REACTION OF ACYL CARBAZATES WITH PROTEOLYTIC ENZYMES

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SUMMARY

Acyl carbazates (1) with appropriate substituents are analogs of acyl amino acids in which the α-methine group has been replaced by a nitrogen atom. The synthesis of three new carbazates Ac-Ala-Bzc-ONp, Ac-Ala-Mec-ONp and Z-Ala-Ala-Pro-Mec-ONp(Bzc= -NHN($CH_2C_6H_5$)CO-, Mec= -NHN(CH_3)CO-) was accomplished. The reaction of chymotrypsin, subtilisin BPN', and elastase(porcine and human leukocyte) with the carbazates resulted either in a spectrophotometric burst of p-nitrophenol or no reaction depending on the specificity of the enzyme. Carbazyl chymotrypsin A_α crystals were isomorphous with the native protein. Acyl carbazates should find utility as inhibitors and active site titrants for serine proteases.

Carbazates (1) with the appropriate substituents are analogs of amino acids in which the α-methine group has been replaced by a nitrogen atom. They would thus be expected to acylate a serine protease with the appropriate specificity in much the same fashion as simple synthetic peptide substrates. The acylated enzymes (carbazyl enzymes) should be considerably more stable toward deacylation than a normal "acyl enzyme" due to the influence of the adjacent nitrogen atom. A similar derivative, Ser-195 carbamyl chymotrypsin is completely stable toward deacylation (1). Carbazyl derivatives of serine proteases should thus be suitable for crystallographic study of the structure of the "acyl enzyme" intermediate in the mechanism of peptide bond hydrolysis catalyzed by this class of enzymes.

We now report the synthesis of several new peptide carbazates and investigation of their reaction with four serine proteases. Previous workers have shown that ethyl 3-acetyl-2-benzyl carbazate was a predominately competitive inhibitor of chymotrypsin while the corresponding p-nitrophenyl ester (2) can be used as an active site titrant for chymotrypsin and trypsin (2). In

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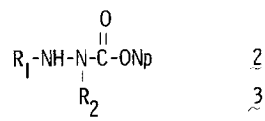

	R_1	R_2
2	Ac	Bz
3	Ac-Ala	Bz
4	Ac-Ala	Me
5	Z-Ala-Ala-Pro	Me

addition, a peptide inhibitor of renin containing a carbazyl residue has been synthesized (4)

Coupling of Ac-Ala-OH† with benzylhydrazine using the mixed anhydride method method (5) gave a mixture of Ac-Ala-NHNHBz (6, 27%, m.p. 157.5 - 159°, pmr (DMSO-d_6):N-CH_2- 3.82δ) and the undesired isomeric hydrazide Ac-Ala-NH(Bz)NH_2 (13%, oil, pmr ($CDCl_3$):N-CH_2-4.62δ) which were separated by silica gel chromatography. The desired isomer (6) was independently synthesized by reduction ($NaBH_4$, EtOH) of the benzylidene derivative Ac-Ala-NHN=CHC_6H_5 (m.p. 198.5 - 200.5°) which was prepared from Ac-Ala-$NHNH_2$(6). Reaction of p-nitrophenyl chloroformate with 6 in $CHCl_3$ containing $(i\text{-Pr})_2$NEt gave p-nitrophenyl 3- (N-acetyl-L-alanyl)-2-benzyl carbazate 3 (38%, m.p. 148°(d), pmr ($CDCl_3$):N-CH_2- 4.82δ. Coupling of Ac-Ala-OH with t-butyl 2-methylcarbazate(7) gave t-butyl 3-(N-acetyl-L-alanyl)-2-methyl-carbazate (38% m.p. 175-177°, pmr ($CDCl_3$):N-CH_3 3.07δ). Deblocking with CF_3-CO_2H followed by reaction with p-nitrophenyl chloroformate ($(i\text{-Pr})_2$NEt in $CHCl_3$) gave p-nitrophenyl 3-(N-acetyl-L-alanyl)-2-methylcarbazate 4 (51%, m.p. 162-167(d), pmr (CF_3COCF_3·1.6 D_2O):N-CH_3 3.38δ). Coupling of Z-Pro-OH (as the dicyclohexylamine salt)(8) with t-butyl 2-methylcarbazate followed by hydrogenolysis (5% Pd on C, 1 atm) and addition of methanolic HCl yielded HCl·Pro-NHN$(CH_3)CO_2C(CH_3)_3$ (76%, m.p. 185.5(d), pmr (D_2O):N-CH_3 3.05δ). Coupling with Z-Ala-Ala-OH by either a mixed anhydride or dicyclohexylcarbodiimide method yielded, after silica gel chromatography, Z-Ala-Ala-Pro-NHN$(CH_3)CO_2C(CH_3)_3$ (82%, oil). Deblocking with CF_3CO_2H followed by reaction with p-nitrophenyl chloroformate ($(i\text{-Pr})_2$NEt in $CHCl_3$) and silica gel chromatography

†Standard abbreviations are used for amino acid derivatives and peptide except for Bzc (2-benzylcarbazic acid) and Mec (2-methylcarbazic acid).

yielded p-nitrophenyl 3-(N-benzyloxycarbonyl-L-alanyl-L-alanyl-L-prolyl)-2-methylcarbazate 5 (38%, m.p. 104-108°, pmr ($CDCl_3$):N-CH_3 3.03δ).

The reactions of the acyl carbazates were studied spectrophotometrically at 347.5 nm. Typically carbazate in acetonitrile was added to 0.5 - 2.0 ml of the appropriate buffer solution. After measuring the slow rate of carbazate hydrolysis, 50 - 500 µg of enzyme in buffer was added. The final reaction mixture contained 5 or 10% acetonitrile and a 24 to 1000 fold excess of carbazate over enzyme. The kinetics were treated according to the following scheme where E·S is the enzyme carbazate complex, E-S is the carbazyl enzyme, HONp is p-nitrophenol and P is product (9).

$$E+S \underset{}{\overset{K_S}{\rightleftharpoons}} E\,S \xrightarrow{K_2} E\text{-}S + HONp \xrightarrow{K_3} E+P$$

In the majority of the cases where reaction was observed between the enzyme and carbazate, a burst of p-nitrophenol occurred followed by an extremely slow (if any) turnover of the carbazyl enzyme. The results are listed in Table I.

The reactivity of the serine proteases toward the four carbazates studied is in general that expected on the basis of the substrate specificity of the individual enzymes. Elastase only reacts with the methyl carbazate Z-Ala-Ala-Pro-Mec-ONp(5) and not with the two benzyl carbazates or the dipeptide carbazate Ac-Ala-Mec-ONp(4). Elastase has been shown to require an extended peptide chain and a small amino acid residue (e.g. Ala) as the P_1 residue(10) in a substrate or inhibitor(11). Subtilisin BPN' which has a rather broad substrate specificity, reacts with all of the carbazates. Chymotrypsin A_γ as expected reacted rapidly with both of the benzyl carbazates (2 and 3), however it was also inactivated, although more slowly, by the methylcarbazate (5). Possibly the binding of the extended peptide chain of 5 to the extended substrate binding region of chymotrypsin A(12) overpowers the lack of an aromatic side chain in the P_1 residue. This would also account for the lack of reactivity of the dipeptide carbazate 4.

The reaction of Ac-Ala-Bzc-ONp(3) with either chymotrypsin A_γ,A_α or subti-

TABLE I. Reaction of Acyl Carbazates With Serine Proteases

		Ac-Bzc-ONp		Ac-Ala-Bzc-ONp		Ac-Ala-Mec-ONp		Z-Ala-Ala-Pro-MecONp	
Enzyme	pH	k_{ac}[a] (sec^{-1})	k_{cat}[b] (sec^{-1})	k_{ac} (sec^{-1})	k_{cat} (sec^{-1})	k_{ac} (sec^{-1})	k_{cat} (sec^{-1})	k_{ac} (sec^{-1})	k_{cat} (sec^{-1})
chymotrypsin A_γ	5.0[c]	0.20	slow[d]	fast[e]	slow[d]	0	-	0.056	slow[d]
	5.8[g]	fast[e]	-	fast[e]	2×10^{-6}[f]	0	-	fast[e]	-
subtilisin BPN'	5.0[c]	0.03	4.6×10^{-5}	fast[e]	7.2×10^{-5}	0.27	6.5×10^{-4}	fast[e]	6.1×10^{-3}
	5.8[g]	fast[e]	-	fast[e]	3.1×10^{-3}	fast[e]	-	fast[e]	-
porcine elastase	5.0[c]	0	-	0	-	0	-	fast[e]	1.1×10^{-3}
	5.8[h]	0	-	0	-	0	-	fast[e]	-
human leukocyte elastase	6.5[e]	0	-	0	-	0	-	fast[i]	-
trypsin	5.8			0	-				

[a] $k_{ac} = ((k_2 + k_3)[S]_o + k_3K_s) / (K_s + [S]_o)$ which is equal to k_2 in the cases where $k_2 > k_3$ and $[S]_o >> K_M$. The first condition is probably met in most of the experiments.

[b] $k_{cat} = k_2k_3/(k_2 + k_3)$ which is equal to k_3 when $k_2 > k_3$.

[c] 0.1M acetate, 5% acetonitrile.

[d] Slow indicates that little or no turnover of the carbazoyl enzyme was observed in 15-30 min., i.e. k_{cat} <ca. 10^{-4} sec^{-1}.

[e] Fast indicated the burst of p-nitrophenol was > 90% complete in the 9 sec. it took to mix enzyme and carbazate, i.e. k_{ac} > ca. 0.2 sec^{-1}.

[f] This is a true deacylation rate (k_3) which was obtained by measuring the recovery of enzyme activity of the inactivated chymotrypsin after removal of the excess carbazate by gel filtration.

[g] 0.1M citrate, 5% acetonitrile.

[h] 0.1M phosphate, 5% acetonitrile.

[i] Due to the small quantity of enzyme available, this experiment was performed by the addition of carbazate to a solution of enzyme and Boc-Ala-ONp and observing the rate of p-nitrophenol release.

lisin BPN, which had been titrated with 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone[9c], was shown to give a stoichiometric burst of p-nitrophenol. The carbazyl derivative of chymotrypsin A_γ was isolated by quenching the reaction mixture to pH 3 followed by gel filtration on Sephadex G-25. Regeneration of chymotryptic activity was followed using an Ac-Tyr-OEt assay and deacylation rates (k_3) at 26.0°C of 2 x 10^{-6} sec^{-1} (pH 5.8) and 2 x 10^{-5} sec^{-1} (pH 7.45) were obtained. The observation that $K_2 >> k_3$ in many of the reactions, shows that acyl carbazates can be utilized as extremely effective and sometimes specific inhibitors of serine proteases. In addition several of the carbazates should find utility as active site titrants for serine proteases and we are currently performing the appropriate kinetic experiments to define their usefulness.

Reaction of crystals of chymotrypsin A_γ with Ac-Ala-Bzc-ONp(3) yielded inactivated crystals with unit cell dimension a=b=69.61 A° and c=97.70 A° (native[13]: a=b=69.6, c=97.7). A crystallographic investigation of this derivative is in progress.

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