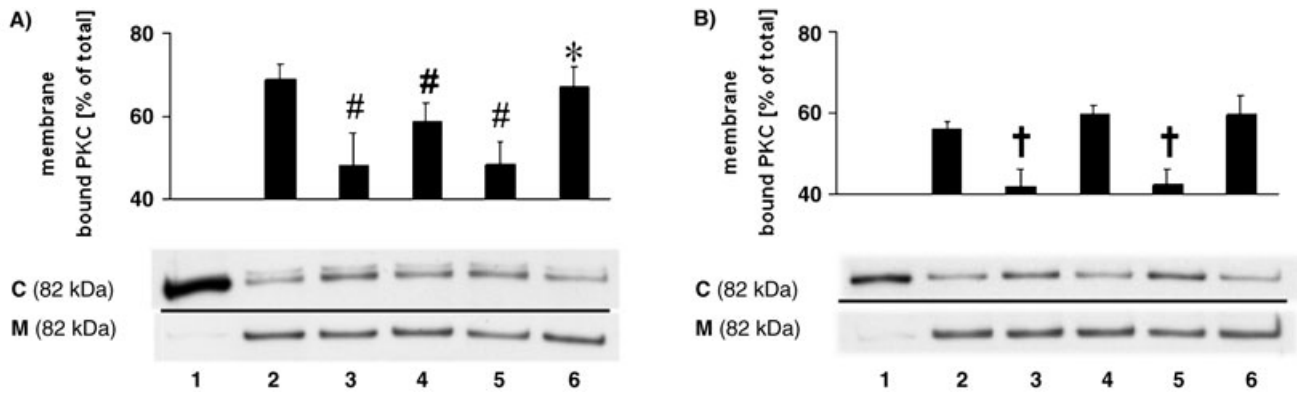

Figure 3. *A) Immunoblotting analysis of PKC-α translocation. HepG2 cells were left untreated (1) or were stimulated as follows: 2) PMA (750 nM, 30 min); 3) PMA (750 nM, 30 min) followed by treatment with Cer (30 μM, 90 min); 4) PMA (750 nM, 30 min) followed by treatment with IFN-γ (600 U mL^{-1}, 90 min) in the presence of 1 mM Mg^{2+}. For monitoring of the inhibitory effects of* ***14****, cells were incubated with analogue* ***14*** *(10 μM) for 30 minutes before treatment with either 5) PMA (750 nM, 30 min) followed by Cer (30 μM, 90 min) or 6) PMA (750 nM, 30 min) followed by IFN-γ (600 U mL^{-1}, 90 min). Pelleted cells were fractionated in the cytosolic (C^{+Mg}) or membrane fraction (M^{+Mg}); equal amounts of protein (3 μg) were electrophoresed, immunoblotted and visualised by using specific antibodies against PKC-α. Proof of equal protein content is given by reprobing of the cytosolic fraction with antibodies against α-tubulin (not shown). Immune-reactive bands showed reasonable molecular weights. The amount of detected protein was densitometrically quantified by using the Advanced Image Data Analyzer software (AIDA, Raytest) after scanning on an image scanner from BioRad. The data for protein distribution are scaled as the ratio between the cytosolic and membrane fractions, calculated as a proportion of normalised arbitrary units in both fractions [%]. For comparison, the value of untreated control samples was 0.76%. B) Results of an identical experimental setting to that in (A) without Mg^{2+} supplementation in the cell-culture media. After subcellular fractionation, for quantification of PKC-α, the cytosolic (C^{-Mg}) and the membrane (M^{-Mg}) fraction are analysed after reprobing the α-tubulin-reactive protein of the cytosolic fraction (not shown). The relative subcellular distribution is also depicted. (untreated control 1.5%). # indicates significance $p < 0.05$ versus PMA-treated cells; * indicates significance $p < 0.05$ versus cells treated with PMA followed by IFN-γ without analogue* ***14****; † indicates significance $p < 0.05$ versus PMA-treated cells without Mg^{2+} supplementation. Representative results of three independent experiments with ANOVA testing are given.*

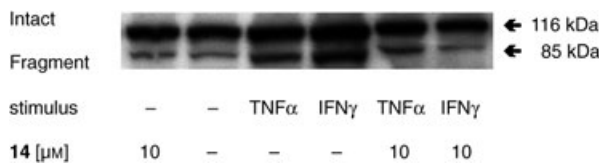

Figure 4. *Protective effects of* ***14*** *on PARP fragmentation. Human monocytes (day 7 after isolation) were preincubated with* ***14*** *(10 μM, 30 min) as indicated and exposed to TNF-α (10 ng mL^{-1}) or IFN-γ (600 U mL^{-1}) for four hours. After incubation, monolayers were washed with ice-cold phosphate-buffered saline (PBS) and cells collected by scraping on ice in lysis buffer. Equal amounts of cytosolic proteins (40 μg) were resolved by 7.5% sodium dodecyl sulfate PAGE. After immunoblotting, analysis was carried out by using monoclonal anti-PARP antibodies. Intact (116 kDa) and cleaved PARP protein (fragment, 85 kDa) were visualised by using polyclonal-specific horseradish peroxdiase (POD) conjugated antibodies. Immune-reactive bands showed reasonable molecular weights. Representative results of three independent experiments are shown; the results of densitometric analyses are given in text.*

the impact of the analogue **14** on IFN-γ-mediated effects by using an EGFP-tagged effector protein of Cer. Under experimental conditions specified for monitoring effects mediated by the neutral, Mg^{2+}-dependent SMase, we clearly recorded an inhibitory effect of the synthetic analogue. This result was confirmed by immunoblotting demonstrating an inhibition of IFN-γ-mediated reverse translocation. Our observations can be explained by specific inhibition of cellular enzyme isotype(s). However, it can also be speculated that **14** predominantly interferes with cellular enzyme activation. For example, recent studies have suggested that glutathione determines nSMase activity by reversibly inhibiting the enzyme,[35] thereby acting as a regulator of the intracellular redox state. Alternatively, the adapter protein FAN (the factor associated with nSMase activation), interacting with the nSMase activation domain on the TNF receptor, is thought to provide a link to the stimulation of nSMase[36,37] and could be a hypothetical target. Interestingly, in cultured cerebellar neurons, Cer generated by nSMase is involved in nerve growth factor (NGF) mediated neuronal cell death and glutamate release, whereas the application of scyphostatin (0.1 or 1.0 μM) significantly blocked the NGF-dependent glutamate release, as well as the NGF-induced Cer production.[38]

In the course of our study, we probed the importance of the presence of an epoxide moiety located at the carbon centre activated by the α,β-unsaturated enone in the natural compound. While this unique structure is rare—for example, the family of manumycin antibiotics comprises a comparable epoxyquinone moiety[39]—it remains to be determined whether its presence is required for biological activity. Potentially reactive intermediates such as epoxides[40,41] are subjected to fast biotransformation. The renouncement of the epoxide moiety leads to a chemically stable compound with potentially increased metabolic stability, thus circumventing rapid inactivation and/or clearance, prerequisites to obtaining sufficient plasma levels for inhibitory effects in vivo. In a most recent study, several *o*-quinol acetate derivatives were described as scyphostatin analogues and potent irreversible inhibitors of rat brain nSMase.[42] Our experimental setting, varying in preincubation of the cell lysate with compound **14**, provides information on properties of the compound interacting with the active site of the tested enzyme. The lack of a significant difference between both approaches indicates that the compound acts as a reversible enzyme inhibitor. In summary, the observed

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biological activities of **14** and the experimental data of others[42] suggest that the enone-activated epoxide displays no essential structural contribution in the inhibitory activity of scyphostatin analogues.

Analogues optimised for both physicochemical properties, critically determined by the length and the degree of saturation of the *N*-acyl side chain, and for inhibitory activities will provide ideal tools to characterise the role of the enzyme in cell-based experiments.

Conclusion

In this report, for the first time, we provide clear evidence of interactions of a scyphostatin analogue with selected targets in a cell-based assay system. SMase inhibitors have recently attracted considerable interest because they are believed to be promising candidates for the treatment of various diseases. While natural scyphostatin is one such compound, limited supply hampers its use in current and further investigations. Access to substantial quantities of the scyphostatin desepoxy analogue **14** by the synthetic route described above paves the way to greater use of stable compounds structurally related to the natural compound scyphostatin. Our synthesis of **14** allows multiple variations in stereochemistry and in the functionalisation of future products and, thereby, allows variations in physicochemical properties related to pharmacokinetics and pharmacodynamics, such as lipophilicity. These, however, are preconditions for subsequent quantitative structure–activity relationship (QSAR) studies to gain further information on the inhibitory mechanism(s), to explore the function(s) of nSMase on a molecular level and to select drug candidates.[43] Selective inhibition of neutral SMases may provide new insights into the effects of ceramide-induced alteration, for example, of ventricular myocyte contractile function and myocardial depression, as well as antiapoptotic effects of ischemic preconditioning in the heart.[43]

Experimental Section

Syntheses:

(*R*)-4-[Hydroxy-(2-oxo-cyclohexyl)methyl]-2,2-dimethyl-oxazolidine-3-carboxylic acid *tert*-butyl ester (3): LDA (5.00 mL, 10.0 mmol, 2 M solution in hexane) was added dropwise to THF (50 mL) at −78 C. After 5 minutes, cyclohexanone (980 mg, 1.05 mL, 10.0 mmol) in THF (5 mL) was added and the solution was stirred for 90 minutes at −78 °C. (*R*)-4-Formyl-2,2-dimethyl-oxazolidine-3-carboxylic acid *tert*-butyl ester (**2**; 916 mg, 4.00 mmol) in THF (5 mL) was then added at −78 °C, and the solution was stirred for 30 minutes at −78 °C and for 1 hour at RT. Afterwards, 5% hydrochloric acid (25 mL) was added and the solution was stirred for 20 minutes. The phases were separated and the aqueous phase was extracted three times with ethyl acetate (50 mL). The organic phases were washed with saturated sodium hydrogen carbonate solution (50 mL) and brine (50 mL), dried over magnesium sulphate and filtered, then the eluent was removed. After column-chromatography purification (silica gel, diethyl ether/*n*-hexane (2:1)), the product was isolated as a colourless oil (yield = 1.10 g, 84%). R_f = 0.55; ^{1}H NMR ($CDCl_3$): δ = 1.49 (br, 15H), 1.53–2.18 (m, 6H), 2.25–2.48 (m, 3H), 3.88 (t, 1H; ^3J = 7.6 Hz), 3.90 (t, 1H; ^3J = 7.6 Hz), 3.97 (m, 1H; ^3J = 7.5, ^3J = 6.0 Hz), 4.18 (dd, 1H; ^3J = 7.5, ^2J = −8.5 Hz), 4.20 (dd, 1H; ^3J = 6.7, ^2J = −8.5 Hz), 4.24 ppm (m, 1H; ^3J = 7.5, ^3J = 6.0 Hz); ^{13}C NMR ($CDCl_3$): δ = 15.1, 15.5 (rotamers, 2×), 21.3, 21.6 (diastereomers, 2×), 24.0, 24.2, 24.7 (diastereomers, 2×), 27.0, 27.5, 28.2, 28.3, 42.0, 42.5 (diastereomers, 2×), 51.0, 53.0 (diastereomers, 2×), 58.4, 59.7 (diastereomers, 2×), 64.5, 65.0 (rotamers, 2×), 68.5, 80.3, 93.7, 94.1 (rotamers, 2×), 153.3, 212.1, 216.2 ppm (diastereomers, 2×); MS (CI+, *i*-butane): *m/z*: 328 [M^+], 314, 210, 200, 196, 174, 116, 99; IR (Film): $\tilde{\nu}$ = 3491 (s), 2977 (s), 2935 (s), 1702 (s), 1693 (s), 1450 (m), 1376 (s), 1254 (s), 1173 (s), 1100 (s), 1055 (s), 846 cm^{-1} (s); HRMS (CI+): *m/z* calcd for $C_{17}H_{30}NO_5$: 328.2123; found: 328.2123.

(*R*)-4-[Methanesulfonyloxy-(2-oxo-cyclohexyl)methyl]-2,2-dimethyl-oxazolidine-3-carboxylic acid *tert*-butyl ester (4): Triethylamine (3.68 g, 5.04 mL, 36.48 mmol) was added at 0 °C to a solution of **3** (3.85 g, 11.8 mmol) in dichloromethane (50 mL). This was followed by addition of methanesulfonyl chloride (2.70 g, 1.83 mL, 23.5 mmol). The solution was stirred for 2 h at 0 °C, diluted with diethyl ether (30 mL) and washed with saturated ammonium chloride solution (50 mL) and brine (50 mL). The organic layer was dried over magnesium sulphate and filtered, then the solvent was removed. After column-chromatography purification (silica gel, diethyl ether/*n*-hexane (3:1)), the product was isolated as a colourless oil (yield = 4.68 g, 98%) R_f = 0.63; ^{1}H NMR ($CDCl_3$): δ = 1.40–1.85 (m, 21H), 1.87–2.19 (m, 2H), 2.22–2.67 (m, 3H), 3.04 (s, 3H), 3.91–4.14 (m, 2H), 4.31 ppm (m, 1H); ^{13}C NMR ($CDCl_3$): δ = 24.1, 26.5, 24.6, 25.2, 24.4, 24.6, 27.0, 27.2, 28.4, 30.6, 31.6 (rotamers, 2×), 37.6, 38.7 (diastereomers, 2×), 41.7, 42.2 (diastereomers, 2×), 52.1, 53.7 (diastereomers, 2×) 58.3, 58.9 (rotamers, 2×), 63.5, 63.8 (rotamers), 80.9, 81.1 (rotamers), 78.0, 81.2 (diastereomers, 2×), 94.4, 154.2, 208.9, 209.6 ppm (diastereomers); MS (CI+, *i*-butane): *m/z*: 406 [$M+H^+$], 350, 310, 306, 292, 288, 254, 210, 196; IR (Film): $\tilde{\nu}$ = 2976 (s), 2871 (s), 1701 (s), 1693 (s), 1366 (s), 1175 (s), 1101 (m), 935 cm^{-1} (s); HRMS (CI+): *m/z* calcd for $C_{18}H_{32}NO_7S$: 406.1900; found: 406.1899.

(*S*)-2,2-Dimethyl-4-[2-oxo-cyclohexylidenemethyl]oxazolidine-3-carboxylic acid *tert*-butyl ester (5): DBU (3.69 g, 3.62 mL, 24.3 mmol) at 0 °C was added dropwise to a solution of **4** (4.68 g, 11.9 mmol) in dichloromethane (50 mL). The solution was stirred overnight, then diluted with diethyl ether (50 mL) and washed with saturated ammonium chloride solution (50 mL) and brine (50 mL). The organic layer was dried over magnesium sulphate and filtered, then the solvent was removed. After column-chromatography purification (silica gel, diethyl ether/*n*-hexane (3:1); R_f = 0.77), the product was isolated as a colourless oil, which crystallised after a few hours (yield = 2.61 g, 73%; m.p. = 66 °C). ^{1}H NMR ($CDCl_3$): δ = 1.40, 1.47 (s, 9H; rotamers, 2×), 1.51, 1.55, 1.59, 1.61 (s, 6H; rotamers, 2×2), 1.72–1.93 (m, 4H), 2.44 (dd, 2H; ^3J = 6.5, ^3J = 6.3 Hz), 2.64–2.76, 2.79–2.91 (m, 2H; diastereomers), 3.64–3.72 (m, 1H; diastereomers, rotamers), 4.07 (d, 1H; ^3J = 6.2 Hz, rotamer), 4.09 (d, 1H; ^3J = 6.5 Hz, rotamer), 4.50–4.59, 4.61–4.70 (m, 1H), 6.49 ppm (dt, 1H; ^3J = 9.1, ^4J = 2.2 Hz); ^{13}C NMR ($CDCl_3$): δ = 23.2, 23.5, 26.7, 24.3, 25.2, 26.1, 27.2 (rotamers, 4×), 26.7, 28.4, 40.3, 54.7, 67.5, 80.0, 80.4 (diastereomers, 2×), 93.8, 94.6 (diastereomers, 2×), 135.7, 137.0 (diastereomers, 2×), 136.8, 137.8 (diastereomers, 2×), 151.6, 152.0 (rotamers, 2×), 200.4, 200.9 ppm (diastereomers, 2×); MS (CI+, *i*-butane): *m/z*: 310, 254, 228, 210, 183, 153; IR (Film): $\tilde{\nu}$ = 3109 (m), 2977 (s), 2936 (s), 2868 (s), 1700 (s), 1691 (s), 1624 (s), 1388 (s), 1173 (s), 1091 (m), 835 cm^{-1} (s); HRMS (CI+): *m/z* calcd for $C_{17}H_{28}NO_4$: 310.2018; found: 310.2018.

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(*S*)-2,2-Dimethyl-4-(2-oxo-cyclohexylmethyl)oxazolidine-3-carboxylic acid *tert*-butyl ester (6): A suspension of **5** (1.39 g, 4.52 mmol) and palladium on charcoal (100 mg) in ethanol (50 mL, 96%) was hydrogenated for 30 minutes. The catalyst was filtered off over celite and the solvent was removed. After column-chromatography purification (silica gel, diethyl ether/*n*-hexane (3:1)), the product was isolated as a colourless oil (yield = 1.41 g, 90%) R_f = 0.72; ^{1}H NMR ($CDCl_3$): δ = 1.32–1.77 (m, 17H), 1.78–2.21 (m, 4H), 2.21–2.51 (m, 3H), 3.69 (m, 1H), 3.91 ppm (m, 2H); ^{13}C NMR ($CDCl_3$): δ = 24.6, 25.2, 23.2, 24.5, 26.9, 27.6, (rotamers, 2×2), 28.0, 28.2 (diastereomers, 2×), 28.4, 41.7, 42.2 (diastereomers, 2×), 48.3, 55.7, 67.8, 79.9, 93.3, 152.5, 212.1, 212.6 ppm (diastereomers, 2×); MS (CI+, *i*-butane): *m/z*: 312 [$M+H^+$], 254, 196, 180; IR (Film): $\tilde{\nu}$ = 2930 (s), 2858 (s), 1711 (s), 1699 (s), 1449 (s), 1388 (s), 1175 (s), 1094 (m), 921 cm^{-1} (m); HRMS (CI+): *m/z* calcd for $C_{17}H_{29}NO_4$: 312.2175; found: 312.2174.

(*S*)-2,2-Dimethyl-4-(2-trimethylsilanyloxy-cyclohex-1-enylmethyl)oxazolidine-3-carboxylic acid *tert*-butyl ester (7): Hexamethyldisilazane (806 mg, 1.05 mL, 5.00 mmol) was added to a solution of **6** (1.03 g, 3.30 mmol) in *n*-pentane (50 mL), then the solution was cooled to −20 °C and stirred for 30 minutes. During a period of 15 minutes, trimethylsilyl iodide (800 mg, 585 μL, 4.00 mmol) was added dropwise. The solution was stirred for 2 hours at room temperature. The solution became cloudy and was diluted with *n*-pentane (25 mL), filtered and washed twice with saturated sodium hydrogen carbonate solution (50 mL). The organic layer was dried over magnesium sulphate and filtered, then the solvent was removed. After column-chromatography purification (silica gel, diethyl ether/*n*-hexane (3:1); R_f = 0.56) the product is isolated as a pale-yellow oil (yield = 1.13 g, 89%). ^{1}H NMR ($CDCl_3$): δ = 0.34 (s, 9H), 1.47–1.74 (m, 17H), 1.83–2.14 (m, 6H), 2.56–2.77 (m, 2H), 3.70 (m, 2H), 3.91 ppm (m, H); ^{13}C NMR ($CDCl_3$): δ = 0.8, 23.1, 23.5, 23.4, 25.4, 26.9 (rotamers, 2×2), 28.5, 27.7, 30.2, 33.0, 33.8 (rotamers, 2×), 56.2, 56.4 (rotamers, 2×), 66.4, 79.2, 79.6 (rotamers, 2×), 93.1, 93.5 (rotamers, 2×), 111.8, 112.2 (rotamers, 2×), 145.3, 145.5 (rotamers, 2×) 151.6, 152.0 ppm (rotamers, 2×); MS (EI, 50 °C): *m/z* (%): 383 (7.1) [M^+], 327 (4.4), 183 (48.2), 100 (59.5), 57 (100); IR (Film): $\tilde{\nu}$ = 3155 (m), 2974 (s), 2863 (s), 1699 (s), 1620 (m), 1378 (s), 1251 (s), 1098 (s), 942 (s), 844 cm^{-1} (s); HRMS (CI+): *m/z* calcd for $C_{20}H_{37}NO_4Si$: 384.2570; found: 384.2571.

(*S*)-4-((*R*)-1-Hydroxy-2-oxo-cyclohexylmethyl)-2,2-dimethyl-oxazolidine-3-carboxylic acid *tert*-butyl ester (9): A solution of AD mix β (3.73 g), sodium hydrogen carbonate (672 mg, 8.00 mmol), methanesulphonamide (253 mg, 2.66 mmol) and potassium osmate dihydrate (40 mg, 109 μmol) in a mixture of water and *tert*-butanol (20 mL; 1:1) was cooled to 0 °C. **7** (1.02 g, 2.66 mmol) in a mixture of water and *tert*-butanol (5 mL; 1:1) was added dropwise. The originally orange solution turned green after a few hours. The solution was stirred for a further 36 hours at room temperature, a saturated solution of sodium thiosulphate in water (30 mL) was added and the solution was stirred for a further 30 minutes. The layers were separated and the aqueous phase was extracted three times with ethyl acetate (50 mL). The combined organic layers were washed with diluted hydrochloric acid (50 mL), saturated sodium hydrogen carbonate solution (50 mL) and brine (50 mL). The organic layer was dried over magnesium sulphate and filtered, then the solvent was removed. After column-chromatography purification (silica gel, diethyl ether/*n*-hexane (3:1)), the product was isolated as a colourless oil, which crystallised after a few days if cooled to −20 °C (yield = 504 mg, 58%). R_f = 0.77; m.p. = 58 °C; ^{1}H NMR ($CDCl_3$): δ = 1.22–2.08 (m, 23H), 2.54–2.68 (m, 2H), 3.53–3.67 (m, 1H), 3.84–4.15 ppm (m, 2H); ^{13}C NMR ($CDCl_3$): δ = 22.5, 24.5, 27.9, 28.7, 29.0, 38.1, 39.9, 43.5, 54.1, 68.4, 78.7, 80.2, 92.9, 152.4, 214.8 ppm; MS (CI+, *i*-butane): *m/z*: 328 [M^+], 312, 310, 272, 270, 258, 254, 252, 228, 214, 198, 196, 170; IR (Film): $\tilde{\nu}$ = 3473 (s), 2977 (s), 2937 (s), 2868 (s), 1713 (s), 1688 (s), 1391 (s), 1255 (s), 1173 (s), 1100 (s), 853 cm^{-1} (m); HRMS (CI+): *m/z* calcd for $C_{17}H_{30}NO_5$: 328.2124; found: 328.2123.

(*S*)-4-((*R*)-3-Bromo-1-hydroxy-2-oxo-cyclohexylmethyl)-2,2-dimethyl-oxazolidine-3-carboxylic acid *tert*-butyl ester: **9** (185 mg, 570 μmol) in THF (5 mL) was added dropwise at −78 °C to a solution of LDA (0.57 mL, 1.15 mmol, 2 M in THF) in THF (10 mL). After 30 minutes, bromine (93 mg, 580 μmol) in dichloromethane (2 mL) was added. The solution was stirred for 2 minutes and diluted with diethyl ether (5 mL), then a saturated sodium carbonate solution (30 mL) was added. The layers were separated and the aqueous phase was washed three times with diethyl ether (30 mL). The combined organic layers were washed with brine (30 mL) and dried over magnesium sulphate. After removal of the solvent, the product was isolated as a pale-yellow oil, which could be purified if necessary by column chromatography (silica gel, diethyl ether/*n*-hexane (3:1); yield = 228 mg, 98%). R_f = 0.64; ^{1}H NMR ($CDCl_3$): δ = 1.38 (s, 6H), 1.51 (s, 9H), 1.40–2.13 (m, 8H), 2.98 (br, 1H), 4.19 (d, 2H; ^3J = 7.6 Hz), 4.37–4.46 ppm (m, 2H); ^{13}C NMR ($CDCl_3$): δ = 18.9, 27.3, 28.2, 33.0, 36.1, 39.0, 48.1, 53.8, 66.0, 76.1, 80.4, 94.1, 152.6, 208.2 ppm; MS (CI+, *i*-butane): *m/z*: 408/406 [$M+H^+$], 326, 292, 270, 254, 252, 214, 198, 196, 170; IR (Film): $\tilde{\nu}$ = 3474 (s), 2979 (s), 2945 (s), 2873 (s), 1704 (s), 1694(s), 1396 (s), 1261 (s), 1178 (s), 1095 (s), 1056 (s), 858 cm^{-1} (m); HRMS: *m/z* calcd for $C_{17}H_{28}NO_5Br$: 405.1202/407.1181; found: 405.1202/407.1181.

(*S*)-4-((*R*)-1-Hydroxy-2-oxo-cyclohex-3-enylmethyl)-2,2-dimethyl-oxazolidine-3-carboxylic acid tert-butyl ester (10): A solution of the intermediate (*S*)-4-((*R*)-3-bromo-1-hydroxy-2-oxo-cyclohexylmethyl)-2,2-dimethyl-oxazolidine-3-carboxylic acid *tert*-butyl ester (228 mg, 560 μmol) in DBU (4 mL) was heated to 115 °C for 5 h. After cooling to room temperature, the solution was diluted with diethyl ether (20 mL) and washed with brine (20 mL). The aqueous layer was extracted with diethyl ether (20 mL) twice and the combined organic layers were dried over magnesium sulphate. The solvent was removed and the product was purified by column chromatography (silica gel, diethyl ether/*n*-hexane (3:1)). **10** was obtained as a colourless oil (yield = 114 mg, 62%). R_f = 0.68; ^{1}H NMR ($CDCl_3$): δ = 1.42–1.92 (m, 17), 1.93–2.26 (m, 2H), 2.54–2.68 (m), 3.52–3.67 (m, 1H), 3.84–4.06 (m, 2H), 6.11 (d, 1H), 6.98 ppm (m, 1H); ^{13}C NMR ($CDCl_3$): δ = 22.8, 24.8, 27.7, 28.6, 38.2, 40.0, 55.5, 68.7, 78.9, 80.5, 93.1, 125.8, 143.1, 152.0, 214.6 ppm; MS (CI, *i*-butane): *m/z*: 326 [$M+H^+$], 308, 270, 252, 196, 178; IR (Film): $\tilde{\nu}$ = 3455 (s), 3012 (m), 2981 (s), 2956 (s), 2858 (s), 1707 (s), 1693 (s), 1644 (s), 1557 (s), 1391 (s), 1258 (s), 1199 (s), 1175(s), 1090 (s), 823 cm^{-1} (m); HRMS (CI+): *m/z* calcd for $C_{17}H_{28}NO_5$: 326.1967; found: 326.1966.

(*R*)-6-((*S*)-2-Amino-3-hydroxy-propyl)-6-hydroxy-cyclohex-2-enone (13): 10 (88 mg, 0.27 mmol) was added dropwise as a solution in THF (2 mL) to TFA (5 mL) in dichloromethane (5 mL). The mixture was stirred for 4 hours at room temperature and afterwards neutralised with a saturated solution of sodium hydrogen carbonate. The layers were separated and the aqueous layer was washed with ethyl acetate (20 mL) twice. The combined organic layers were washed with water (20 mL) and dried over magnesium sulphate. The solvent was removed and the product was purified by column chromatography (silica gel, diethyl ether/*n*-hexane (1:1); yield = 45 mg, 89%). R_f = 0.58; ^{1}H NMR ($CDCl_3$): δ = 1.66 (d, 2H; ^3J = 6.43 Hz), 2.08 (m, 4H), 3.37 (m, 1H), 3.54 (br m, 4H), 6.22 (d, 1H; ^3J = 10.4 Hz), 7.05 ppm (m, 1H); ^{13}C NMR ($CDCl_3$): δ = 27.1, 36.0,

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40.8, 51.1, 65.0, 77.4, 125.9, 148.3, 203.2 ppm; MS (EI, 78 °C): *m/z* (%): 185 (3.7) [M^+], 169 (17.9), 111 (7.5), 95 (12.1) ppm; IR (Film): $\tilde{\nu}$ = 3414 (s), 3048 (m), 2981 (s), 2928 (s), 1706 (s), 1648 (s), 1511 (m), 1250 (s), 1113 (s), 1092 (s), 866 cm^{-1} (m); HRMS: *m/z* calcd for $C_9H_{15}NO_3$: 185.2983; found: 185.2985; elemental analysis: calcd (%) for $C_9H_{15}NO_3$ (185.22 g mol^{-1}): C 58.36, H 8.16, N 7.56; found: C 58.21, H 8.34, N 7.32).

Hexadecanoic acid [(*S*)-2-hydroxy-1-((*R*)-1-hydroxy-2-oxo-cyclohex-3-enylmethyl)ethyl]amide (14): Triethylamine (1.00 mL) was added to a solution of **13** (145 mg, 0.79 mmol) in THF (50 mL) and the resulting solution was cooled to −78 °C. Palmitic acid chloride (217 mg, 0.79 mmol, 0.24 mL) in THF (5 mL) was added very slowly and the solution was stirred for 15 minutes at −78 °C. The reaction was stopped with a saturated solution of sodium hydrogen carbonate (25 mL), then the aqueous layer was extracted with ethyl acetate (30 mL) three times and the combined organic layers were dried with magnesium sulphate. The solvent was evaporated and the crude product was isolated as a pale-yellow solid. Washing with diethyl ether results in pure colourless **14** (yield = 302 mg, 91 %): ^{1}H NMR ($CDCl_3$): δ = 0.90 (t, 3H; ^3J = 6.6 Hz); 1.24–2.21 (m, 34H), 3.27 (br, 2H), 3.48 (d, 1H; ^3J = 9.7 Hz), 4.18 (m, 1H), 5.88 (br, 1H), 6.10 (d, 1H; ^3J = 9.7 Hz), 7.01 ppm (m, 1H); ^{13}C NMR ($CDCl_3$): δ = 13.9, 22.3–40.2 (17C), 49.4, 65.2, 78.9, 129.0, 147.3, 200.9 ppm; MS (EI, 58 °C): *m/z* (%): 424 (0.82) [M^+], 407 (2.31), 254 (14.82), 239 (27.88), 185 (7.46), 94 (8.95); IR (Film): $\tilde{\nu}$ = 3436 (s), 3051 (m), 2977 (s), 2919 (s), 1710 (s), 1635 (s), 1467 (m), 1219 (s), 1122 (s), 1087 (s), 870 cm^{-1} (m); elemental analysis: calcd (%) for $C_{25}H_{45}NO_4$ (423.63 g mol^{-1}): C 70.88, H 10.71, N 3.31; found: C 70.63, H 10.58, N 3.53).

(*S*)-4-((*S*)-1-Hydroxy-2-oxo-5-phenylselenyl-cyclohex-3-enylmethyl)-2,2-dimethyl-oxazolidine-3-carboxylic acid *tert*-butyl ester (11): LDA (830 μL, 1.65 mmol, 2 M in THF) was added to THF (15 mL) at −78 °C. To this solution, **10** (250 mg, 768 μmol) dissolved in THF (5 mL) was added dropwise. The solution was stirred for 45 minutes at the same temperature and then phenylselenyl chloride (156 mg, 810 μmol) in THF (5 mL) was added in one portion. The cooling bath was removed and the reaction mixture was added to a stirred mixture of dichloromethane (50 mL) and saturated sodium carbonate solution (50 mL). The aqueous layer was extracted twice with dichloromethane (50 mL) and the combined organic layers were washed with water (50 mL). After drying over magnesium sulphate, the solvent was evaporated and the residue was purified by column chromatography (silica gel, diethyl ether/*n*-hexane (1:1)) to yield **11** (281 mg, 584 μmol, 76 %) as a yellowish oil. R_f = 0.69; ^{1}H NMR ($CDCl_3$): δ = 1.42–1.82 (m, 15H), 1.93–2.56 (m, 4H), 3.35 (m, 1H), 3.45–3.76 (m, 1H), 4.10–4.22 (m, 2H), 6.38 (d, 1H; ^3J = 10.0 Hz), 6.78 (m, 1H), 7.14–7.52 ppm (m, 5H); ^{13}C NMR ($CDCl_3$): δ = 24.9, 27.6, 28.2, 37.6, 38.7, 39.0, 53.6, 65.4, 80.0, 80.5, 95.3, 125.2, 125.6, 126.6, 128.8, 133.7, 140.1, 152.1, 212.0 ppm; MS (CI+, *i*-butane): *m/z*: 482 [M+H^+], 480 [M+H^+], 325, 266; IR (Film): $\tilde{\nu}$ = 3438 (s), 3051 (m), 2977 (s), 2933 (s), 2863 (s), 1712 (s), 1699 (s), 1638 (s), 1576 (m), 1504 (m), 1390 (s), 1255 (s), 1169 (s), 1092 (s), 1071 (s), 847 (m), 739 cm^{-1} (s); HRMS (CI+): *m/z* calcd for $C_{23}H_{32}NO_5Se$: 482.1446; found: 482.1446.

(*S*)-4-((*S*)-1-Acetoxy-2-oxo-5-phenylselenyl-cyclohex-3-enylmethyl)-2,2-dimethyl-oxazolidine-3-carboxylic acid *tert*-butyl ester (15): **11** (100 mg, 208 μmol) was dissolved in dichloromethane (30 mL) and pyridine (5 mL), than DMAP (10 mg, 81.8 μmol) was added. After 5 minutes, acetyl chloride (27 mg, 350 μmol) was added and the mixture was stirred for 4 hours at room temperature. The solution was diluted with diethyl ether (100 mL), extracted three times with hydrochloric acid (1 N), washed three times with a saturated sodium hydrogen carbonate solution (30 mL), dried over magnesium sulphate and evaporated to dryness. The residue was purified by column chromatography (silica gel, diethyl ether/*n*-hexane (1:2)) to yield **15** (101 mg, 193 μmol, 93 %). R_f = 0.59; ^{1}H NMR ($CDCl_3$): δ = 1.43–1.85 (m, 15H), 1.93–2.54 (m, 4H), 2.06 (s, 3H), 3.36 (m, 1H), 3.38–3.68 (m, 1H), 4.22–4.34 (m, 2H), 6.39 (d, 1H; ^3J = 10.0 Hz), 6.81 (m, 1H), 7.15–7.53 ppm (m, 5H); ^{13}C NMR ($CDCl_3$): δ = 20.8, 24.8, 27.7, 28.1, 37.4, 38.5, 38.8, 54.2, 62.6, 80.4, 88.1, 95.2, 125.6, 126.0, 126.7, 128.9, 133.4, 139.6, 152.3, 170.6, 204.5 ppm; MS (CI+, *i*-butane): *m/z*: 524 [M+H^+], 522 [M+H^+], 462, 325, 266; IR (Film): $\tilde{\nu}$ = 3048 (m), 2976 (s), 2928 (s), 2858 (s), 1710 (s), 1700 (s), 1678 (s), 1653 (s), 1578 (m), 1502 (m), 1391 (s), 1253 (s), 1092 (s), 1070 (s), 843 cm^{-1} (m); HRMS (CI+): *m/z* calcd for $C_{25}H_{34}NO_6Se$: 524.1551; found 524.1553.

(*S*)-4-((*S*)-6-Acetoxy-8-phenylselenyl-1,4-dioxa-spiro[4.5]dec-9-en-6-ylmethyl)-2,2-dimethyl-oxazolidine-3-carboxylic acid *tert*-butyl ester (16): **15** (60 mg, 115 μmol) and 1,2-ethanediol bis-trimethylsilyl ether (24 mg, 117 μmol) were added dropwise to a 2.30 mM solution of trimethylsilyl trifluoromethanesulphonate in absolute dichloromethane (500 μL, 1 mol %). The reaction mixture was stirred overnight at the same temperature, then the reaction was terminated by addition of dry pyridine (50.0 μL) and the solution was added to a saturated sodium carbonate solution (10 mL). The mixture was extracted three times with diethyl ether (20 mL) and the combined organic layers were dried over a mixture of sodium carbonate and sodium sulphate (1:1). The solvent was evaporated and the residue was purified by column chromatography (silica gel, diethyl ether/*n*-hexane (1:3)) to yield **16** (41.0 mg, 72.0 μmol, 63 %). R_f = 0.56; ^{1}H NMR ($CDCl_3$): δ = 1.44–1.86 (m, 15H), 1.98 (s, 3H), 2.10–2.47 (m, 4H), 3.22 (m, 1H), 3.27–3.59 (m, 1H), 4.03–4.23 (m, 6H), 5.61 (m, 1H), 5.93 (d, 1H; ^3J = 10.0 Hz), 7.01–7.35 ppm (m, 5H); ^{13}C NMR ($CDCl_3$): δ = 21.7, 24.7, 27.8, 28.0, 36.0, 36.3, 39.3, 54.1, 64.1, 65.3, 78.1, 80.5, 95.4, 104.4, 107.2, 126.1, 127.6, 128.4, 129.0, 132.3, 151.9, 169.1 ppm; MS (CI+, *i*-butane): *m/z*: 568 [M+H^+], 566 [M+H^+], 508, 412, 266; IR (Film): $\tilde{\nu}$ = 3024 (m), 2970 (s), 2852 (s), 1697 (s), 1654 (s), 1580 (m), 1506 (m), 1393 (s), 1254 (s), 1158 (s), 1110 (s), 942 cm^{-1} (m); HRMS (CI+): *m/z* calcd for $C_{27}H_{38}NO_7Se$: 568.1813; found: 568.1817.

(*S*)-4-((*S*)-6-Acetoxy-1,4-dioxa-spiro[4.5]deca-7,9-dien-6-ylmethyl)-2,2-dimethyl-oxazolidine-3-carboxylic acid *tert*-butyl ester (17): Hydrogen peroxide (3.00 mmol, 341 μL of 30 % H_2O_2 in 500 μL water) was added to a stirred solution of **16** (159 mg, 281 μmol) in dichloromethane (2.00 mL) and pyridine (50 μL, 619 μmol). The resulting mixture was stirred at room temperature for 2 hours and subsequently added to a stirred solution of diethyl ether (10 mL), *n*-pentane (40 mL) and saturated sodium hydrogen carbonate solution (50 mL). The organic layer was separated, washed with water (10 mL) and brine (10 mL), dried over magnesium sulphate and filtered. A few crystals of hydroquinone were added, the solvent was evaporated and the residue was purified by column chromatography (silica gel, diethyl ether/*n*-hexane (1:2)) to yield **17** (53.0 mg, 129 μmol, 46 %). R_f = 0.55; ^{1}H NMR ($CDCl_3$): δ = 1.43–1.87 (m, 15H), 2.03 (s, 3H), 2.12–2.31 (m, 2H), 3.30–3.67 (m, 1H), 4.05–4.35 (m, 6H), 5.89 (d, 1H; ^3J = 9.9 Hz), 6.23 (d, 1H; ^3J = 10.1 Hz), 6.75–6.89 (m, 1H), 6.92–7.08 ppm (m, 1H); ^{13}C NMR ($CDCl_3$): δ = 21.4, 24.9, 28.3, 28.8, 36.8, 54.9, 65.0, 66.1, 80.6, 84.1, 96.1, 106.5, 128.8, 132.7, 134.2, 139.2, 152.4, 168.7 ppm; MS (CI+, *i*-butane): *m/z*: 410 [M+H^+], 366, 352, 350, 306, 240; IR (Film): $\tilde{\nu}$ = 3018 (m), 2979 (s), 2854 (s), 1703 (s), 1659 (s), 1253 (s), 1163 (s), 1112 (s), 944 cm^{-1} (m); HRMS (CI+): *m/z* calcd for $C_{21}H_{32}NO_7$: 410.2179; found: 410.2176.

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(*S*)-4-((*S*)-6-Hydroxy-1,4-dioxa-spiro[4.5]deca-7,9-dien-6-ylmethyl)-2,2-dimethyl-oxazolidine-3-carboxylic acid *tert*-butyl ester (18): **17** (26.0 mg, 63.0 μmol) was dissolved in THF (10 mL), and water (10 mL) was added. 1 N NaOH (126 μL, 126 μmol, 200 mol%) was added and the solution was stirred at room temperature for 30 minutes. Afterwards, Amberlite IR 120 was added until pH 5 was reached, then the mixture was filtered and the filtrate was evaporated to dryness to give the pure product **18** (yield = 23.0 mg, 63.0 μmol, quant.): ^{1}H NMR ($CDCl_3$): $\delta = 1.44$–1.86 (m, 15H), 2.06–2.28 (m, 2H), 3.35–3.51 (m, 1H), 4.12–4.43 (m, 6H) 6.07 (d, 1H; ^3J = 9.9 Hz), 6.24 (d, 1H; ^3J = 10.1 Hz), 6.41–6.60 (m, 1H), 6.90–7.03 ppm (m, 1H); ^{13}C NMR ($CDCl_3$): $\delta = 24.7$, 28.0, 28.4, 38.3, 54.1, 63.2, 68.3, 75.2, 80.1, 96.2, 106.2, 126.9, 131.9, 134.5, 138.4, 152.3 ppm; MS (CI+, *i*-butane): *m/z*: 368 [M^+], 350, 324, 310, 248; IR (Film): $\tilde{\nu}$ = 3030 (m), 2945 (s), 2878 (s), 1705 (s), 1689 (s), 1232 (s), 1173 (s), 1116 (s), 934 cm^{-1} (m); HRMS (CI+): *m/z* calcd for $C_{19}H_{30}NO_6$: 368.2073; found: 368.2071.

Biological experiments: For cellular experiments, **14** was dissolved in a dimethylsulfoxide (DMSO) stock solution (10 mM). In all experiments, the DMSO concentration added up to 0.1% for the maximal value. *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl (BODIPY) sphingosyl phosphocholine (BODIPY-SM, BODIPY-FL_{C5}-sphingomyelin, D-3522), 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoic acid (BODIPY-FL_{C5}, D-3834) and YOPRO-1 stain were obtained from Molecular Probes–Invitrogen Detection Technologies (Karlsruhe, Germany). Media, buffers and plasticware for cell culture were obtained from GIBCO Life Technologies (Eggenstein, Germany) and NUNC (Wiesbaden, Germany).

Fluorescently labelled BODIPY-FL_{C5}-sphingomyelin was used in all experiments dealing with labelled SM. All other reagents (PMA, 1,4-dithiothreitol (DTT), TNF-α, IFN-γ, bacterial sphingomyelinase, etc.) were from Sigma–Aldrich (Deisenhofen, Germany) in the highest available quality unless otherwise stated. The protease inhibitor cocktail I from Calbiochem (Bad Soden, Germany) was used.

Minimally modified LDL was prepared as previously described immediately after isolation of LDL from peripheral blood of a pool of at least three healthy volunteers.[3]

Apoptosis assays: Human peripheral blood mononuclear cells (HBMC) were isolated, cultivated and stimulated, and the rate of apoptotic cells was determined by YOPRO-1 staining as recently described.[3] Macrophages (MΦ) were incubated with **14** for 30 minutes prior to mmLDL (27 μg mL^{-1} for 4 h) or TNF-α (10 ng mL^{-1} for 4 h) addition. Apoptosis is expressed as the percentage of YOPRO-1 (2.5 μg mL^{-1}) stained cells relative to Hoechst stained cells.[3] Later, induction of apoptosis in CD14-positive cells was also studied by using flow cytometry. Cells were incubated with **14** for 30 minutes prior to addition of TNF-α (10 ng mL^{-1} for 4 h), washed, and resuspended in Hanks buffered saline solution (HBSS). Subsequently, HBMCs were incubated for 15 minutes with anti-CD14 fluorescein isothiocyanate (FITC; 5μL; BD Bioscience, Heidelberg, Germany). After gating of positive cells, the gate was fixed for the determination of monocytic cells. Analyses were performed with a fluorescence-activated cell sort scan (FACScan) and the CellQuest software (BD Bioscience) by using FL1 channel annexin(V) FITC for 10 minutes and Fl2 for propidium iodide detection for 20 minutes. The number of analysed cells in the CD14-positive gate was 5000. The amount of positive signals in untreated cells was set at about 1%.

Intracellular SM turnover: 1.5×10^6 freshly isolated human monocytes were labelled overnight with BODIPY-SM/BSA complex (1.5 mL; 4 nmol BODIPY-SM in MeOH) prepared with defatted BSA according to Martin and co-workers[28] and diluted in HEPES-buffered minimal essential medium (HMEM; HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; 10 mM, pH 7.4) after dialysis and sterile filtration (final volume = 12 mL). Monolayers were then washed with fresh media, preincubated with **14** as indicated and exposed to mmLDL (27 μg mL^{-1}) for 4 hours. After washing with ice-cold PBS (2×), cellular lipids were extracted by the method of Bligh and Dyer[44] and the organic phase was evaporated in vacuo and dissolved in MeOH. The following HPLC protocol was used in all subsequent experiments: HPLC analysis for separation and quantification was performed on RP-18 columns (LiChroCART 125–4, LiChrospher 100 RP-18 (Merck, Darmstadt, Germany)) with 90:10 MeOH/100 mM tris(hydroxymethyl)aminomethane (Tris; pH 7.5) as the eluent and with a flow rate of 1.85 mL min^{-1}. The turnover rate was determined by calculation of the SM/Cer ratio by monitoring the emission at 505/515 nm (Em/Ex) in the eluent by using a Shimadzu RS-535 detector.

In vitro SMase assay: 1.5×10^6 human monocytes at day 7 after isolation were preincubated with **14** (0.01, 0.1 and 1.0 μM) for 30 minutes at 37°C and then exposed to mmLDL (80 μg mL^{-1}) for 2 hours. The cells were collected by scraping on ice and were centrifuged at 250*g*. After washing of the pellets once with ice-cold PBS, the cells were lysed in nSMase reaction buffer (50 mM Tris (pH 7.4), 10 mM DTT, 10 mM $MgCl_2$, 0.05% Triton X-100)[43] and the sphingolytic activity was determined in a 5.0-μL aliquot by addition of labelled SM at pH 7.4 under nonlimiting substrate conditions. After incubation (37°C for 120 min), the reaction was stopped by freezing in liquid nitrogen. After addition of the internal standard (BODIPY-FL_{C5}), sample lipids were separated by reversed-phase HPLC (RP-18) and the ceramide content determined by 505/515 (Em/Ex) analysis under appropriate conditions. Plain **14** was used as a negative control.

Preincubation assay: 100×10^6 freshly isolated HBMCs were cultivated overnight under standard conditions,[3] washed with ice-cold PBS, lysed in nSMase reaction buffer (400 μL)[45] and centrifuged (10 min, 14000*g*, 4°C), then the sphingolytic activity was determined in 20-μL aliquots of the supernatant after preincubation (37°C for 60 min) of compound **14** at a final concentration of 100 μM. For control experiments, plain DMSO was added prior to incubation. Mg^{2+}-independent activity was assayed by not supplementing Mg^{2+} in the reaction buffer. After addition of labelled substrate, the reaction was continued at 37°C for another 120 minutes and analysed as described above.

Determination of nSMase activity of prokaryotic origin: nSMase of *B. cereus* (1.0 mg mL^{-1}, 24 U mg^{-1} protein) was diluted with reaction buffer (1:10000), preincubated with **14** as indicated and incubated with SM substrate for 15 min at 37°C. The SM/Cer ratio was determined by HPLC (RP-18) separation as described above.

Cytotoxicity: The cytotoxicity of **14** was assayed in the cell-culture media of freshly isolated human monocytes by incubation over 4 orders of magnitude up to 10 μM for 16 hours. In cell-culture supernatant, release of lactate dehydrogenase from the cytosol of damaged cells was determined by using the "cytotoxicity test" of Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions, by comparison of the optical density (OD) produced by using 0.1% Triton X-100 as a positive and untreated control as well as that with DMSO-treated cells as negative control.

PKC-α translocation: For stable transfection, HepG2 cells were seeded in 100-mm culture dishes and grown to 60% confluence in Rosewell Park Memorial Institute (RPMI) medium supplemented

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with 10% foetal bovine serum (FBS). Transfection was performed in OptiMEM medium with Lipofectamin reagent (Life Technologies, Eggenstein, Germany) by using 10 μg of PKC-α–EGFP plasmide (BD Clontech, Heidelberg, Germany) per dish. After 5 hours, cells were washed with culture medium and grown for 2 days. Selection of transfected cells was achieved by addition of the anitbiotic G418 (0.4 mg mL^{-1}). The stable transfected clones were transferred to 25-cm^2 culture flasks. After passaging of the cells for several weeks, the concentration of G418 was reduced to 0.2 mg mL^{-1}.

Transfected HepG2 cells were cultivated in RPMI 1640 medium supplemented with 10% FBS at 37 °C and with 5% CO_2. For Western blot experiments, 8×10^5 HepG2 cells were seeded in 35-mm culture dishes, grown for 2 days in RPMI medium supplemented with 10% FBS and starved in RPMI medium without serum for 2 hours before treatment.

For microscopy, cells were seeded at 1×10^5 cells per 4 wells on cover slips coated with 0.2% gelatine for 6 hours and cultivated for 2 days in RPMI medium supplemented with 10% FBS. Before treatment, cells were starved for 2 hours in RPMI medium without FBS.

Cytosol and membrane fractionation: Stable transfected HepG2 cells as mentioned above were separated into cytosolic and particulate fractions by using an adaptation of a previously published method with minor modifications.[46]

Immediately after treatment with the analogue **14**, the PMA, C_6-Cer or INF-γ cells were washed twice with ice-cold Dulbecco's PBS. Cells were recovered from tissue-culture plates by scraping and suspending in lysis buffer (80 μL) containing a protease inhibitor cocktail (leupeptin, benzamidin, soja trypsin inhibitor, DTT and phenylmethylsulfonyl fluoride (PMSF)). All subsequent procedures were performed at 4 °C. Cells were lysed by sonicating for 2 seconds and incubated for 10 minutes on ice. After centrifugation at 100 000 *g* for 30 minutes, supernatants were collected (cytosolic fraction) and stored at −80 °C. Pellets were resuspended in lysis buffer (45 μL) and centrifuged again at 100 000 *g* for 30 minutes at 4 °C. The supernatants were discarded as a wash fraction to remove residual cytosolic proteins. The remaining pellets were resuspended in lysis buffer (55 μL) supplemented with 0.2% Triton X-100 and sonicated once for 15 seconds. After incubation for 10 minutes on ice, extracts of the particulate fraction were centrifuged at 100 000 *g* for 30 minutes. Supernatants were collected (particulate fraction) and stored at −80 °C. Protein concentrations of both fractions were determined by using Bradford reagent and the albumin fraction V as a standard.

For studying PKC-α translocation by Western blotting, equal amounts of protein (3 μg) from cell extracts were diluted in Roth 4× loading buffer, boiled at 95 °C for 2 minutes and separated on a 7.5% SDS PAGE gel with a stacking gel of 7.1% polyacrylamide. Proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Eschborn, Germany) by using a wet-blot system (BioRad Laboratories, München, Germany). The PVDF membranes were blocked for 8 h at 4 °C in 1% BSA incubation buffer containing 0.05% Tween 20 and were incubated at 4 °C overnight with primary antibody (anti-PKC-α mouse monoclonal antibody; Biomol, Hamburg, Germany) diluted 1:4 000 in incubation buffer with 1% BSA and 0.05% Tween 20. Membranes were washed several times in incubation buffer containing 0.05% Tween 20 and incubated for 1 hour at room temperature with secondary antibody (anti-mouse POD-conjugated antibody; Sigma, Deisenhofen, Germany) diluted 1:4 000 in incubation buffer with 0.05% Tween 20. After extensive washing, membranes were developed by using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Freiburg, Germany). These membranes were stored at 4 °C and stripped according to the manufacturer's protocol for reprobing with anti-α-tubulin monoclonal antibody (Dianova, Hamburg, Germany) to ensure equal amounts of utilised extract. For detection of PARP fragmentation, membranes were incubated after protein transfer with primary antibody (anti-PARP mouse monoclonal antibody; Biomol, Hamburg, Germany; 1:4 000) and extensively washed. Visualisation was performed by using secondary anti-mouse immunoglobulin G (IgG)/POD-conjugated antibody (Sigma; 1:4 000) and the ECL detection system from Amersham Biosciences.

Microscopy: Stable transfected HepG2 cells were treated with PMA, IFN-γ, C_6-Cer and the analogue **14** as indicated, washed with PBS, fixed in 2% paraformaldehyde in PBS for 20 minutes and transferred to a microscope slide coated with Dako fluorescent mounting medium. The slides were observed with a Axioplan-2 imaging microscope (Zeiss, Jena, Germany) with a Zeiss AxioCam attached.

Statistical analyses: Results are expressed as the mean values ± standard error of the mean (SEM) of at least three experiments. Statistical procedures were performed by the impaired Student's t test or the Mann–Whiney test by using the SIMSTAT program (Provalis Research, Montreal, Canada). A p value of 0.05 or less was chosen for statistical significance.

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