Potent and selective inactivation of cysteine proteinases with
N-peptidyl-O-acyl hydroxylamines

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A series of N-peptidyl-O-acyl hydroxylamines was synthesized and tested as inactivators of cysteine proteinases. Depending on the structure of the peptidyl residue of the inhibitors, rapid and complete irreversible inactivation of the lysosomal cathepsins, B, L and S, may be achieved. The most effective inhibitors display second-order rate constants of the inactivation in the range 10^2–10^6 M^{-1}·s^{-1}. By contrast, the activity of the aminopeptidase cathepsin H is only negligibly affected by the N-terminal-protected peptidyl inhibitors.

INTRODUCTION

The lysosomal cathepsins B, H, L and S are well characterized cysteine proteinases and play an important role in intracellular protein breakdown (Kirschke & Barrett, 1987). Furthermore, it has been shown previously that cathepsins B and L are involved in the degradation of extracellular-matrix proteins such as collagen and elastin (Burleigh et al., 1974; Kirschke et al., 1982; Mason et al., 1986) and may take part in osteoporosis (Delaisé et al., 1984), pulmonary emphysema (Mason, 1988) and tumour invasion (Denhardt et al., 1987).

The design of specific irreversible inhibitors could therefore be a useful contribution in studying the role of these proteinases in disease.

Favoured inhibitors should be specific for one type of proteinase and should not induce non-specific side reactions. This goal may be reached by using enzyme-activated inhibitors that generate their inhibiting power only during the catalytic action of the target enzyme.

Searching for new inactivators of dipeptidyl peptidase IV (DP IV, EC 3.4.15.5) we developed N-peptidyl-O-acyl hydroxylamines as potential mechanism-based inhibitors of serine and cysteine proteinases (Fischer et al., 1982, 1983; Demuth et al., 1988, 1989a,b). Diacyl hydroxylamine derivatives (R₂-CO-NH-O-CO-R₁) permit variations of the N-acetyl and O-acyl residues and thus a selective control of their affinity and reactivity towards the enzymes.

Using DP IV as model enzyme we studied the inactivation reactions, enzyme-catalysed turnover and spontaneous degradation of a series of substrate-analogous N-Xaa-Pro-O-benzoyl hydroxylamines (Demuth et al., 1988). The inhibitors have been successfully used in biological investigations to evaluate the participation of DP IV in human T-lymphocyte activation (Schön et al., 1984, 1987). Recently, Smith et al. (1988) have demonstrated the potential of this new inhibitor class to inactivate the cysteine proteinase cathepsin B.

In the present paper we report the rapid and selective inactivation, by several N-peptidyl-O-acyl hydroxylamines, of several lysosomal cysteine proteinases exhibiting different substrate specificities.

EXPERIMENTAL

Enzymes

Cathepsin B (EC 3.4.22.1), cathepsin L (EC 3.4.22.15) and cathepsin H (EC 3.4.22.16) were prepared from the lysosomal fraction of rat liver as described by Kirschke et al. (1977) and Barrett & Kirschke (1981). Bovine spleen cathepsin S (EC 3.4.22.-) was isolated as described by Kirschke et al. (1986). All cathepsins used were in an electropheretically homogeneous form.

Substrates and inhibitors

Z-Phe-Arg-NHMec, Arg-NHMec·HCl and Z-Val-Arg-NHMec were synthesized as in Brömme et al. (1989a,b).

N-Peptidyl-O-acyl hydroxylamines have been used from previous experiments or synthesized as described (Fischer et al., 1983; Demuth et al., 1988, 1989a). Boc- and Z-protected peptidyl methyl esters were built up according to standard procedures. Treatment of the ester with hydroxylamine in sodium methanolate solution gave the appropriate hydroxamic acids. Acylation using acid chlorides in dry tetrahydrofuran or in Schotten-Baumann reactions with N-methylmorpholine or triethylamine as base resulted the diacyl hydroxylamines. Yields of the final reaction step were between 60 and 85%. Final products, usually crystallized from ethanol/ethyl acetate or ethyl acetate/light petroleum (b.p. 30–60 °C), gave correct elemental (C,H,N) analyses, single spots on t.l.c. and were partially characterized by 13C-
Table 1. N-Peptidyl-O-acyl hydroxylamines: analytical parameters

Under ‘M.p. (°C)’, (D) means that the compound decomposes. Under ‘Elemental analysis’, ‘F’ means ‘found’ and ‘R’ means ‘requires’.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( M_r )</th>
<th>Formula</th>
<th>M.p. (°C)</th>
<th>Elemental analysis (( % ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boc-Phe-Gly-NHO-Bz</td>
<td>441.48</td>
<td>( C_{23}H_{21}N_5O_6 )</td>
<td>118-119</td>
<td>F: 62.26 R: 61.5 C: 8.80</td>
</tr>
<tr>
<td>Z-Phe-Phe-NHO-Bz</td>
<td>565.63</td>
<td>( C_{24}H_{21}N_6O_6 )</td>
<td>192-194</td>
<td>F: 69.54 R: 5.96 C: 7.51</td>
</tr>
<tr>
<td>Z-Phe-Phe-NHO-Ma</td>
<td>530.60</td>
<td>( C_{29}H_{24}N_6O_6 )</td>
<td>180-182</td>
<td>F: 66.24 R: 5.5 C: 7.43</td>
</tr>
<tr>
<td>Boc-Phe-Pro-Ala-NHO-Nbz</td>
<td>597.62</td>
<td>( C_{29}H_{33}N_5O_6 )</td>
<td>111-113</td>
<td>F: 57.43 R: 5.9 C: 11.09</td>
</tr>
<tr>
<td>Boc-Ala-Phe-Leu-NHO-Nbz</td>
<td>582.65</td>
<td>( C_{29}H_{38}N_6O_6 )</td>
<td>115 (D)</td>
<td>F: 59.47 R: 5.9 C: 10.28</td>
</tr>
<tr>
<td>Boc-Gly-Phe-Phe-NHO-Nbz</td>
<td>633.66</td>
<td>( C_{29}H_{39}N_6O_6 )</td>
<td>128-129</td>
<td>F: 61.84 R: 5.7 C: 9.62</td>
</tr>
</tbody>
</table>

Table 2. Spontaneous degradation of N-peptidyl-O-acyl hydroxylamines in aqueous solution

\( t_1 \) is the half-life. The standard deviation (s.d.) is given as a percentage value and is the mean of three experiments. For further details, see the Experimental section.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( t_1 ) (min)</th>
<th>( k ) (min(^{-1}))</th>
<th>s.d. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Boc-Ala-Pro-NHO-Nbz</td>
<td>209</td>
<td>0.0032</td>
<td>2.5</td>
</tr>
<tr>
<td>2 Boc-Ala-Phe-NHO-Nbz</td>
<td>178</td>
<td>0.0039</td>
<td>12.9</td>
</tr>
<tr>
<td>3 Boc-Phe-Ala-NHO-Nbz</td>
<td>365</td>
<td>0.0019</td>
<td>12.1</td>
</tr>
<tr>
<td>4 Boc-Phe-Gly-NHO-Ma</td>
<td>900</td>
<td>0.00077</td>
<td>5.9</td>
</tr>
<tr>
<td>5 Z-Phe-Phe-NHO-Bz</td>
<td>16</td>
<td>0.0439</td>
<td>2.2</td>
</tr>
<tr>
<td>6 Z-Phe-Phe-NHO-Ma</td>
<td>124</td>
<td>0.0056</td>
<td>9.8</td>
</tr>
<tr>
<td>7 Boc-Phe-Pro-Ala-NHO-Nbz</td>
<td>133</td>
<td>0.0052</td>
<td>7.3</td>
</tr>
<tr>
<td>8 Boc-Ala-Phe-Leu-NHO-Nbz</td>
<td>248</td>
<td>0.0028</td>
<td>24.0</td>
</tr>
<tr>
<td>9 Boc-Gly-Phe-Phe-NHO-Nbz</td>
<td>45</td>
<td>0.0153</td>
<td>3.4</td>
</tr>
</tbody>
</table>

n.m.r. spectroscopy. All reagents were obtained in Research Grade from commercial sources; organic solvents were dried before use.

Structural parameters of newly synthesized derivatives are listed in Table 1.

Non-enzymic decomposition of the inhibitors

The stability of the compounds in aqueous solution has been tested by Demuth et al. (1988) by means of u.v. spectroscopy. Inhibitors (10–200 \( \mu \)M) were incubated in 0.04 M-Tricine buffer, pH 7.6, adjusted to \( I = 0.125 \). In a typical experiment the total volume was 2.0 ml containing 5% (v/v) acetonitrile.

Kinetic runs were recorded in the range of 225–300 nm by using a Carl Zeiss (Jena, German Democratic Republic)-microprocessor-controlled spectrophotometer (M 40) equipped with a thermostatically controlled cell compartment at 30 ± 0.1 °C. The data were collected and stored in an internal RAM buffer and analysed by using software packages provided for the instrument on the ROM card REACTION KINETICS.

Parameters of decomposition reactions with half-times longer than 200 min were calculated by fitting the data collected at 30 min time intervals by non-linear regression programs running on an IBM-PC-compatible computer. The pseudo-first-order rate constants of the decompositions are compiled in Table 2.

Inactivation measurements

The inactivation of proteinases with substrate-analogue inhibitors proceeds according to eqn. (1):

\[
E + I \xrightleftharpoons{k_1}{k_{-1}} E\cdot I \xrightarrow{k_{inact}} E-I
\]

where \( E \cdot I \), \( E-I \), \( k_1 \) and \( k_{-1} \) represent the enzyme–inhibitor complex, the inactivated enzyme and the rate constants of non-covalent reaction steps respectively. \( k_{inact} \) is the rate constant of the formation of covalent modified enzyme. This rate constant may be determined as described by Kitz & Wilson (1962) by preincubation of enzyme and inhibitor and subsequent estimation of residual activity in an essay with substrate. Tian & Tsou (1982) introduced a more convenient method of evaluation of inactivation rates in the presence of a substrate according to eqn. (2):

\[
\begin{align*}
E + I \xrightleftharpoons{k_1}{k_{-1}} E\cdot I \xrightarrow{k_{inact}} E-I \\
E + S \xrightleftharpoons{k_1}{k_{-1}} E\cdot S \xrightarrow{k_{inact}} E+S
\end{align*}
\]

where substrate and inhibitor are competing for the enzyme’s binding site. The decrease in enzyme concentration during incubation with inhibitor follows pseudo-first-order kinetics. Applying chromogenic or fluorogenic substrates and establishing steady-state conditions during the inactivation time, first-order rate constants may be obtained by fitting absorbance and time values to an exponential function. Measurements at constant inhibitor and different substrate concentrations give different \( k_{obs} \) values. Extrapolation of these values gives the rate constant at zero substrate concentration.
By fitting these rate constants evaluated at different inhibitor concentrations to a hyperbola, the inactivation parameters \( k_i \) (\( \approx k_{inact}/k_i \)) and \( k_{inact} \) may be obtained. This method was applied here.

The progress curves for the inactivation of the proteinases in the presence of substrate were monitored at 25 °C by using a Shimadzu spectrophotometer (UV-300) equipped with a fluorescence-detection unit at an excitation wavelength of 383 nm and with an emission filter of 450 nm. The kinetic experiments were performed with a constant enzyme concentration in 50 mM-acetate buffer, pH 5.5, for cathepsin L; in 50 mM-phosphate buffer containing 0.01 % (v/v) Triton X-100, pH 6.5, for cathepsin S; in 50 mM-phosphate buffer, pH 6.5, for cathepsin H; and in 50 mM-phosphate buffer, pH 6.0, for cathepsin B. In all experiments the enzyme concentrations were 0.7 nM, 2.3 nM, 2.4 nM and 0.9 nM for cathepsins L, S, H and B respectively. For the activation of the proteinases the cathepsins L, B and H were incubated for 5 min at 25 °C with 2.5 mM-dithioerythritol, 2.5 mM-EDTA·Na\(_4\) and 0.005 % (v/v) Brij-35 in the assay buffer, whereas cathepsin S was activated for 15 min with 5 mM-dithioerythritol, 5 mM-EDTA·Na\(_4\) and 0.01 % (v/v) Triton X-100 in 50 mM-phosphate buffer, pH 6.5. The reaction was started by the addition of the activated enzyme (0.5 ml) to 1 ml of substrate in assay buffer containing different inhibitor concentrations (seven per substrate concentration).

Substrates were Z-Phe-Arg-NHMec (3 \( \mu \)M and 8 \( \mu \)M) for cathepsin L, Z-Phe-Arg-NHMec (10 \( \mu \)M and 50 \( \mu \)M) for cathepsin B, Z-Val-Val-Arg-NHMec (10 \( \mu \)M and 50 \( \mu \)M) for cathepsin S and Arg-NHMec (8 \( \mu \)M and 25 \( \mu \)M) for cathepsin H.

The exponential progress curves recorded were analysed by the method described above using non-linear regression programs running on an IBM-PC-compatible computer.

In cases where no saturation of the enzyme by the inhibitor was achieved ([I] \( \ll \) \( K_i \)), the second-order rate constants were calculated from \( k_{obs.}/[I]/(1+[S]/K_m) \) as described by Crawford et al. (1988).

**RESULTS AND DISCUSSION**

N- Peptidyl-O-acyl hydroxylamines were shown to react irreversibly with different lysosomal cysteine proteinases. After incubation of cathepsin B with Boc-Phe-Ala-NHO-Nbz (compound 3 in Table 3) and cathepsin S with Boc-Ala-Phe-Leu-NHO-Nbz (compound 8) for 1 h in molar enzyme/inhibitor ratios of 1:1000 and 1:100 respectively, the enzymes were completely inactivated. No regeneration of activity within 15 h could be observed after removal of excess inhibitor by exhaustive ultrafiltration (up to an enzyme/free inhibitor ratio of <1:0.05) or by chromatography on Sephadex G-10.

Typical progress curves for the cathepsin S-catalysed hydrolysis of Z-Val-Val-Arg-NHMec in the presence of the inhibitor Boc-Gly-Phe-Phe-NHO-Nbz are shown in Fig. 1.

The pseudo-first-order rate constants \( k_{obs.} \) obtained at fixed substrate and inhibitor concentrations are independent of proteinase concentrations varied over the range of one order of magnitude (cathepsin S: 2.3–23 nm, cathepsin B: 0.9–9 nm). This implies that the inactivation of both enzymes is not (or only to a very small extent)

**Table 3. Inactivation of the cathepsins S, L, B and H with N-peptidyl-O-acyl hydroxylamines**

<table>
<thead>
<tr>
<th>Compound</th>
<th>( k_i ) (( \mu )M)</th>
<th>( K_i ) (( \mu )M)</th>
<th>( k_{inact}/K_i ) (s(^{-1}))</th>
<th>( k_{inact} ) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Bac-Ala-Phe-NHO-Nbz</td>
<td>100-200(^{+})</td>
<td>50-100(^{+})</td>
<td>0.48±0.06</td>
<td>0.02±0.001</td>
</tr>
<tr>
<td>2 Bac-Ala-Phe-NHO-Nbz</td>
<td>50-100(^{+})</td>
<td>0.48±0.06</td>
<td>0.02±0.001</td>
<td>42.000 (^{+})</td>
</tr>
<tr>
<td>3 Bac-Ala-Phe-NHO-Nbz</td>
<td>50-100(^{+})</td>
<td>0.48±0.06</td>
<td>0.02±0.001</td>
<td>19.000 (^{+})</td>
</tr>
<tr>
<td>4 Bac-Ala-Phe-NHO-Nbz</td>
<td>50-100(^{+})</td>
<td>0.48±0.06</td>
<td>0.02±0.001</td>
<td>2.000 (^{+})</td>
</tr>
<tr>
<td>5 Z-Val-Val-Arg-NHO-Nbz</td>
<td>50-100(^{+})</td>
<td>0.48±0.06</td>
<td>0.02±0.001</td>
<td>22.000 (^{+})</td>
</tr>
<tr>
<td>6 Z-Val-Val-Arg-NHO-Nbz</td>
<td>50-100(^{+})</td>
<td>0.48±0.06</td>
<td>0.02±0.001</td>
<td>22.000 (^{+})</td>
</tr>
<tr>
<td>7 Boc-Ala-Phe-Phe-NHO-Nbz</td>
<td>50-100(^{+})</td>
<td>0.48±0.06</td>
<td>0.02±0.001</td>
<td>26.000 (^{+})</td>
</tr>
<tr>
<td>8 Boc-Ala-Phe-Phe-NHO-Nbz</td>
<td>50-100(^{+})</td>
<td>0.48±0.06</td>
<td>0.02±0.001</td>
<td>26.000 (^{+})</td>
</tr>
</tbody>
</table>

\(^{+}\) Rate observed (\( v/v \))
\(^{*}\) No time-dependence observed
\(^{\dagger}\) N.D., not determined

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accompanied by enzyme-catalysed degradation of the inhibitor. Smith et al. (1988) found a partition ratio ($k_{cat}/k_{inact}$) of 5.5 in the reaction between cathepsin B and N-Z-Phe-Gly-O-mesitoyl hydroxylamine. In contrast, DP IV hydrolyses substrate-analogous diacyl hydroxylamines to a remarkable extent, exhibiting partition ratios between 8000 to 200000 (Demuth et al., 1988).

The compounds 1, 2 and 7 (Table 4) are not suited to an effective inactivation of the lysosomal cathepsins. This is due to the non-specific peptide sequence of the inhibitors. Proline especially, in the P$_1$ or P$_2$ position prevents or disturbs interactions between the inhibitor and the target enzyme (Mason et al., 1984; Brömme et al., 1987). The same holds true for small amino acid residues in the P$_2$ position. A substitution of the P$_2$ residue by the specific subsite residue phenylalanine gives very potent and rapid-acting inhibitors of cathepsins S, L and B (compounds 3–6, 8 and 9). The preference for aromatic hydrophobic residues in the S$_2$ subsite by cathepsins B and L had already been shown with various other inhibitors and substrates (Barrett & Kirschke, 1981; Kirschke & Shaw, 1981; Crawford et al., 1988; Kirschke et al., 1988).

Introduction of an additional hydrophobic residue such as phenylalanine or leucine into the P$_1$ position gives the most potent inhibitors of cathepsin L (compounds 6, 8 and 9). This is in agreement with the well-known high affinity of cathepsin L for bulky hydrophobic side chains in its subsites, S$_1$ and S$_2$ (Kirschke et al., 1988). Z-Phe-Phe-NHO-Ma (compound 6 in Table 3; Fig. 2) reacts extremely rapidly with this enzyme ($k_{inact}/K_i = 1222000 \text{m}^{-1} \cdot \text{s}^{-1}$), in contrast with the rather slow inactivation of cathepsin B by this compound ($k_{inact}/K_i = 2800 \text{m}^{-1} \cdot \text{s}^{-1}$). This diacyl hydroxylamine should be well-suited to differentiate between cathepsins L and B.

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**Fig. 1.** Progress curves of the cathepsin S-catalysed hydrolysis of Z-Val-Val-Arg-NHMec in the presence of Boc-Gly-Phe-Phe-NHO-Nbz

The substrate concentration was 10 $\mu$M and the following inhibitor concentrations were used: ■, 66 nM; ▲, 100 nM; △, 116 nM; ○, 133 nM; and ●, 200 nM.

**Fig. 2.** Dependence of the rate constant of inactivation ($k_{inact}$) of cathepsin L on the concentration of Z-Phe-Phe-NHO-Ma

Cathepsin L was inactivated in 50 mM-acetate buffer, pH 5.5, containing 0.83 mM-dithioerythritol and 0.83 mM-EDTA at 25°C in the presence of substrate (Z-Phe-Arg-NHMec; 3 and 8 $\mu$M). $k_{inact}$ values were obtained by extrapolation as described in the Experimental section.

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**Table 4. Comparison of second-order rate constants of inactivation of serine and cysteine proteinases**

Abbreviations used: PSE, proline-specific endopeptidase; Ser, serine proteinase or serine peptidase; Cys, cysteine proteinase.

References: a, Demuth et al. (1988); b, Demuth et al. (1989); c, the present work.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{inact}/K_i$ (M$^{-1} \cdot $s$^{-1}$)</th>
<th>Enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-Ala-Pro-NHO-Nbz</td>
<td>1.9</td>
<td>DP IV (Ser)</td>
<td>a</td>
</tr>
<tr>
<td>Boc-Ala-Pro-NHO-Nbz</td>
<td>2.5</td>
<td>PSE (Ser)</td>
<td>b</td>
</tr>
<tr>
<td>Boc-Ala-Ala-NHO-Nbz</td>
<td>12</td>
<td>Elastase (Ser)</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>166</td>
<td>Thermitase (Ser)</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>226</td>
<td>Subtilisin (Ser)</td>
<td>b</td>
</tr>
<tr>
<td>Boc-Gly-Phe-NHO-Bz</td>
<td>652</td>
<td>Thermitase (Ser)</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>1020</td>
<td>Subtilisin (Ser)</td>
<td>b</td>
</tr>
<tr>
<td>Z-Phe-Phe-NHO-Ma</td>
<td>1222000</td>
<td>Cathepsin L (Cys)</td>
<td>c</td>
</tr>
<tr>
<td>Boc-Ala-Phe-Leu-NHO-Nbz</td>
<td>696000</td>
<td>Cathepsin L (Cys)</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>229000</td>
<td>Cathepsin S (Cys)</td>
<td>c</td>
</tr>
</tbody>
</table>
Cathepsin S was inactivated in 50 mm-phosphate buffer, pH 6.5, containing 1.66 mm-dithioerythritol and 1.66 mm-EDTA at 0.01 % Triton X-100 at 25 °C in the presence of substrate (Z-Val-Val-Arg-NHMec; 10 μM and 50 μM). k_{app.} values were obtained by extrapolation as described in the Experimental section.

The best inhibitors of cathepsin S are the tripeptide derivatives Boc-Ala-Phe-Leu-NHO-Nbz (Fig. 3) and Boc-Gly-Phe-Phe-NHO-Nbz, exhibiting one order of magnitude higher second-order inactivation rate constants compared with the most effective dipeptide derivatives. However, they do not allow one to differentiate between cathepsins S and L.

Compared with cathepsins L and S, cathepsin B is inhibited relatively slowly by the peptide inhibitors tested. Similar ratios of second-order inactivation rate constants have been found when cathepsins B and L were inactivated by peptidyl diazomethanes (Crawford et al., 1988).

Smith et al. (1988) obtained somewhat higher second-order inactivation rate constants for comparable N-peptidyl-O-acyl hydroxylamines as inactivators of cathepsin B. This may be due to lower binding constants (K), whereas the saturation inactivation rate constants (k_{inact.}) are similar to the values obtained in our experiments. The reason for this discrepancy may lie in the use of different N-protecting groups.

Cathepsin H, acting as aminoendopeptidase (Barrett & Kirschke, 1981), is only negligibly inhibited by the most potent inhibitors of the cathepsins B, L and S (compounds 3, 6 and 8: Table 3). Obviously those peptidyl derivatives are not able to bind productively to this enzyme. The same behaviour was found with peptidyl methyl ketones, known as potent reversible inhibitors of papain and the cathepsins B and L (Brömmle et al., 1989a). However, N-terminal unprotected aminomethyl ketones act as reversible inhibitors of cathepsin H. It is likely that unprotected N-amino acid-O-acyl hydroxylamines could serve as cathepsin H inhibitors.

Besides the N-peptidyl residue, the O-acyl part of the hydroxylamine inhibitor may also efficiently alter the second-order inactivation rate constants. Substitution of the benzoyl-O-acyl residue with the methacryl one (compounds 5 and 6, Table 3) leads to 5–20-times higher values for the inactivation of the cathepsins B, L and S.

REFERENCES

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