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SYNTHESIS OF MODEL COMPOUNDS RELEVANT TO THE ACTIVE-SITE-DIRECTED INACTIVATION OF L-ASPARAGINASE BY 5-DIAZO-4-OXO-L-NORVALINE*

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Earlier work has shown that 5-diazo-4-oxo-L-norvaline (DONV) irreversibly inactivates the L-asparaginase from E. coli by formation of a covalent bond in the region of the active site. Model compounds have been prepared to study this acid-labile covalent bond tentatively assigned to a serine or possibly a threonine residue in a decapeptide isolated from ^{14}C-DONV-inactivated enzyme. Appropriately blocked DONV was found to alkylate methanol, and the hydroxyl function of blocked serine or threonine in the presence of boron trifluoride. The labile β-ketoethers thus formed were reduced to the more stable β-hydroxyethers. Facile lactonization of these 5-substituted-4-hydroxy-L-norvalines was observed. The diastereoisomers of both the lactonized and open forms of 5-methoxy-4-hydroxy-L-norvaline and related 4-hydroxy-L-2-amino acids of similar length were distinguishable on the amino acid analyzer. The β-hydroxyethers derived from serine and threonine were hydrolyzed with acid and yielded the expected cleavage products. When the β-ketoether was reduced by sodium borohydride prior to deblocking, in addition to the β-hydroxyether, N-blocked amino alcohols were also formed, yielding a complex mixture of products.

Key words: active-site-directed inactivation; L-asparaginase; 5-diazo-4-oxo-L-norvaline (DONV); β-hydroxyether; β-ketoether.

It has been shown that the asparagine analog, 5-diazo-4-oxo-L-norvaline (DONV) irreversibly inactivates the L-asparaginase (Jackson & Handschumacher, 1970) from *E. coli*, an enzyme used in the treatment of human leukemia (Hill *et al.*, 1967). Using DONV-5-^{14}C, it has been demonstrated that the inactivation involves the formation of a covalent bond in the active site region. Subsequent isolation and sequence analysis of a ^{14}C-labeled decapeptide have indicated that the DONV residue is attached to the serine-9 or threonine-8 of the peptide (Peterson *et al.*, 1977). Since diazoketones have been known to alkylate alcohols in the presence of suitable catalysts (Newman & Beal, 1950), it appears reasonable that the covalent bond formation during the inactivation of the enzyme could involve the alkylation of

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the hydroxyl group of either a serine or a threonine residue by DONV in the substrate-binding site. To evaluate this possible alkylation mechanism we have alkylated a variety of hydroxyl functions by appropriately blocked DONV.

MATERIALS AND METHODS

Melting points were determined in capillary tubes using a Gallenkamp melting point apparatus and are uncorrected. Elemental analyses were performed by Baron Consulting Co., Orange, Conn. N.m.r. spectra were recorded on a Bruker 270HX spectrometer or a Varian T-60 spectrometer. Mass spectra were recorded on an AEI MS-9 mass spectrometer. T.l.c. were run on Eastman chromatograms 13181 (silica gel) and 13245 (cellulose). A JEOL 5 AH amino acid analyzer was used for amino acid analyses. 2-Amino-1,3-propanediol and 2 - amino 1,3 - butanediol were purchased from Research Organic/Inorganic Chemical Corp.

Benzyl N-*carbobenzoxy-L-2-amino-4-oxo-5-methoxypentanoate* (2)

To a solution of benzyl *N*-carbobenzoxy-L-2-amino-4-oxo-5-diazopentanoate (Liwschitz *et al.*, 1959) (*1*, 0.6 g, 1.6 mmol) in Et_2O (5 ml) and MeOH (15 ml) was added redistilled $BF_3 \cdot Et_2O$ (0.08 ml) under N_2. The reaction mixture was allowed to stand at 25° for 2 h until the evolution of N_2 ceased. After removal of the solvents *in vacuo*, the orange oily residue was extracted with Et_2O. The extract was concentrated *in vacuo* to an oil (0.44 g), which was now repeatedly extracted with light petroleum (4 × 40 ml). The combined light petroleum extracts were reduced to yield the crude *2* as a colorless oil, which was triturated with light petroleum to render it solid. The analytical sample was recrystallized from Et_2O-light petroleum: m.p. = 52–54°; n.m.r. ($CDCl_3$) δ 3.35 (s, 3 H, OCH_3), 3.9 (s, 2 H, $COCH_2O$), 5.1 (2 × s, 4 H, OCH_2Ar), 7.3 (s, 10 H Ar*H*); t.l.c., Rf 0.46 in 3:7 EtOAc-hexanes system on silica gel.

Anal. calc. for $C_{21}H_{23}NO_6$: C, 65:44; H, 6.02; N, 3.63. Found: C, 64.69; H, 5.84; N, 3.46.

L-2-Amino-4-hydroxy-5-methoxypentanoic acid (5)

A solution of crude *2* (0.34 g, 0.87 mmol) in MeOH (50 ml) was hydrogenated over 10% Pd-C (0.2 g) at 50 psi for 3 h. The catalyst was removed by filtration and the filtrate evaporated *in vacuo* to yield the crude L-2-amino-4-oxo-5-methoxypentanoic acid (0.18 g). To the solution of the crude β-ketoether in MeOH (15 ml) was added $NaBH_4$ (90 mg) in several portions. The reaction mixture was allowed to stand at 25° for 30 min and the solvent removed *in vacuo*. The residue was treated with H_2O (3 ml), and the solution, after adjusting to pH 2 with 90% formic acid, was applied to a column of Dowex-50 (H^+) (1 × 45 cm). It was eluted with 1 *N* NH_4OH to yield the crude *5* (70 mg). The analytical sample was recrystallized from EtOH: m.p. = 185° (dec); n.m.r. (D_2O) δ 3.25 (s, 3 H, OCH_3); t.l.c., Rf 0.36 in 15:13:12 *n*-ButOH-pyridine-H_2O system on cellulose.

Anal. calc. for $C_6H_{13}NO_4$: C, 44.16; H, 8.03; N, 8.58. Found: C, 43.31; H, 7.69; N, 8.51.

Treatment of *5* (5 mg) by 1 *N* HCl (2 ml) at 60° for 1 h produced the lactone *6*, which may be reconverted to *5* by 0.1 *N* NaOH at 37° for 3 h. Aliquots (50 μl) of these solutions were applied to the amino acid analyzer, which contained a single 1 × 30 cm column. The elution was performed with Hamilton sodium citrate buffers at pH 3.49 (0.2 *N*), pH 4.3 (0.2 *N*), and pH 6.18 (1 *N*).

Reaction of 1 *with* N-*carbobenzoxy-L-serine benzyl ester (or the corresponding blocked threonine)*

To a solution of *1* (0.42 g, 1.1 mmol) and *N*-carbobenzoxy-L-serine benzyl ester (0.33 g, 1 mmol) in Et_2O (40 ml) was added $BF_3 \cdot Et_2O$ (0.08 ml) under N_2. The reaction mixture was allowed to stand for 3 h at 25° until the evolution of N_2 ceased. The clear supernatant solution, which contained only the ketoether *3* (as evidenced by t.l.c., Rf 0.50 in 9:1 EtOAc-hexanes system on cellulose), was decanted from the deposits. It was concentrated *in vacuo* to an oil (0.74 g), which was immediately taken up in MeOH (25 ml) and treated with $NaBH_4$ (90 mg). After 30 min, the MeOH was removed *in vacuo* and the residue, suspended in H_2O (30 ml), was extracted with EtOAc (5 × 25 ml).

After drying over Na_2SO_4, the extract was concentrated *in vacuo* to an oil (0.62 g), which was now composed of several substances (*7, 9, 10,* and *12* for the serine series; and *8, 9, 11,* and *13* for the threonine series) as evidenced by t.l.c. in 1:1 EtOAc-hexanes system on silica gel.

N-*Carbobenzoxy-L-serinol* (7)

(a) From the reaction products of *1* and *N*-carbobenzoxy-L-serine benzyl ester: The crude, oily product mixture of *7, 9, 10,* and *12* obtained above was recrystallized first from EtOAc-light petroleum, then benzene to yield *7* (80 mg, 35%). The analytical sample was recrystallized once more from benzene: m.p. = 97–100°; n.m.r. ($CDCl_3$) δ 2.45 (s, 2 H, O*H*), 3.77 (s, 1 H, C*H*), 3.83 (d, 4 H, CH_2OH), 5.11 (s, 2 H, OCH_2Ar), 5.5 (s, 1 H, N*H*), 7.35 (s, 5 H, Ar*H*); mass spectrum m/e 225 (M^+); t.l.c., Rf 0.2 in 1:1 EtOAc-hexanes system on silica gel.

Anal. calc. for $C_{11}H_{15}NO_4$: C, 58.65; H, 6.71; N, 6.21. Found: C, 58.73; H, 6.80; N, 6.07.

(b) From *N*-carbobenzoxy-L-serine benzyl ester: A solution of *N*-carbobenzoxy-L-serine benzyl ester (329 mg, 1 mmol) in MeOH (20 ml) was treated with $NaBH_4$ (50 mg). After 30 min, the same work-up as above gave pure *7* (145 mg, 65%; m.p. = 106–109°; i.r., n.m.r., t.l.c. and mass spectrum of this material were identical with those of *7* obtained in (a).

Anal. calc. for $C_{11}H_{15}NO_4$: C, 58.65, H, 6.71; N, 6.21. Found: C, 58.87; H, 6.74; N, 5.98.

N-*Carbobenzoxy-L-threoninol* (8)

A solution of *N*-carbobenzoxy-L-threonine benzyl ester (343 mg, 1 mmol) in MeOH (20 ml) was treated with $NaBH_4$ (50 mg) as described in procedure (b) above to yield 220 mg of crude *8* as an oil. A solution of *8* (155 mg) in Me_2CO (2 ml) was applied to a preparative t.l.c. plate (8 x 8 inch, Analtech 2-mm silica gel GF) and developed with EtOAc-hexanes (1:1). The major band was extracted with Me_2CO (7 x 30 ml) and the extract filtered, and concentrated *in vacuo* to an oil (100 mg), which crystallized on standing: m.p. = 51–54°; n.m.r. ($CDCl_3$) δ 1.2 (d, 3 H, CH_3), 1.76 (s, 1 H, CHO*H*), 2.7 (s, 1 H, CH_2O*H*), 3.6 (m, 1 H, NC*H*), 3.8 (d, 2 H, CH_2OH), 4.15 (m, 1 H, OC*H*), 5.11 (s, 2 H, OCH_2Ar), 5.5 (d, 1 H, N*H*), 7.35 (s, 5H Ar*H*); mass spectrum m/e 239 (M^+); t.l.c., Rf 0.25 in 1:1 EtOAc-hexanes system on silica gel.

Anal. calc. for $C_{12}H_{17}NO_4$: C, 60.23; H, 7.16; N, 5.85. Found: C, 60.11; H, 7.06; N, 5.68.

1, 3-Di-O-benzoyl-2-N-benzoylserinol

A solution of *7* (120 mg, 1.3 mmol) in MeOH (60 ml) was hydrogenated over 10% Pd-C (60 mg) at 45 psi for 3 h. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo* to yield the crude serinol* (62 mg); mass spectrum m/e 92 ($M + H)^+$; its position on the amino acid analyzer was identical with that of 2-amino-1, 3-propanediol.

A solution of the crude serinol (44 mg, 0.48 mmol) in pyridine (4 ml) was treated with benzoyl chloride (264 mg, 1.8 mmol) dropwise. The pyridine hydrochloride formed was removed by filtration and the filtrate was concentrated *in vacuo*. The oily residue was extracted with benzene (5 x 2 ml) and the extract was concentrated *in vacuo* to yield the crude tribenzoyl derivative (98 mg, 48%). After one recrystallization from MeOH-H_2O, the analytical sample was recrystallized from MeOH: m.p. = 129–131°; n.m.r. ($CDCl_3$) δ 4.56–4.76 (2 x dd, 4 H, CH_2OBz), 4.95 (m, 1 H, C*H*NBz), 6.88 (d, 1 H, N*H*), 7.44 (t, 6 H, *m*-Ar*H*), 7.5 (d, 1 H, *p*-BzN), 7.58 (d, 2 H, *p*-BzO), 7.78 (d, 2 H, *o*-BzN), 8.04 (d, 4 H, *o*-BzO); mass spectrum m/e 403 (M^+); t.l.c., Rf 0.81 in EtOAc on silica gel.

Anal. calc. for $C_{24}H_{21}NO_5$: C, 71.45; H, 5.25; N, 3.47. Found: C, 71.57; H, 5.24; N. 3.66.

The i.r., n.m.r., t.l.c. and mass spectrum of the tribenzoyl derivative of 2 - amino - 1,3 - propanediol were identical with those recorded above.

Hydrolysis of reaction product mixtures from 4 *(or 3)*

The crude mixture of *8, 9, 11,* and *13* was deblocked by catalytic hydrogenolysis to give the amino acids A and B, and the amino alcohol C and threoninol. The deblocked mixture was taken up in 15:13:12 *n*-BuOH-pyridine-H_2O and chromatographed on a column of cellulose;

* Crude threoninol was isolated in the same manner: mass spectrum m/e 106 $(M + H)^+$; its position on the amino acid analyzer was identical with that of 2-amino-1, 3-butanediol.

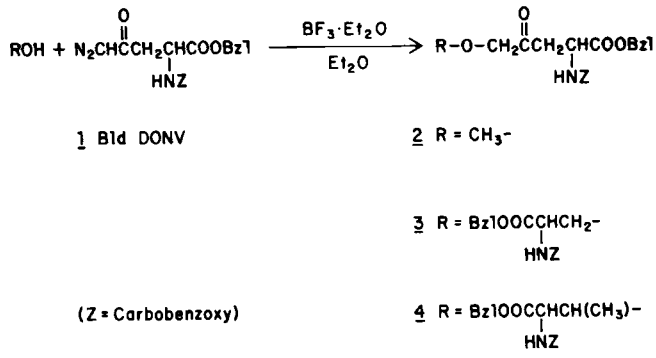

FIGURE 1

Scheme for the boron trifluoride catalyzed reaction of blocked DONV with methanol, blocked serine or blocked threonine.

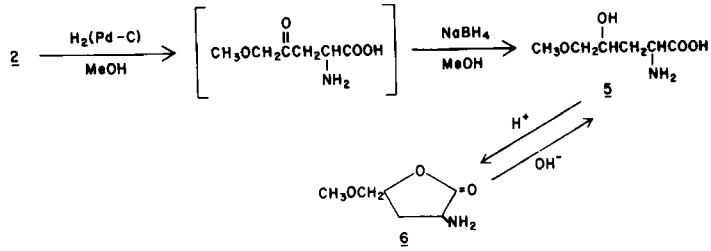

FIGURE 2

Scheme for the deblocking and sodium borohydride reduction of the β-ketoether *2* prepared by the reaction of blocked DONV with methanol (Fig. 1).

A and C co-chromatographed, followed by a mixture of B and threoninol. For acid hydrolysis: 0.5 ml of the *n*-BuOH-pyridine-H_2O eluate containing the mixture was concentrated *in vacuo*. The residue was taken up in 6*N* HCl (1 ml) and kept *in vacuo* at 110° for 3 h in the manner of peptide hydrolysis. The HCl was removed by evaporation. The components in an aqueous solution of the residue were resolved on the amino acid analyzer as described earlier for compounds *5* and *6*, using the Hamilton sodium citrate buffers.

RESULTS AND DISCUSSION

The chemical reaction of blocked DONV with methanol was evaluated as the prototype reaction of a diazoketone with an alcohol. Benzyl *N*-carbobenzoxy-L-2-amino-4-oxo-5-diazopentanoate (Liwschitz *et al.*, 1959) (*1*, a blocked DONV) was found to react with methanol in the presence of a catalytic amount of boron trifluoride etherate (Fig. 1). The β-ketoether *2* was sufficiently stable† to give the correct elemental analysis and n.m.r. data; however, the deblocked derivative, 5-methoxy-4-oxo-L-norvaline, was extremely unstable and had to be reduced to the β-hydroxyether (*5*) immediately, as shown in Fig. 2. As expected, compound *5* forms the lactone *6* under acidic conditions. The diastereoisomers of either *5* or *6* are observed as a pair of adjacent but separate peaks of approximately equal heights on the amino acid analyzer, with the lactone peaks appearing in the basic region (Fig. 3). This separation of diastereoisomers on the amino acid analyzer has also been observed with the 4-hydroxynorvalines derived from 4-oxo-L-norvaline (Chang *et al.*, 1973) and 5-hydroxy-4-oxo-L-norvaline (Jackson & Handschumacher, 1970). In the latter case, although the possibility exists for the compound to lactonize through either the 4- or 5-hydroxyl, only one set of diastereomeric lactones was observed. Evidence favors formation of the lactone through the 4-hydroxyl, in agreement with the findings of others (Wieland *et al.*, 1968). Wieland *et al.* also observed separation of diastereoisomers of a series of 4-hydroxynorvalines, including some 4,5 - dihydroxynorvalines and their lactones, not only on the amino acid analyzer but also in certain t.l.c. systems.

† A solution of *2* in chloroform was found to decompose slowly on standing. Catalytic hydrogenolysis of the decomposed material yielded 5-hydroxy-4-oxo-L-norvaline.

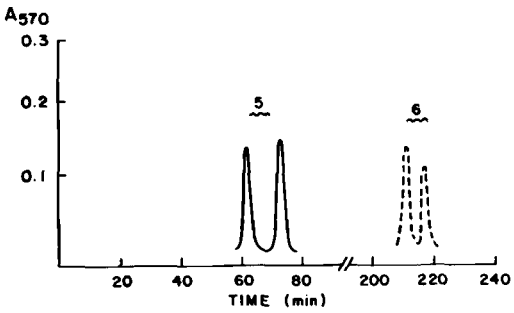

FIGURE 3

Separation of the diastereoisomer of *5* and *6* (Fig. 2) on the amino acid analyzer.

The β-ketoethers *3* and *4* were even less stable than the ketoether *2*. Prior to work-up, the ketoether (either *3* or *4*) did appear as a single spot in the monitoring t.l.c. system, with the complete disappearance of starting materials. However, during the work-up, approximately one third of the ether degraded to other compounds. This unusual lability of the ether linkage may be attributed to the presence of the boron trifluoride etherate, which not only catalyzes the alkylation of a hydroxyl by a diazoketone, but also serves as an effective agent for the cleavage of ethers (Mooney & Qaseem, 1967), especially as its concentration increases during the removal of solvent. This observation made it necessary to reduce these compounds as soon as possible to the more stable β-hydroxyether prior to deblocking.

When the crude β-ketoether *3* was reduced with sodium borohydride in methanol, the only crystalline product isolated was characterized to be the hitherto unreported *N*-carbobenzoxy-L-serinol (*7*), identical to the sodium borohydride reduction product of *N*-carbobenzoxy-L-serine benzyl ester. Compound *7* was further identified by deblocking to the known serinol. Treatment of the amino alcohol with benzoyl chloride gave a product identical with the tribenzoyl derivative of authentic 2-amino-1,3-propanediol. Since it is generally assumed that sodium borohydride will not reduce carboxylic esters, the formation of *7*, presumably by action of the excess sodium borohydride on one of the cleavage products of the β-ketoether *3*, *N*-carbobenzoxy-L-serine benzyl ester, was unexpected. However, cases have been reported in which reduction of esters to alcohols by sodium borohydride does occur (Brown & Rapoport, 1963). As for amino acid esters, only the reduction of selected *N*, *O*-blocked-L-serine methyl esters to the corresponding derivatives of 2-amino-1,3-propanediol has been recorded (Farbenfabriken Bayer, 1959).

Since the amount of *7* that was isolated accounted for only 30% of the total reduction products, efforts were made to characterize the remainder of the mixture. Attempts to separate the components by silica gel column chromatography caused cleavage of the existing β-hydroxyether *3* to form additional amounts of *N*-carbobenzoxy-L-serine benzyl ester. Therefore, the crude mixture of the reduction products was deblocked without purification. In addition to serinol, there were three new major ninhydrin-positive compounds detected by the amino acid analyzer. They are thought to be the hydroxyethers A, B, and C from the blocked precursors *10* and *12* (Fig. 4).

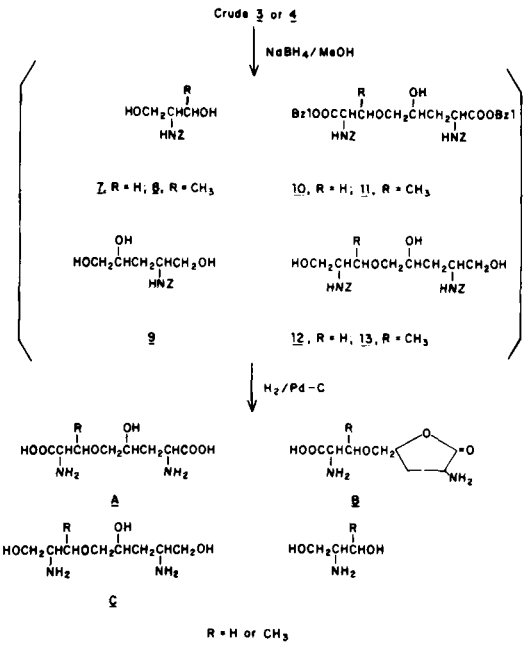

FIGURE 4

Scheme for the sodium borohydride reduction of β-ketoether *3* or *4* (Fig. 1) followed by deblocking of the resultant β-hydroxyethers.

Analogous results were obtained with the crude reduction mixture of the β-ketoether *4*. The amino acid (A), the lactone (B), and the amino alcohol (C) elute in appropriate positions on the amino acid analyzer. The basicity increases from that of A (tyrosine region) to that of B (the region after phenylalanine) to that of C (NH_3 region). When the reduction mixture from *4* was chromatographed on a column of cellulose; A and C were unresolved and eluted before the mixture of B and threoninol. Attempts to effect preparative separation of A from C, and B from threoninol were not successful. Evidence that peak B was the desired α-amino-δ-(*O*-L-threonyl-γ-valerolactone (B, R = CH_3) was obtained by acid hydrolysis which released equivalent amounts of threonine and 4,5 - dihydroxy - norvaline lactone; the amount of threoninol already present in this sample was unchanged and B completely disappeared (Fig. 5). Acid hydrolysis completely converted the mixture of A and C (R = CH_3 in Fig. 4) to equivalent amounts of threonine, 4,5-dihydroxynorvaline lactone, threoninol and a new peak in the basic region, which could be an amino alcohol* derivative of 4,5-dihydroxynorvaline (Fig. 4, the deblocked derivative of *9*). The cleavage of amino acids A and B by acid hydrolysis to yield the expected components serine (or threonine) and 4,5-dihydroxynorvaline lactone clearly demonstrates that the diazo function of DONV is indeed capable of alkylating these hydroxyl groups under appropriate catalytic conditions.

N-Carbobenzoxythreoninol (*8*) could not be isolated from the reduction mixture as a crystalline solid. However, reduction of *N*-carbobenzoxy-L-threonine benzyl ester with sodium borohydride in methanol did yield *8* as a low melting solid with the correct n.m.r., i.r., and mass spectrum. It was further characterized as threoninol by deblocking and comparing its mass spectrum as well as its position on the amino acid analyzer with those of authentic 2-amino-1, 3-butanediol.

* It may be of interest to note that the identical amino alcohol (the deblocked derivative of *9*, Fig. 4) appeared on the amino analyzer when *2* was first reduced by sodium borohydride, then deblocked and subjected to acid hydrolysis.

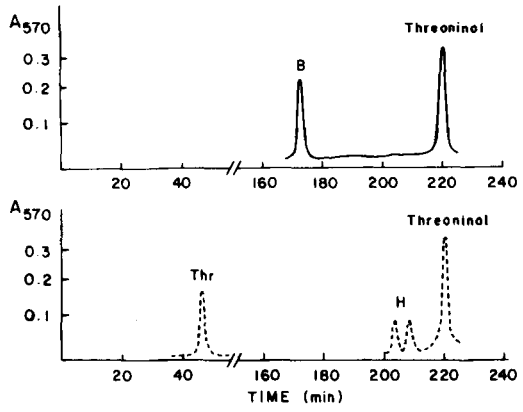

FIGURE 5

Amino acid analyzer chromatogram; (———) a mixture of B (Fig. 4; R = CH_3) and threoninol; (– – – –) after acid hydrolysis of the above mixture (H = diastereoisomers of 4, 5-dihydroxynorvaline lactone).

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