ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION NO. 2623 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA]

The Interaction of Ethyl 1-Acetyl-2-benzylcarbazate with *alpha*-Chymotrypsin[1]

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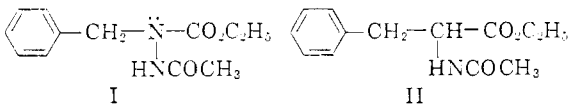
RECEIVED OCTOBER 13, 1960

Ethyl 1-acetyl-2-benzylcarbazate, an analog of ethyl acetyl-D and L-phenylalaninate, is not a substrate of α-chymotrypsin. Although the carbazate inhibits the α-chymotrypsin catalyzed hydrolysis of methyl chloroacetyl-L-valinate, it is less effective in this action than ethyl acetyl-D-phenylalaninate. The significance of these observations relative to the mechanism of action of α-chymotrypsin is discussed.

The purpose of this investigation was to determine the kinetic consequences of replacement of the α-methine group, in a "typical" substrate of α-chymotrypsin, by a nitrogen atom. The compound chosen for study was ethyl 1-acetyl-2-benzylcarbazate[3] (I), an analog of ethyl acetyl-D and L-phenylalaninate (II). The former com-



I II

pound possessed three of the four structural features of a "typical" substrate of α-chymotrypsin, *i.e.*, an α-benzyl side chain, an α-acetamido group and a carbethoxy function, but with these groups disposed about a nitrogen atom instead of a methine group.

An aqueous solution containing ethyl 1-acetyl-2-benzylcarbazate and α-chymotrypsin was maintained at 25° and *p*H 7.95 for one week. During this time the amount of base consumed, in maintaining the *p*H constant, was less than that required for a comparable system containing only enzyme. The intended substrate was recovered from the reaction mixture in 94% yield. Thus, replacement of the α-methine group by a nitrogen atom, in a molecule that possessed all of the other requisite structural features, led to a compound that was incapable of functioning as a substrate.

The preceding observation raised the question as to whether ethyl 1-acetyl-2-benzylcarbazate is exceptionally resistant to hydrolysis.

It may be recalled that whereas 2-methyl-4-benzyl-5-oxazolone is hydrolyzed at room temperature,[4] the base catalyzed ethanolysis of the analogous 2-methyl-4-benzyl-1,3,4-oxadiazolone-5 requires refluxing conditions.[3] The lesser reactivity of the latter compound probably is due to a reduction in the electrophilic character of the carbon atom in position 5 by the unshared pair of electrons of the nitrogen atom in position 4. The same situation would prevail in the base catalyzed hydrolysis of ethyl 1-acetyl-2-benzylcarbazate, and it was anticipated that this compound would be hydrolyzed at a slower rate than ethyl acetyl-D or L-phenylalaninate. The question was, how much slower?

In aqueous solutions at 25.0 ± 0.1° and 0.1 *M* in sodium chloride and over a *p*H range of 7.5 to 9.0, the base catalyzed hydrolysis of ethyl 1-acetyl-2-benzylcarbazate was described by the relation $v = k_B[RCO_2C_2H_5][OH^-]$ and a value of $k_B = 3.42 \pm 0.04$ l. mole^{-1} min.$^{-1}$ was obtained. For ethyl acetyl-DL-phenylalaninate $k_B = 17.7 \pm 1.6$ l. mole^{-1} min.$^{-1}$.

If steric factors are excluded and if the electronic factors encountered in the base catalyzed hydrolysis are similar to those operative in the enzyme catalyzed hydrolysis, the five-fold difference in the rates of the base catalyzed hydrolysis of ethyl 1-acetyl-2-benzylcarbazate and ethyl acetyl-DL-phenylalaninate does not afford a satisfactory explanation as to why ethyl acetyl-L-phenylalaninate can function as a substrate of α-chymotrypsin and ethyl 1-acetyl-2-benzylcarbazate cannot. While the unshared pair of electrons on the α-nitrogen atom will lead to depolarization of the adjacent carbonyl group, it does not appear reasonable that this effect can account for the loss in substrate activity, particularly when the rate of the base catalyzed hydrolysis of ethyl 1-acetyl-2-benzylcarbazate is only one-fifth as rapid as that of ethyl acetyl-DL-phenylalaninate.

The hydrolysis of β-N-benzyl-β-N-carbethoxy-N′-benzyl-N′-carbethoxycarbamylhydrazine with concd. hydrochloric acid to give benzylurethan and a 98% yield of ethyl 2-benzylcarbazate[3] provides an instructive, but biased, example of the hydrolytic stability of a carbalkoxy group in an alkyl 1-acyl-2-alkylcarbazate under conditions where the acid catalyzed hydrolysis of the 1-acyl group may proceed in high yield. The example cited is particularly favorable to the point being made because of the nature of the 1-acyl group, *i.e.*, it is an N-alkyl-N-acyl carbamyl group. However, with any simple alkyl 1-acyl-2-alkylcarbazate one would expect the rate of an acid catalyzed deësterification to be slow because protonation of the nitrogen atom alpha to the carbethoxy group would cause a marked decrease in the apparent basicity of the adjacent carboxyl oxygen atom.

If the mechanism of enzyme catalysis is analogous to that of acid catalysis and, in addition, is such as to permit protonation of the α-nitrogen atom, in ethyl 1-acetyl-2-benzylcarbazate, it is possible that the cation so formed would not be able to function as a substrate even though all other factors were favorable for substrate activity.

It is probable that the bond between the α-

(1) Supported in part by a grant from the National Institutes of Health, U. S. Public Health Service.

(2) To whom inquiries regarding this article should be sent.

(3) A. N. Kurtz and C. Niemann, *J. Org. Chem.*, in press.

(4) M. Bergmann, F. Stern and C. Witte, *Ann.*, **449**, 277 (1926).

nitrogen and adjacent carbonyl carbon atoms in ethyl 1-acetyl-2-benzylcarbazate has a higher internal barrier of rotation than the bond between the α-carbon and adjacent carbonyl carbon atoms in ethyl acetyl-D- or L-phenylalaninate. This singular structural feature and the inability of ethyl 1-acetyl-2-benzylcarbazate to function as a substrate led us to examine the latter compound as an inhibitor of the α-chymotrypsin catalyzed hydrolysis of chloroacetyl-L-valine methyl ester. The inhibition studies were conducted in aqueous solutions at 25.0 ± 0.1°, *p*H 7.90 ± 0.01 and 0.10 *M* in sodium chloride. The experimental procedure was that described by Applewhite, Martin and Niemann.[5] Chloroacetyl-L-valine methyl ester[6,7] was used at substrate concentrations which varied from 4.50 to 56.0 × 10^{-3} *M*, ethyl 1-acetyl-2-benzylcarbazate at inhibitor concentrations of 0, 4.41, 8.83 and 18.1 × 10^{-3} *M* and α-chymotrypsin at an enzyme concentration of 0.1464 mg. protein–nitrogen per ml. For each inhibitor concentration 9 to 12 experiments were performed at 8 or 9 different substrate concentrations.

The initial velocities were determined by the method of Booman and Niemann,[8] using in this instance a program written for the Datatron 205 digital computer,[9] and were corrected for enzyme and substrate blank reactions by the method of Martin and Niemann.[10] The constants K_S' and k_3 were then evaluated, with a Datatron 205, using a program written for a least squares fit to the equation

$$1/v_0 = (K_S'/k_3[E])(1/[S]_0) + (1/k_3[E]) \text{ where } K_S' = K_S(1 + ([I]/K_I))$$

It will be seen from the data summarized in Table I that the values of k_3 exhibit a small and possibly significant drift toward lower values with increasing inhibitor concentration. If this drift is ignored and the mean value of k_3 used to calculate a set of K_S'' values, a value of $K_I'' = 20 \pm 6 \times 10^{-3}$ *M* is obtained. This value may be taken as the enzyme-inhibitor dissociation constant of α-chymotrypsin–ethyl 1-acetyl-2-benzylcarbazate, under the conditions specified, if the latter compound is a totally competitive inhibitor and the drift in k_3 values is ignored.

An attempt was made to determine whether the drift in k_3 values noted above was or was not significant. Our interest in this matter arose from the possibility that α-chymotrypsin, chloroacetyl-L-valine methyl ester and ethyl 1-acetyl-2-benzylcarbazate were not functioning as a totally competitive system but as one in which a ternary complex involving enzyme, substrate and inhibitor was formed,[11] or, as Blum has suggested,[12] one in which two or more conformational forms of the enzyme were being generated by interaction of the enzyme with the inhibitor (modifier).

TABLE I

INHIBITION OF THE α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF CHLOROACETYL-L-VALINE METHYL ESTER BY ETHYL 1-ACETYL-2-BENZYLCARBAZATE[a]

$[I]_0$[b]	K_S'[b,c]	K_S''[b,d]	k_3[e]	K_I''[b,f]
0	46.9 ± 2.9	43.2 ± 2.9	1.65 ± 0.09	
4.41	55.3 ± 4.8	53.8 ± 5.5	1.55 ± .11	
8.83	56.5 ± 5.5	60.0 ± 6.8	1.42 ± .13	
18.1	83.0 ± 7.7	85.0 ± 9.2	1.49 ± .13	
		Mean value	1.54 ± 0.14	20 ± 6

[a] In aqueous solutions at 25.0°, *p*H 7.90 ± 0.01 and 0.1 *M* in sodium chloride. [b] In units of 10^{-3} *M*. [c] Value based upon value of k_3 determined simultaneously. [d] Value based upon mean value of k_3. [e] In units of 10^{-3} *M*/min./mg. protein–nitrogen per ml. [f] Derived from values of K_S'' assuming competitive inhibition.

The constants given in Table I were evaluated from initial velocities computed by the orthogonal polynomial procedure[8] using a $t_{0.10}$ significance level to determine the degree of polynomial to be used for representation of the d*P*/d*t* relationships, which in 38 out of 43 experiments required polynomials of order substantially greater than unity for the 1 to 9 min. reaction period. Since the total extent of reaction rarely exceeded 10% one might expect more linear relationships. Therefore, several alternative procedures for testing the reliability of individual initial velocities were examined.

The first method was based upon the proposition that as $[S]_0$ approaches zero, Michaelis–Menten kinetics approach first order kinetics; hence, values of the initial velocities observed at low substrate concentrations are less reliable for the evaluation of K_S and k_3 than those observed at high substrate concentrations. For each individual point a new slope was computed between the limits K_S'/k_3 and that point. From this new slope a new value, k_{3i}, was calculated and compared with the k_3 value derived from all of the points. The assembled k_{3i} values were then compared by the analysis of variances at a 99% significance level for rejection. By selecting a *t* test value for a 99% confidence limit only those runs that were in serious error were made evident. Following this step K_S' and k_3 were recalculated using the acceptable data. The values so obtained are given in the first and second columns Table II. In three out of four cases data derived from experiments conducted at the lowest level of $[S]_0$ were rejected.

The second method assumed that all inherent errors were associated with the ordinate $[S]_0 \cdot [E]/v_0$ and that the basis of rejection should be a difference between the observed ordinate value and the ordinate value calculated from a least squares solution of the slope and intercept. The differences between the measured $[S]_0[E]_0/v_0$ values and those calculated from the slope of the least squares line based on all of the data were

(5) T. H. Applewhite, R. B. Martin and C. Niemann, *J. Am. Chem. Soc.*, **80**, 1457 (1958).

(6) T. H. Applewhite, H. Waite and C. Niemann, *ibid.*, **80**, 1465 (1958).

(7) In ref. 6 a value of K_S = 47.1 ± 0.8 × 10^{-3} *M* and k_3 = 1.755 ± 0.028 × 10^{-3} *M*/min./mg. protein–nitrogen per ml. is given for chloroacetyl-L-valine methyl ester in aqueous solutions at 25.0 ± 0.1°, *p*H 7.90 ± 0.01 and 0.1 *M* in sodium chloride. Re-evaluation of the original data using a more reasonable estimate of the extent of the enzyme blank reaction gave a value of K_S = 39.3 ± 0.7 × 10^{-3} *M* and k_3 = 1.61 ± 0.03 × 10^{-3} *M*/min./mg. protein–nitrogen per ml.

(8) K. A. Booman and C. Niemann, *ibid.*, **78**, 3642 (1956).

(9) H. I. Abrash, A. N. Kurtz and C. Niemann, *Biochem. et Biophys. Acta*, **45**, 378 (1960).

(10) R. B. Martin and C. Niemann, *ibid.*, **26**, 634 (1957).

(11) H. T. Huang and C. Niemann, *J. Am. Chem. Soc.*, **75**, 1395 (1953).

(12) J. J. Blum, *Arch. Biochem. Biophys.*, **55**, 486 (1955).

TABLE II

RE-EVALUATION OF VALUES OF K_S' AND k_3

I		II		III		
K_S'[a]	k_3[b]	K_S'[a]	k_3[b]	K_S'[a]	k_3[b]	$[I]_0$[a]
50.6 ± 2.9	1.74 ± 0.09	50.6 ± 2.9	1.74 ± 0.09	56.7 ± 2.4	1.85 ± 0.07	0
59.2 ± 4.8	1.61 ± .12	59.2 ± 4.8	1.61 ± .12	60.0 ± 1.1	1.59 ± .03	4.41
63.1 ± 5.7	1.53 ± .13	58.6 ± 2.4	1.48 ± .05	65.1 ± 2.2	1.51 ± .05	8.83
82.0 ± 9.9	1.45 ± .16	82.0 ± 9.9	1.45 ± .16	83.8 ± 3.4	1.37 ± .05	18.1

[a] In units of 10^{-3} M. [b] In units of 10^{-3} M/min./mg. protein-nitrogen per ml.

computed and compared by the analysis of variances to a 99.5% confidence level. After rejection of the data not passing the test, K_S' and k_3 were recalculated to give the values listed in the third and fourth columns of Table II.

The third method used for re-evaluation assumed that the orthogonal polynomial procedure was biased in favor of a non-linear relationship during the first one or two minutes of an experiment. The data obtained during the 1 to 9 minute interval were used to calculate an initial velocity by fitting the data to a first order equation by the method of least squares and then using this value to evaluate K_S' and k_3. These values are given in fifth and sixth columns of Table II.

The values of K_S' and k_3 given in Table II are characterized by a uniform increase in values of K_S' and a uniform decrease in values of k_3 with increasing inhibitor concentration and thus confirm the trend in values noted in Table I. The significance in the downward trend in k_3 values listed in Table II was tested at a 95% chi square level from which it was concluded that no differences in precision existed amongst the values obtained at the different inhibitor concentrations. Following this analysis a 95% F test for homogeneity of the four k_3 values of each set was performed with the result that to a 95% confidence limit the different inhibitor concentrations gave significantly different k_3 values.

The preceding results suggest that the system α-chymotrypsin–chloroacetyl-L-valine methyl ester–ethyl 1-acetyl-2-benzylcarbazate may be other than totally competitive.[13] However, for the purpose at hand it is reasonable to regard this system as one approximating totally competitive inhibition.

Ethyl acetyl-D-phenylalaninate has not been evaluated as a competitive inhibitor. However, the corresponding methyl ester is known to have a K_I value of $2.4 \pm 0.4 \times 10^{-3}$ M.[14] On the basis of the K_I values of methyl and ethyl acetyl-D-tryptophanate, 0.09 and 0.25×10^{-3} M[15] and those of ethyl acetyl-D-tyrosinate, $5.0 \pm 1.0 \times 10^{-3}$ M,[14] acetyl-D-tyrosinamide, $12 \pm 2 \times 10^{-3}$ M[14] and acetyl-D-phenylalaninamide, $12 \pm 3 \times 10^{-3}$ M,[14] it is unlikely that the K_I value of ethyl acetyl-D-phenylalaninate is greater than 10×10^{-3} M and is probably *ca.* $5 \pm 2 \times 10^{-3}$ M. From this K_I value and that of ethyl 1-acetyl-2-benzylcarbazate, *i.e.*, $20 \pm 6 \times 10^{-3}$ M it follows that the active site of α-chymotrypsin is more accessible to ethyl acetyl-D-phenylalaninate than it is to the analogous carbazate.

A system involving α-chymotrypsin and ethyl acetyl-L-phenylalaninate has been reported[16] to have a K_S value of $1.1\text{–}1.2 \times 10^{-3}$ M, which is not markedly different from the value of $K_S = 1.80 \times 10^{-3}$ M recently given for methyl acetyl-L-phenylalaninate.[17] The fact that the K_S value for ethyl acetyl-L-phenylalaninate is smaller than the K_I value of ethyl acetyl-D-phenylalaninate and the latter value is smaller than the K_I value for ethyl 1-acetyl-2-benzylcarbazate suggests that accessibility to the active site of the enzyme is in the order ethyl acetyl-L-phenylalaninate > ethyl acetyl-D-phenylalaninate > ethyl 1-acetyl-2-benzylcarbazate.

The implication that the active site of α-chymotrypsin is less accessible to ethyl 1-acetyl-2-benzyl carbazate than to ethyl acetyl-L-phenylalaninate does not in itself provide an explanation as to why the latter compound is a substrate and the former is not. However, it does appear that there is a singular structural feature of the carbazate that impedes its access to the active site of the enzyme irrespective of whether the comparison is made with ethyl acetyl-L-phenylalaninate, which is a substrate, or with ethyl acetyl-D-phenylalaninate, which is not.

The inability of ethyl 1-acetyl-2-benzylcarbazate to function as a substrate could arise as a consequence of several limiting situations.

If the carbazate is present in the enzyme-substrate complex as the base, rather than as the conjugate acid arising from protonation of the α-nitrogen atom, it is clear that the lack of reactivity cannot be explained solely in terms of a greater degree of depolarization of the carbethoxy carbonyl group of the carbazate relative to that of the reactive α-acylamino acid ester. However, the lack of reactivity could arise indirectly from the greater degree of constraint in the bond between the α-nitrogen and adjacent carbonyl carbon atoms, relative to that in the analogous bond of the α-acylamino acid ester, and directly from an inability of the carbazate to assume conformations in the enzyme-substrate complex that are accessible to ethyl acetyl-L-phenylalaninate. In this instance the inertness of the carbazate would arise largely from its unfavorable orientation in the enzyme-substrate complex.

(13) A treatment of this system as one involving ternary complex formation is given in the Ph.D. Thesis of A. N. Kurtz, Calif. Inst. Tech., Pasadena (1960). It is not reproduced here because of the limitations arising from the lack of an unambiguous value of K_I for ethyl 1-acetyl-2-benzylcarbazate in a totally competitive system.

(14) R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, **77**, 3370 (1955).

(15) H. T. Huang and C. Niemann, *ibid.*, **74**, 101 (1952).

(16) B. R. Hammond and H. Gutfreund, *Biochem. J.*, **61**, 187 (1955).

(17) M. L. Bender and W. A. Glasson, *J. Am. Chem. Soc.*, **82**, 3336 (1960).

The factors leading to unfavorable orientation of the carbazate in the enzyme-substrate complex might be eliminated by protonation of the α-nitrogen atom of the carbazate. However, in the conjugate acid of the carbazate the apparent basicity of the carbethoxy carbonyl oxygen atom would be diminished to such a degree that even if the conjugate acid were able to assume those conformations accessible to ethyl acetyl-L-phenylalaninate in its enzyme-substrate complex, it is probable that it would still be unable to function as a substrate.

If the conjugate acid of the carbazate is able to assume those conformations accessible to ethyl acetyl-L-phenylalaninate in its complex with the enzyme, it should also be able to assume those conformations available to ethyl acetyl-D-phenylalaninate in its complex with the enzyme. This situation in itself would not prevent the carbazate from functioning as a substrate. However, the fact that the K_I value of the carbazate is substantially greater than that of ethyl acetyl-D-phenylalaninate does not support the view that the carbazate is present in its complex with the enzyme in the form of the conjugate acid unless one invokes the added hypothesis that protonation is possible only in those conformations that are analogous to those assumed by ethyl acetyl-L-phenylalaninate.

The most direct explanation of the inability of ethyl 1-acetyl-2-benzylcarbazate to function as a substrate of α-chymotrypsin is that it is unable to assume an orientation in the enzyme substrate complex that can lead to reaction products.

Experimental

Attempted Hydrolysis of Ethyl 1-Acetyl-2-benzylcarbazate by α-Chymotrypsin.—A solution of 3.06 g. of ethyl 1-acetyl-2-benzylcarbazate[3] and 0.275 g. of α-chymotrypsin in 230 ml. of carbon dioxide free water was adjusted to *p*H 7.95 with 0.1 *N* aqueous sodium hydroxide, the resulting solution made up to 250 ml., thermostatted at 25° and the *p*H held at 7.95 by the addition of 0.1 *N* aqueous sodium hydroxide. After 70 min. 2.35 ml. of base had been added, after 275 min. a total of 3.50 ml., after 16.5 hr. a total of 4.00 ml., after which time no further addition of base was required to maintain the *p*H at 7.95 for a period of one week. The total amount of base added corresponded to a maximum extent of hydrolysis of 1.3%, a value less than that observed for a comparable system containing only enzyme.

At the end of the reaction period the system was extracted with three 100 ml. portions of carbon tetrachloride and three 100 ml. portions of ethyl ether. The combined extracts were dried over anhydrous sodium sulfate and the solvent removed *in vacuo* at 25°[3] to give 2.88 g. (94%) of a viscous, non-crystallizable oil, $n^{25}D$ 1.5131. The infrared spectrum in carbon tetrachloride was identical with that of ethyl 1-acetyl-2-benzyl carbazate.[3]

Base Catalyzed Hydrolyses of Ethyl 1-Acetyl-2-benzyl Carbazate and Ethyl Acetyl-DL-phenylalaninate.—Stock solutions 0.040 *M* in substrate were prepared from carbon dioxide free water. Aliquots of these solutions were used to prepare reaction systems 0.1 *M* in sodium chloride and with a total volume of 10.0 ml. All hydrolyses were conducted at 25.0 ± 0.1° in an atmosphere of nitrogen at a constant *p*H maintained with a *p*H-stat. Aqueous sodium hydroxide, 0.0107 *N*, was used as the titrant and each hydrolysis was followed for a period of 8 min. The schedule used for each substrate is given in Table III.

TABLE III

SCHEDULE OF EXPERIMENTS FOR DETERMINATION OF k_B

Molarity of substrate *M*	*p*H
0.024	7.50
.024	7.90
.024	8.50
.036	8.50
.004	9.00
.016	9.00
.024	9.00
.028	9.00

The recorder traces of extent of reaction *vs.* time were linear and the velocities were computed from the chart coordinates. The data so obtained were described by the relation $v = k_B[RCO_2R'][OH^-]$ to within ± 10% and the second order constant k_B evaluated by a least squares fit of *v vs.* the product $[RCO_2R'][OH^-]$ using a program written for the Datatron 205 digital computer.

Inhibition Studies.—The inhibition of the α-chymotrypsin catalyzed hydrolysis of chloroacetyl-L-valine methyl ester in aqueous solutions at 25.0 ± 0.1°, *p*H 7.90 ± 0.01 and 0.1 *M* in sodium chloride by ethyl 1-acetyl-2-benzylcarbazate[3] was studied with the aid of a *p*H-stat as described previously.[5] The only departure from the previous procedure was in the use of equal time intervals of one min. from t = 1 min. to t = 9 min. instead of t = 0 to t = 8 min. In this particular study some difficulty was experienced in adjusting the *p*H of the enzyme solution so as to minimize "hunting" during the initial minute of reaction. Crystalline α-chymotrypsin, Armour lot No. 283, was used throughout.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA 14, MD.]

Synthetic Kanosamine

BY HANS HELMUT BAER

RECEIVED DECEMBER 17, 1960

A methyl 3-amino-3-deoxy-β-D-hexopyranoside that has recently become readily available by a new synthesis was proved to belong to the *gluco* series, *i.e.*, to be the methyl β-glycopyranoside of kanosamine. Acid hydrolysis and subsequent N-acetylation afforded kanosamine hydrochloride and N-acetylkanosamine, respectively. The latter was degraded by means of periodate to give 2-acetamido-2-deoxy-4-*O*-formyl-D-arabinose and N-acetyl-D-arabinosamine.

Recently we have described a synthesis that afforded, *via* a nitromethane condensation of periodate-oxidized methyl β-D-glycosides and subsequent hydrogenation, a methyl 3-nitro-3-deoxy-β-D-hexopyranoside (I, m.p. 204–205°, $[\alpha]^{20}D$ −12°) and the corresponding methyl 3-amino-3-deoxy-β-D-hexopyranoside (II, m.p. 207–208° dec., $[\alpha]^{20}D$ −34°).[1] Since the course of the synthesis was non-specific with regard to the stereochemistry at carbon atoms 2, 3 and 4, the configuration of the products remained unsettled. Three of the eight

(1) H. H. Baer, *Ber.*, **93**, 2865 (1960).