Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 711–714

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHESIS AND EVALUATION OF A PHOTOLYZABLE DERIVATIVE OF SPHINGOSINE 1-PHOSPHATE—CAGED SPP

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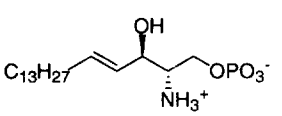
Received 7 January 1998; accepted 11 February 1998

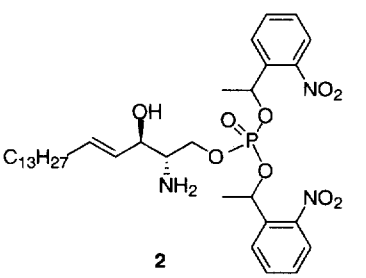
Abstract: The synthesis of a photolyzable sphingosine 1-phosphate derivative is reported via the reaction of *N*-(*tert*-butoxycarbonyl)-2-*N*,3-*O*-isopropylidenesphingosine **7** and bis(α-methyl-*o*-nitrobenzyl) *N*,*N*-diisopropylphosphoramidite. Stimulation of DNA synthesis upon illumination of caged SPP-loaded cells is also described.

Sphingolipid metabolites, such as ceramide, sphingosine and sphingosine-1-phosphate (SPP), are emerging as a novel class of lipid second messengers.[1,2] Recently, Spiegel and coworkers[3–5] have shown that SPP protects cells from apoptosis resulting from elevation of ceramide and proposed that the dynamic balance between levels of ceramide and SPP and consequent regulation of different signaling pathways is an important factor that determines whether a cell survives or dies. Another important downstream effect of SPP is calcium mobilization. In most cell lines tested, SPP-mediated calcium mobilization occurs by an inositol trisphosphate (IP_3)-independent pathway from stores within the endoplasmic reticulum, which also contain the enzyme responsible for the synthesis of SPP, sphingosine kinase.[6,7] Similarly, calcium mobilization resulting from FcεR1 engagement requires activation of sphingosine kinase leading to generation of SPP.[8] In addition to calcium and phosphatidic acid, SPP also regulates levels of another second messenger, cAMP.[9]



1

D-*erythro*-Sphingosine 1-Phosphate



2

Caged D-*erythro*-Sphingosine 1-Phosphate

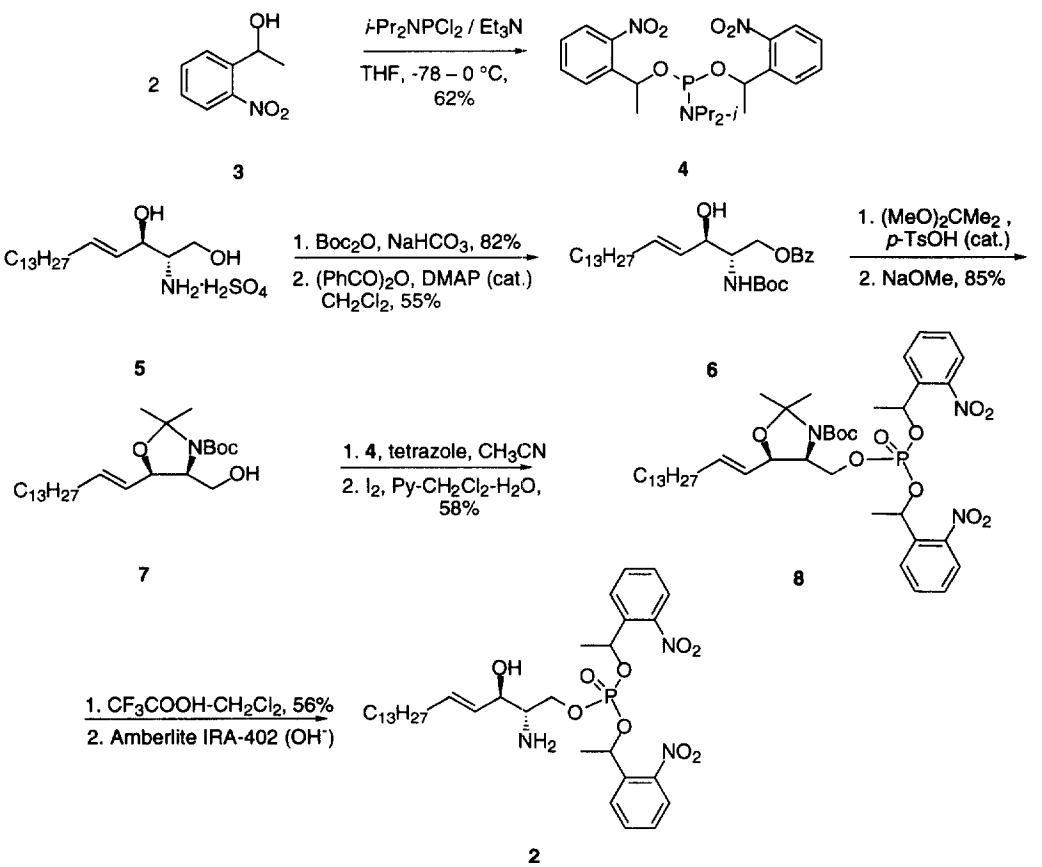
Figure 1

In an effort to learn more about the mechanism by which SPP modulates cell growth and survival and to unravel the signal transduction pathways involved, we synthesized a photolyzable derivative of SPP, termed caged SPP. Caged SPP may allow us to elevate intracellular level of SPP upon ultraviolet irradiation and thus can be utilized as a new tool in studies of SPP-mediated intracellular events. We report herein the synthesis of caged SPP and preliminary studies on its biological activity.

0960-894X/98/$19.00
PII: S0960-894X(98)00112-7

Chemistry

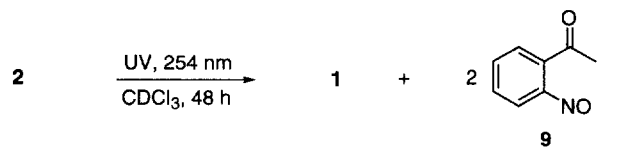
In contrast to the case of caged phosphatidic acid,[10] our initial attempts to make caged SPP by means of alkylation of the ionized phosphate group of SPP with 1-(2-nitrophenyl)diazoethane were unsuccessful because of the poor solubility of SPP in most organic solvents. We then decided to introduce the photolyzable phosphate



Scheme 1

group by a phosphitylation reagent followed by oxidation, a method which has been successfully applied to the synthesis of various inositol phosphates.[11] The requisite phosphitylation reagent **4** was prepared from α-methyl-*o*-nitrobenzyl alcohol **3**[12] with *N,N*-diisopropylphosphoramidous dichloride[13] according to a known procedure (Scheme 1).[14] **3** was employed in this step due to the stability of the *o*-nitrosoacetophenone formed after photorelease.[15] The synthesis of caged SPP started with D-*erythro*-sphingosine sulfate (**5**). First, its amino group was protected as a Boc derivative, and then the primary alcohol was transformed into the corresponding benzoate **6** with benzoic anhydride. Reaction of this intermediate with 2,2-dimethoxypropane and a catalytic amount of *p*-

TsOH in refluxing benzene followed by saponification afforded the alcohol **7** in 85% yield after purification. Coupling of **7** and **2** was carried out in the presence of tetrazole, and the resulting diastereomeric phosphites were then oxidized to the phosphates **8** with I_2 in pyridine-CH_2Cl_2-H_2O 3/1/1 (v/v/v) solution in 58% yield. Subsequent treatment of **8** with trifluoroacetic acid delivered caged SPP trifluoroacetate in 56% yield after column chromatography (SiO_2, ethyl acetate/methanol 95/5 (v/v)). Final removal of trifluoroacetic acid was accomplished by filtration over Amberlite® IRA-402 resin (OH^-).[16]



Scheme 2

The photolysis of caged SPP was observed upon ultraviolet illumination at a wavelength of 254 nm (Scheme 2). The products were purified by TLC and identified as 1-SPP (**1**) and *o*-nitrosoacetophenone (**9**).[17]

Biological Assay

The most well established biological response to SPP is stimulation of proliferation.[4,6,9,18–23] Therefore, we examined the effect of SPP generated intracellularly by photolysis of caged SPP on DNA synthesis.

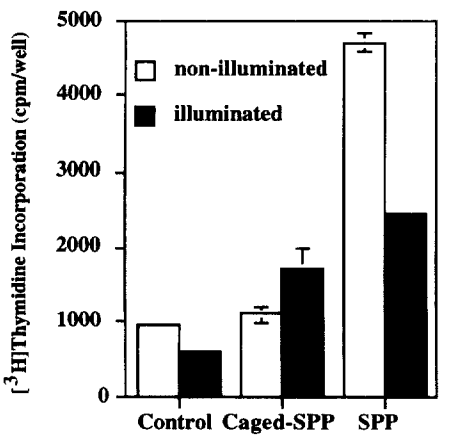


Figure 2. Stimulation of DNA synthesis upon illumination of caged SPP-loaded cells.

Confluent and quiescent cultures of Swiss 3T3 cells were treated with vehicle, caged SPP or SPP (5 μM) in phenol red- and serum-free medium. After 2 h, the medium was removed and plates were washed twice with chemically defined phenol red- and serum-free medium. Caged-SPP was photolyzed with ultraviolet illumination for 30 s (filled bars) while control cells were not illuminated (open bars). Cells were then washed and incubated in DMEM supplemented with 20 μg/mL BSA, 5 μg/mL transferrin and 2 μg/mL insulin for 18 h. Cells were then pulsed with 1 μCi/mL of [^{3}H]thymidine for 6 h and incorporation of [^{3}H]thymidine was measured as described.[6]

Quiescent Swiss 3T3 fibroblasts cultured in chemically defined medium were used for this study since SPP is a very potent mitogen for these cells.[6,18,23] In agreement with previous studies, exogenous SPP (5 μM) markedly stimulated proliferation of quiescent Swiss 3T3 fibroblasts as measured by [^{3}H]thymidine incorporation. Caged SPP had no significant effect on DNA synthesis in non-illuminated cells (Figure 2). However, photolysis of caged SPP-loaded cells caused a 2.8-fold increase in DNA synthesis, which is significant although the effect is smaller than that obtained from exogenously added SPP (4.0-fold). Thus, photolysis of caged SPP can mimic

signal-dependent activation of sphingosine kinase, which is able to experimentally cause very rapid changes in cellular SPP concentrations.

In summary, we have described a route to prepare a photolyzable derivative of SPP. The introduction of caged SPP into cultured cells is a novel approach that should allow rapid and controlled elevation of intracellular SPP levels and eliminate effects mediated by cell surface receptors or caused by complications of uptake.

Acknowledgment. This work was supported by the Department of Defense Research Grant (DAMD17-93-V-3018) to A.P.K. and by Research Grants from the National Institutes of Health (GM43880 and CA61774) to S.S., and by a grant from the American Cancer Society (BE-275) to S.S. We also wish to thank Fidia Pharmaceuticals of Abano Terme, Italy for a generous gift of D-sphingosine sulfate and Margaret E. Schott in the Lombardi Cancer Center of Georgetown University for recording the electrospray mass spectra.

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17. *o*-Nitrosoacetophenone: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.11 (d, 1H, J = 7.5 Hz), 7.73 (t, 1H, J = 7.5 Hz), 7.61 (t, 1H, J = 7.5 Hz), 7.44 (d, 1H, J = 7.5 Hz), 2.56 (s, 3H); MS *m/z* 150 ($M + H^+$, 83%), 135, 123, 43 (100%).
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