Design and Synthesis of a Potent and Selective Peptidomimetic Inhibitor of Caspase-3

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Abstract: In this paper we report the synthesis and characterization of a novel potent and selective inhibitor of caspase-3, a member of the caspase family of cysteine proteases which plays an important role in many human disorders. This molecule represents 3(*S*)-acetylamino-*N*-{1-[(((3*S*)-2-hydroxy-5-oxo-tetrahydrofuran-3-yl)carbamoyl)methyl]-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl}succinamic acid, a monocyclic conformationally constrained form of the tetrapeptide Ac-DEVD-H, in which a 1,4-benzodiazepine nucleus is introduced internally to the peptidic sequence.

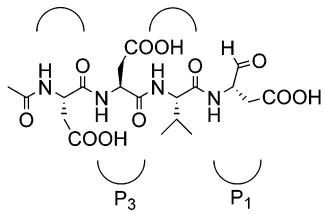
The caspase (*c*ysteinyl-*asp*artate prote*ase*) family represents a class of intracellular proteases playing a critical role in apoptotic cell death pathways and activation of pro-inflammatory cytokines.[1] Their enzymatic properties are governed by a nearly absolute specificity for substrates containing aspartic acid at the P_1 site and by the use of a cysteine side-chain for peptide-bond hydrolysis.[2]

To date, 11 human caspases have been identified.[3] Among them, caspase-3 plays a role of the major executioner in cell death triggered by a variety of pro-apoptotic stimuli.[1c,4]

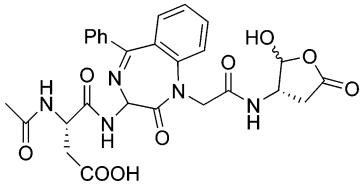
Since activation of caspase-3-dependent apoptotic cell death has been implicated in the etiology of many harmful human disorders, such as immunodeficiency, Alzheimer's, Parkinson's, and Huntington's diseases, as well as ischemia, brain trauma, and amyotrophic lateral sclerosis, inhibition of this caspase is believed to be a valuable therapeutic approach.[3d,5]

Previous peptidomimetic studies[6] resulted in identification of a semispecific inhibitor of caspase-3. This inhibitor is the tetrapeptide Ac-Asp-Glu-Val-Asp-CHO[3a] (Ac-DEVD-H, Chart 1) with an aldehyde functional group that reversibly forms a covalent bond with the thiol of the active-site cysteine, thereby inactivating it.[7] While the Asp residue at P_1 is strictly required for activity, the Asp at P_4 represents the most critical determinant of the inhibitor's specificity.[3a] Liberal substitution is tolerated at P_2 without loss of potency. The P_2 amide nitrogen is not utilized in a hydrogen bonding interaction with the enzyme,[8] unlike the P_1 and P_3 amido hydrogens, which are best retained for high-affinity binding.[7]

Chart 1



Ac-DEVD-H



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Furthermore, one of the most striking features in the design of peptidomimetics is the incorporation of non-peptidic scaffolds into the amino acidic sequences with the perspective to force peptides into bioactive conformations, enhance stability toward degradation by enzymes, and improve biological selectivity.[9]

Taking into account these considerations, we synthesized a monocyclic conformationally constrained analogue of Ac-DEVD-H in which the P_2 amide nitrogen has been "tied back" to the P_3 side chain with the purpose to improve the selectivity for caspase-3, analogously to what was observed by other authors.[10] In this work, by using a 3-amino-2,3-dihydro-2-oxo-5-phenyl-1*H*-benzo[*e*][1,4]diazepin-1-acetic acid moiety[11] as a P_3−P_2 dipeptide replacement, a novel, potent, and specific inhibitor **16** of caspase-3 has been discovered (Chart 1).

The strategy adopted by us to obtain this compound required the construction of the peptidomimetic frame containing the diazepine ring (**8**), and subsequent in situ coupling with the aspartyl aldehyde building block (**13**), followed by coupling with the *N*-Ac-aspartic acid at the P_4 site.

The synthesis of the 5-phenyl-1,4-benzodiazepine ring system was accomplished according to a well-known sequence of reactions shown in Scheme 1 by condensation of 2-aminobenzophenone (**1**) and *N*-(benzyloxycarbonyl)-2-(propylthio)glycine (**2**),[12] a protected α-aminoglycine equivalent, which in turn was obtained in two steps from glyoxylic acid, benzyl carbamate, and 1-propanethiol.[13]

The building block **2** thus formed was converted to its mixed anhydride with isobutyl chloroformate in the presence of *N*-methylmorpholine and reacted in situ with 2-aminobenzophenone (**1**) to give the amide **3** in good yield after extractive workup. In the critical step, the (propylthio)glycinamide **3** was dissolved in dry tetrahydrofuran, and the solution was cooled to 0 °C and saturated with ammonia. Mercuric chloride was then added in one portion to the stirred mixture, while a continuous stream of ammonia gas was bubbled

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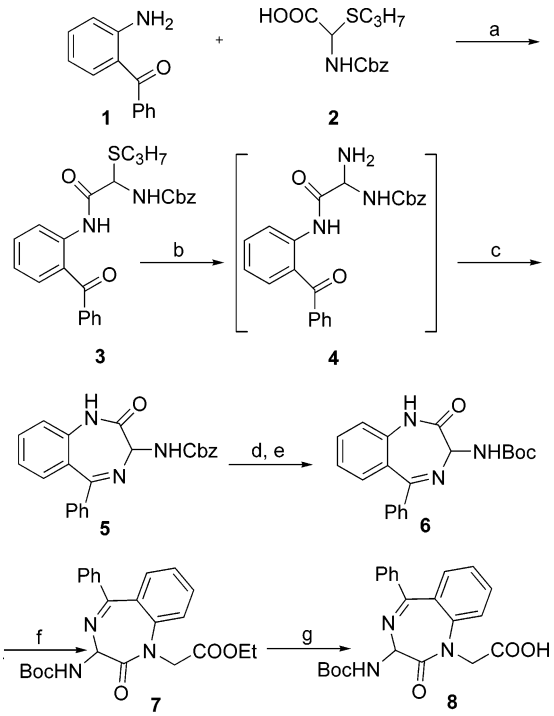
Scheme 1[a]

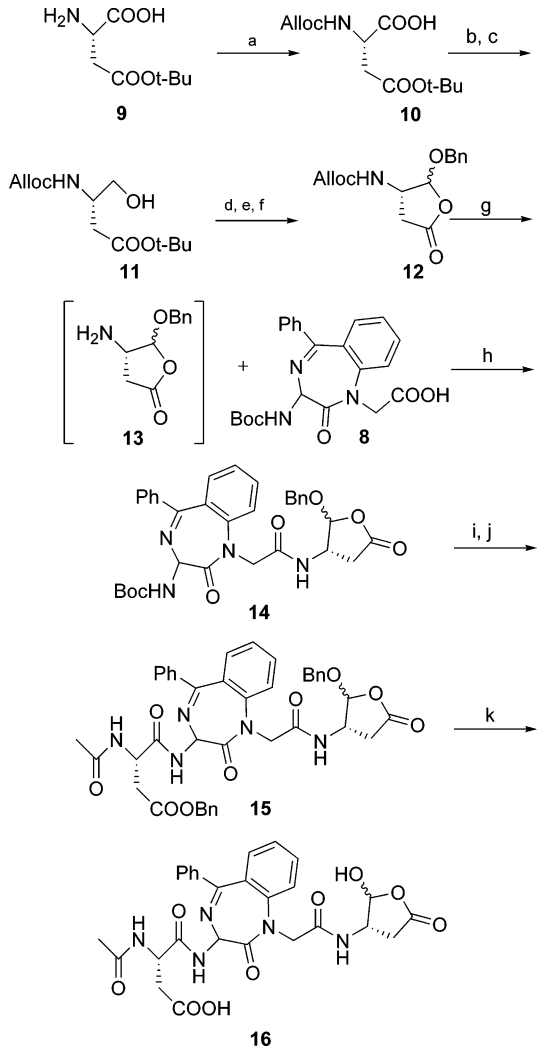


[a] Reagents: (a) *i*-BuOCOCl, *N*-methylmorpholine, CH_2Cl_2, 0 °C, 15 min, then 0 °C − rt, 12 h. (b) NH_3 (g), THF, 0 °C − rt, 3 h. (c) NH_4OAc, HOAc, rt, 12 h. (d) Et_3SiH, $Pd(OAc)_2$, CH_2Cl_2, rt, 3 h. (e) $(Boc)_2O$, CH_2Cl_2, rt, 2 h. (f) NaH, DMF, 0 °C, 30 min, then $BrCH_2COOEt$, 0 °C − rt, 4 h. (g) 1 N LiOH, MeOH, 0 °C − rt, 6 h.

into it. The intermediate α-aminoglycinamide **4** was obtained in essentially quantitative yield and immediately treated with ammonium acetate in glacial acetic acid with stirring at room-temperature overnight to allow the cyclization. In this way, **5** was obtained as a crystalline solid in 70−75% yield. Deprotection of the NH-Cbz group of **5** was carried out with triethylsilane and palladium acetate as catalyst.[14] Quenching with a solution of aqueous ammonium chloride and brine followed by extraction of the reaction mixture removed the excess of silane reagent and provided the crude amine which was reacted with 1.25 equiv of $(Boc)_2O$ without further purification to give **6** in good yield. The side chain at N-1 was introduced by treatment with sodium hydride and ethyl bromoacetate in dimethylformamide at ambient temperature to provide **7** after chromatographic purification. Saponification of the ester group with 1 N LiOH in methanol afforded the dipeptidic N-protected intermediate **8** which is equivalent to the P_3-P_2 sites of Ac-DEVD-H.

The building block **12** was synthesized as described in Scheme 2. First, commercially available aspartic acid *β-tert*-butyl ester **9** was N-protected with allyl chloroformate in the presence of sodium bicarbonate. The product **10** was then converted to a mixed anhydride with isobutyl chloroformate and *N*-methylmorpholine, which was reduced to the corresponding alcohol **11** with sodium borohydride. The alcohol **11** was oxidized to the aldehyde under Swern conditions,[15] and the latter immediately protected with benzyl alcohol and *p*-toluenesulfonic acid in the presence of 3 Å molecular sieves. Trifluoroacetic acid (TFA)-promoted deprotection of the *tert*-butyl ester moiety was accompanied by cyclization

Scheme 2[a]



[a] Reagents: (a) Allyl chloroformate, aq $NaHCO_3$/THF (8/2), rt, 12 h; (b) *i*-BuOCOCl, *N*-methylmorpholine, CH_2Cl_2, 0 °C, 30 min; (c) $NaBH_4$, MeOH/THF (5/1), −78 °C, 4 h; (d) $(COCl)_2$, DMSO, *i*-Pr_2NEt, −65 °C, 1 h; (e) BnOH, TsOH, 3 Å mol. sieves, CH_2Cl_2, rt, 16 h; (f) CF_3CO_2H, CH_2Cl_2, rt, 1 h; (g) $PdCl_2(PPh_3)_2$, *n*-Bu_3SnH, CH_2Cl_2, 0 °C, 30 min; (h) EDCI, HOBt, CH_2Cl_2, rt, 15 h; (i) CF_3CO_2H, CH_2Cl_2, rt, 4 h; (j) Ac−Asp(OBn)-OH, EDCI, HOBt, CH_2Cl_2, rt, 15 h; (k) H_2, $Pd(OH)_2$/C, EtOH, 6 h.

to the desired acylal **12**, which was isolated as a 1:1 mixture of diastereomers.

Cleavage of the *N*-allyloxycarbonyl (Alloc) group[16] gave the intermediate **13** which, being volatile and sensitive to acid and base,[17] was not isolated and was in situ coupled to **8** for an efficient peptide elongation. To incorporate the last aspartate residue at the P_4 position, the Boc group in **14** was first removed by TFA, and the resulting amine was coupled to *N*-acetylaspartic acid *β*-benzyl ester by the procedure reported above. Hydrogenolysis of the tetrapeptide **15** using $Pd(OH)_2$/C as catalyst in ethanol provided the final 3(*S*)-acetylamino-*N*-{1-[(((3*S*)-2-hydroxy-5-oxo-tetrahydrofuran-3-yl)carbamoyl)methyl]-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl}succinamic acid **16** in a reasonable yield.

Potency of the synthesized compound in the inhibition of caspase-3 was tested in vitro using the pure recombinant active human enzyme expressed in *E. coli*. Briefly, 2 Units of caspase-3 was added to the reaction

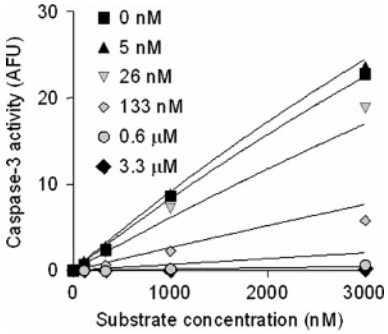


Figure 1. Determination of the inhibitory potency of compound **16** against recombinant human caspase-3. Dissociation constant (K_i) was calculated using the *Graphpad Prism 4* computer program.

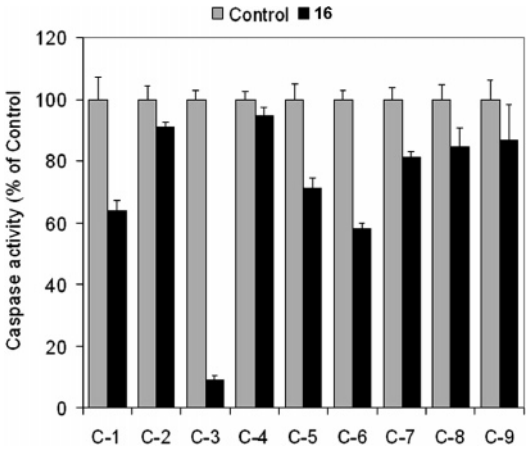


Figure 2. Determination of the inhibitory specificity of compound **16**. Two Units of each recombinant caspase were incubated with their specific substrates in the absence or presence of 210 nM compound **16**. Ten μM concentrations of each Ac-YVAD-AMC for caspase-1, Ac-VDVAD-AMC for caspase-2, Ac-DEVD-AMC for caspases-3 and −7, Ac-LEVD-AMC for caspase-4, Ac-WEHD-AMC for caspase-5, Ac-VEID-AMC for caspase-6, Ac-IETD-AMC for caspase-8, and Ac-LEHD-AMC for caspase-9 were used to analyze activity of the caspases in the fluorometric assay by measuring the accumulation of free AMC. Caspase activities in the presence of compound **16** are expressed as percent of those in the absence of the inhibitor.

mixture containing increasing from 0 to 3 μM concentrations of a semispecific Z-DEVD-AMC substrate and increasing from 0 to 3.3 μM concentrations of compound **16**. Caspase-3 activity results in the cleavage of Z-DEVD-AMC after Asp at P_1 site, thus releasing the fluorescent AMC group. Hence, fluorescence of AMC in a sample reflects actual activity of the enzyme. We performed measurements of AMC liberation as a function of time using a fluorescent plate reader as describe earlier.[3a] Results were expressed as mean values of three independent measurements per each experimental point. A value for the dissociation constant K_i was computed by fit of the reaction rates to the Michaelis−Menten equation. Thus, K_i for **16** was estimated to be as low as 36±9 nM at 95% confidence intervals (Figure 1). Parallel experiments demonstrated that the K_i for Ac-DEVD-CHO, a common tetrapeptide inhibitor of caspases-3 and −7, was equal to 3.2 ± 6 nM under the same experimental conditions (data not shown).

Specificity of compound **16** was estimated in the reactions with a panel of active recombinant human

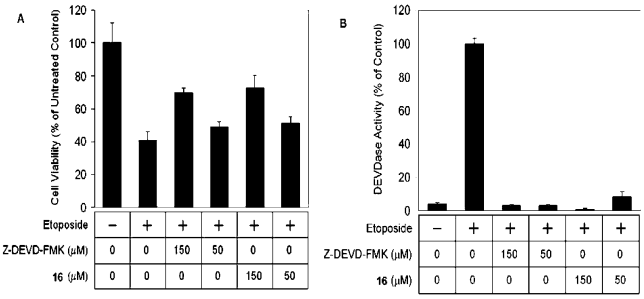


Figure 3. Protective effect of **16** in a model of etoposide-induced apoptosis in SH-SY5Y cells. Apoptosis was induced by addition of 10 μM etoposide to cell cultures. (A) After 24 h of treatment in the absence or presence of indicated concentrations of caspase inhibitors, cells were stained with 5 μM calcein AM, which is deesterified into a fluorescent product by viable cells. Data are represented as the percent-to-control average number of fluorescent cells + STDEV ($n = 4$). (B) Caspase-3-like activity was measured in situ after 6 h of treatment with etoposide. Cleavage of Ac-DEVD-AMC was assayed fluorometrically by measuring the accumulation of free AMC.

caspases-1−9 and the specific substrates for each individual caspase. In these experiments we used 210 nM of the inhibitor, a concentration approximately 6 times higher than the estimated K_i value for caspase-3 inhibition. Control reactions included caspases with their specific substrates in the absence of **16**. Results that are summarized in Figure 2 demonstrate that **16** most potently inhibits caspase-3. Activity of this caspase in the presence of **16** was reduced to 9 ± 2% of the control level, whereas activity of other 8 caspases remained essentially higher, from 58 ± 2% for caspase-6 to 95 ± 4% for caspase-4. Overall, these data demonstrate marked selectivity of the developed compound against human caspase-3.

Relatively poor membrane permeability of the described earlier caspase inhibitors limits possibility of their use as anti-apoptotic drugs. Therefore, our essential goal was to develop a cell permeable compound. Cell permeability of **16** was indirectly assessed by measuring caspase-3 activity and viability of SH-SY5Y human neuroblastoma cells treated with 10 μM etoposide. Z-DEVD-FMK was used as a positive control in parallel to **16**. Results indicate that both compounds demonstrate similar levels of protection, as shown at the level of caspase-3 activity and cell viability (Figure 3). A fact that compound **16** is 10 times less potent than Z-DEVD-FMK and represents a reversible inhibitor, while Z-DEVD-FMK is irreversible, may indicate that compound **16** demonstrates higher cell permeability.

Taken together, we synthesized a conformationally constrained surrogate of the caspase-3 cleavage site DEVD, in which the dipeptide Val-Glu was replaced with a 1,4-benzodiazepine moiety. The results obtained demonstrate that, while the inhibitory activity (K_i) of this new compound is somewhat lower than that of the commonly used tetrapeptide, its selectivity for caspase-3 and the ability to inhibit apoptosis in live cells make it an attractive target for further development.

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Supporting Information Available: Experimental procedures, characterization of new compounds, and references to known procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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