Tetrahedron Vol 39, No 9, pp 1661 to 1666, 1983
Printed in Great Britain

0040-4020/83/091661-06$03.00/0
Pergamon Press Ltd

STRUCTURAL STUDIES OF GLYCOPEPTIDE ANTIBIOTIC A35512B

IDENTIFICATION OF THE DIPHENYL ETHER-TYPE BIS(AMINO ACID)

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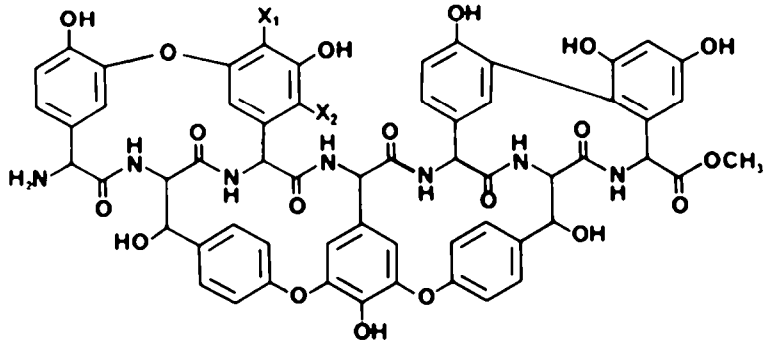
(*Received in USA 24 August* 1982)

Abstract—A35512B is one of the components of the A35512 complex of glycopeptide antibiotics recently isolated from *Streptomyces candidus*. Hydrolysis of the aglycone with 6 N HCl gave actinoidinic acid (**6**), and the previously reported Cl-containing diphenyl ether-type bis(phenylglycine)**2**. Hydrolysis of the aglycone in 57% HI gave **6**, tris(amino acid) **5b** arising from reduction of the β-OH groups of amino acid **5a**, and amino acid **7**, formed by dehalogenation of **2**. Amino acids **2** and **7** were oxidatively degraded to bis(benzoates) **3d** and **10**. The structures of **3d** and **10** were confirmed by independent syntheses. Amino acid **2** is assigned structure **2d** rather than the previously proposed structures **2a** or **2b**. The absolute configuration of **2d** was determined as *R* for the *para*-hydroxylated ring and *S* for the *meta*-hydroxylated ring.

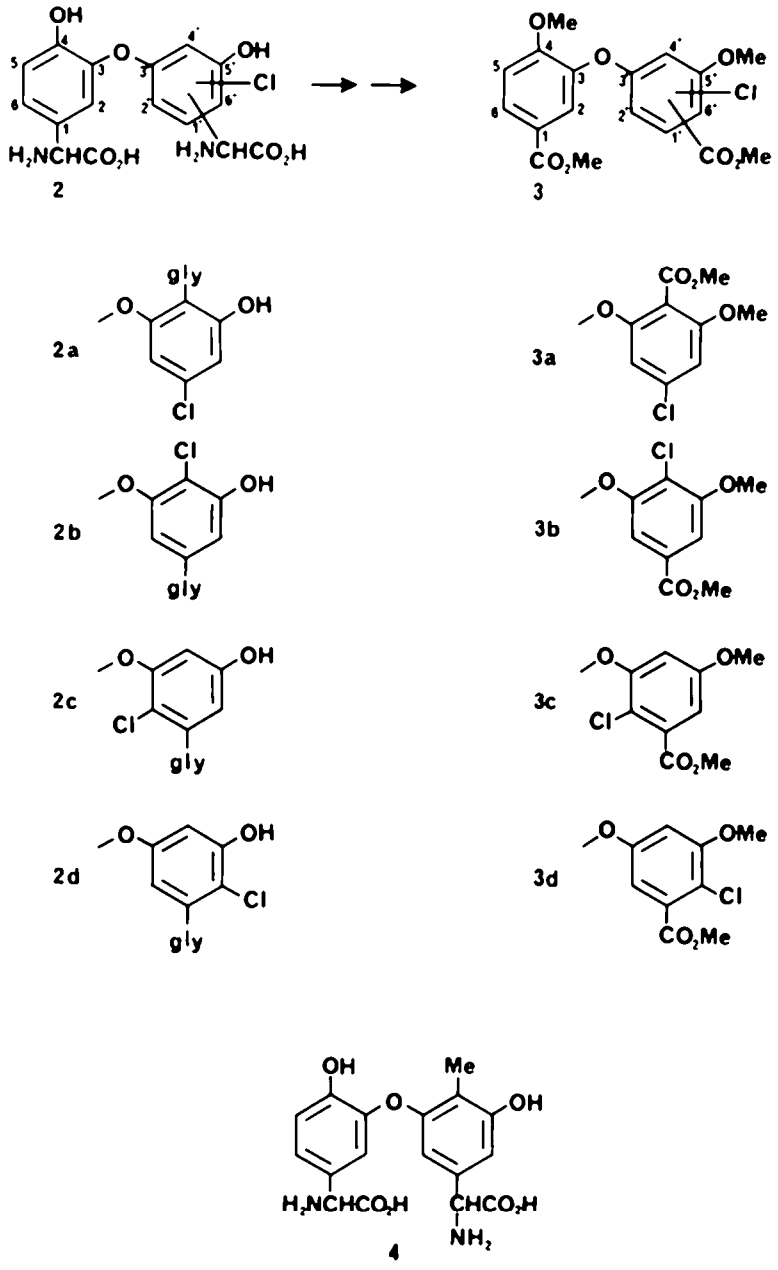
The isolation and characterization of a complex of glycopeptide antibiotics designated A35512 from the fermentation broth of *Streptomyces candidus* NRRL 8156 was recently reported by workers at Eli Lilly.[1] These antibiotics belong to the vancomycin group, which includes ristocetin (ristomycin), avoparcin and others.[2] One member of the new complex, A35512B, has been studied extensively and structure **1a** has been proposed for the aglycone.[3] A specific complexation of A35512B with acetyl-D-ala-D-ala has been demonstrated which is similar to that of other antibiotics in this class.[3d]

The aglycone of A35512B[3c] contains a biphenyl-type bis(amino acid), actinoidinic acid, at positions 5 and 7, which has previously been found in all other members of the vancomycin group and an ether-linked tris(amino acid) at positions 2, 4 and 6, which has also been found in all of the other members, although in some cases possessing a chlorine substituent on one or both β-hydroxytyrosines. A novel diphenyl ether-type bis(amino acid), **2**, comprises residues 1 and 3. Uncertainty has surrounded the structure of this fragment. On the basis of ^{1}H NMR studies of bis(benzoate ester) **3**, derived from oxidative degradation of the antibiotic (Scheme 1), the bis(amino acid) was first assigned structure **2a**[3a] but this structure was modified to **2b** on biosynthetic grounds and by analogy with the corresponding residue, ristomycinic acid (**4**),[4] in ristocetin.[3b,5] We were led to investigate this question further by the reported chemical shift (δ 6.73) of the protons assigned to positions *ortho* to the carbomethoxy group on the chlorinated ring of bis(benzoate ester) **3** which appeared to be too high field for either structure **3a** or **3b**. On the basis of synthetic studies involving the preparation of bis(benzoate esters) **3c** and **3d**, we now assign the structure of the bis(amino acid) as **2d**.

An initial study was carried out to ascertain that the relative locations of the OAr, OH, and glycyl substituents on the right-hand ring of **2** was indeed 1, 3, 5 rather than the previously postulated 1, 3, 2 or yet some other arrangement, before taking up the more difficult question of the location of the chloro substituent. Hydrolysis of the aglycone of A35512B was carried out with 57% HI in the presence of red phosphorus (Scheme 2). These conditions commonly dehalogenate activated aromatic rings. Previous investigations of these conditions with ristocetin had shown that the *para* sub-



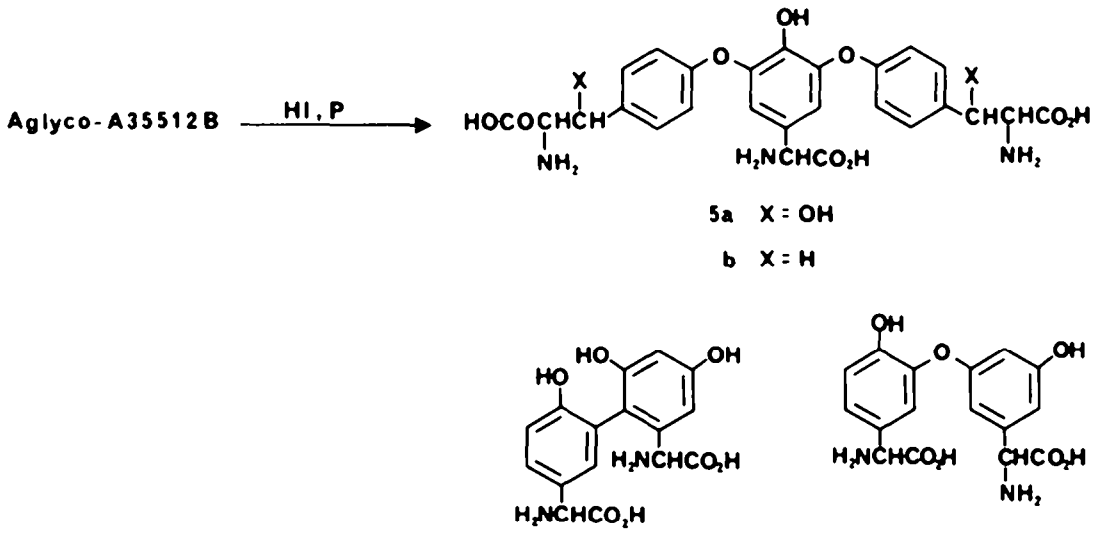
1a X_1 = Cl, X_2 = H
b X_1 = H, X_2 = Cl



Scheme 1.

stituted β-hydroxytyrosines in tris(amino acid) **5a** undergo hydrogenolysis of the β-OH groups.[6,7] The reductive degradation was of value in studies of the structure of ristocetin because the resulting *para* substituted phenylalanine residues of **5b** provide the only direct evidence for the presence of phenylserine moieties in **5a** since the latter undergo degradation under both the acidic and basic conditions normally employed for hydrolysis of the peptide. Separation of the A35512B hydrolysate by ion-exchange chromatography yielded tris(amino acid) **5b**, actinoidinic acid (**6**), and an additional bis(amino acid), the dechloro derivative of **2**, to which structure **7** was assigned on spectroscopic grounds.

The ^{1}H NMR spectrum of **7** was very similar to that of **2** except for the presence of an additional aromatic signal (δ 6.54) indicating that dechlorination had taken place. The ^{13}C NMR spectrum confirmed that one of the substituted aromatic carbons had been transformed into a proton-bearing carbon. Bis(amino acid) **7** was converted to O-methylated bis(benzoate ester) **8** by a sequence involving protection of the amino group (Ac_2O), methylation of the phenolic OH groups (CH_2N_2), hydrolysis back to the O-methylated amino acid (HCl), oxidative decarboxylation to the bis(benzonitrile) (NaOCl), hydrolysis to the bis(benzoic acid) (NaOH), and esterification (CH_2N_2). The chemical shifts and coupling constants of the protons on the right hand ring of **8** established the 1, 3, 5 arrangement of substituents consistent with structures **2b–d** for the native bis(amino acid). Finally the structure of **8** was confirmed by an independent synthesis involving Ullmann condensation

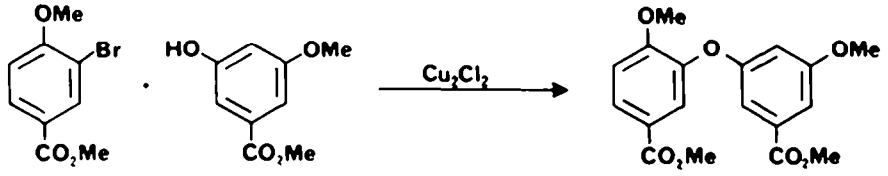


Scheme 2.

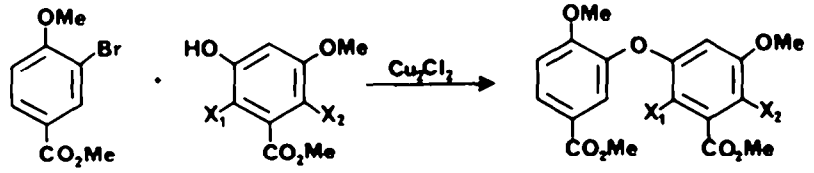
between methyl 3-hydroxy-5-methoxybenzoate and methyl 3-bromoanisate (Scheme 3).

Hydrolysis of aglyco-A35512B with constant boiling HCl gave amino acid **2**, as reported previously,[3b] and actinoidinic acid. Amino acid **2** was degraded to bis(benzoate) **3** as described above for amino acid **7**. NMR studies of **3** failed to lead to an unequivocal structure assignment, necessitating preparation of authentic samples of bis(benzoates) **3c** and **3d**.

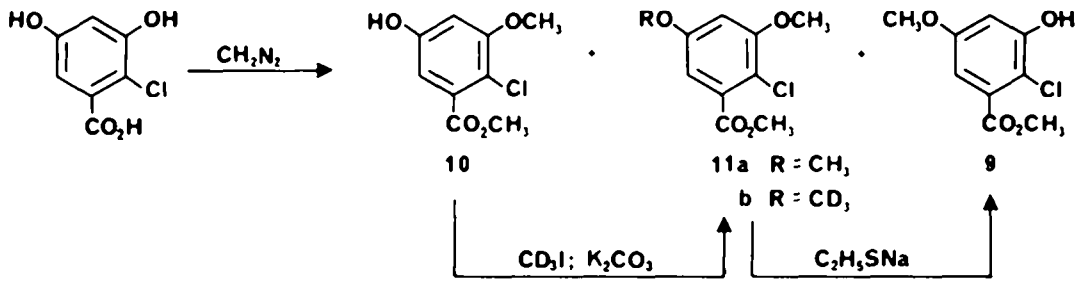
The synthesis of compounds **3c** and **3d** involved Ullmann coupling between methyl 3-bromoanisate and methyl *meta*-hydroxybenzoates **9** and **10**, respectively (Scheme 4). The latter compounds had not been reported previously. 2-Chloro-3,5-dihydroxybenzoic acid, prepared by chlorination of the dihydroxy acid with SO_2Cl_2, was treated with limited quantities of diazomethane to give a mixture of methylation products containing mainly monomethoxy ester **10** and dimethoxy ester **11a** along with a trace of the isomeric monomethoxy ester **9** (Scheme 5). Ester **9** was prepared in fair yield by demethylation of dimethoxy ester **11a** by treatment with sodium ethanethiolate. The structures of **9** and **10** were established by ^1H NMR. The spectrum of dimethoxy ester **11a** in $CDCl_3$(OMe signals at δ 3.79, 3.86, and 3.91 and aromatic signals at 6.61 and 6.82) was assigned with the aid of shift reagent and decoupling studies. The signals at 3.91 and 6.82 were assigned to the ester Me group and to the proton at C-6, respectively, from the fact that only they were shifted down-field significantly by the addition of $Eu(fod)_3$. Irradiation of the OMe at δ



Scheme 3.



Scheme 4.



Scheme 5.

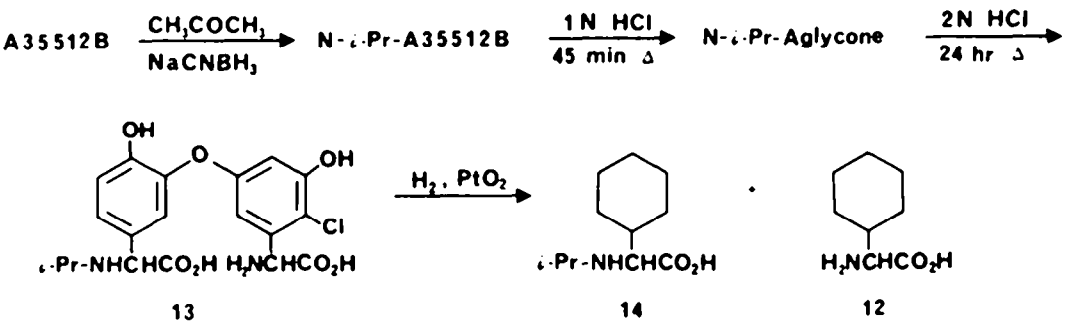
3.79 narrowed and enhanced the signal at 6.82 whereas irradiation of the OMe at δ3.86 had a similar effect on the signal at 6.61. Therefore the signals at δ 3.79, 3.86 and 6.61 can be assigned to the 5-OMe, the 3-OMe and 4-H, respectively. The spectrum of deuterated analog **11b**, prepared by alkylation of **10** with CD_3I, was identical to that of **11a** except that the signal at δ 3.79 ppm was missing. The selectivity observed in the demethylation of **11a** is consistent with that observed previously in the preparation of 2-bromo-3-hydroxy-5-methoxytoluene from 2-bromo-3, 5-dimethoxytoluene.[8]

Ullmann reactions of **9a** and **10** with methyl 3-bromoanisate proved to be very difficult to accomplish. Under all conditions investigated dechlorination and self-condensation competed severely with the cross coupling reactions. A variety of methods were investigated, most of which gave no trace of the desired products. Most satisfactory was the method of Williams *et al.*[9] employing Cu_2Cl_2 in pyridine which gave esters **3c** and **3d** in very low yields along with dechlorinated diester **8**. Bis(ester) **3** obtained from the antibiotic proved to be identical with **3d**. Therefore **2d** is the revised structure for the bis(amino acid) and **1b** the revised structure for the aglycone of A35512B.

The previous assignment of structure for the diester as **3b** by Debono and coworkers rested upon NMR studies.[1b] NOE determinations showed one of the aromatic protons of the right ring to be close to H-2 in the left one and the other proton on the right ring to be adjacent to an OMe group. These measurements were made in $CDCl_3$ in the presence of $Eu(fod)_3$, the presence of which was required to remove spectral degeneracy (in d_6-acetone the aromatic protons are well resolved; see experimental). It should be noted that these results are not inconsistent with structure **3d**. The latter structure should also show a NOE between the second proton (H-4) on the right ring and H-2 on the left one, but signal enhancement may be too small to be detected since the adjacent OMe group also participates in the relaxation process for H-4. The upfield shifts of the protons on the right hand ring of **3d**, which originally attracted our attention, are very similar to those of the corresponding protons in benzoates **9–11**.

The mechanism of action of antibiotics in this class depends not only upon the gross structure of the aglycone but also upon the configurations of the amino acids. The structure of A35512B is very similar to that of ristocetin A, the key difference being that ristocetin contains ristomycinic acid (**4**) as residues 1 and 3, instead of bis(amino acid) **2d**. The two antibiotics also differ in carbohydrate content but this difference is of less relevance to the peptide binding process. The absolute configurations of the two chiral centers of ristomycinic acid have been the subject of dispute. Lomakina *et al.*[10] originally assigned the terminal one as *R* but Williams *et al.* proposed on the basis of NMR studies that both residues 1 and 3 were *S*.[11a] We have obtained chemical evidence showing residues 1 and 3 are *R* and *S*, respectively. Williams, employing another experimental approach, has recently confirmed our results.[11b]

The absolute configurations of the two chiral centers in **2d** were shown to be opposing by reduction of the amino acid with Adams catalyst and hydrogen to give racemic cyclohexylglycine (**12**). A second experiment (Scheme 6) confirmed this result. The N-terminus was reductively alkylated[12] with acetone and $NaCNBH_3$ to give the N-isopropyl derivative of A35512B. Hydrolysis of the peptide with 2 N HCl gave the mono-N-isopropyl derivative of **2d** (**13**) which on reduction gave (S)-cyclohexylglycine and (R)-N-isopropylcyclohexylglycine (**14**).[13] This result in conjunction with NMR data obtained by Hunt[3c] showing close similarity in the pattern of peptide NOE's



Scheme 6.

between A35512B and ristocetin, supports the hypothesis that the two antibiotics have essentially identical peptide sequences, absolute configurations, and conformations.

EXPERIMENTAL

Mps were determined in open capillaries and are uncorrected. ^1H and ^{13}C NMR spectra were recorded with a JEOL FX-90Q spectrometer. Acetonitrile and t-BuOH were used as internal standards for spectra run in D_2O. TMS was used in $CDCl_3$. Optical rotations were measured with an Autopol III automatic spectropolarimeter. TLC was performed on Merck silica gel 60F-254. Open column chromatography was carried out on Merck silica gel 60F-254 or by flash chromatography.[14] Ion-exchange chromatography was carried out on a 0.9 × 50 cm column using Aminex AG-50W-X2 resin and 0.1 M pyridine-acetate buffer, pH 4.50, as the eluant. Elution was monitored either with a Uviscan III or by TLC(BuOH–AcOH–water, 6:2:2). Low resolution mass spectra were obtained on an LKB-9000A mass spectrometer. High resolution mass spectra were obtained on a VG Micromass 7070 mass spectrometer. Only the major (Cl^{35}) isotope peak is reported for Cl-containing fragments.

Preparation of A35512B aglycone. A35512B (1 g) in 1 N HCl (25 ml) was heated in a boiling water bath for 45 min and cooled in ice; the resulting ppt was filtered off, redissolved in water and lyophilyzed to give 0.5 g of aglycone. This probably corresponds to aglycone I described in Ref. 3*b*; i.e. it may contain amino sugar residues.

Hydrolysis of the aglycone in HI. Aglyco-A35512B (0.10 g) was suspended in 57% HI (8 ml) containing red P (80 mg) in a hydrolysis tube, frozen and thawed while being flushed with N_2, and heated at 105° *in vacuo* for 23 hr. The contents of the tube were diluted with water, transferred to a r.b. flask and the HI was removed *in vacuo*. Water was added to the residue, the suspension was filtered, and the filtrate was lyophilyzed. The residue was dissolved in 0.02 N HCl (1 ml) and subjected to ion-exchange chromatography. Actinoidinic acid (**6**, 18.1 mg), amino acid **7** (12.6 mg) and tris(amino acid) **5b** (5.2 mg) were eluted in that order. Actinoidinic acid and **5b** were identical by TLC and NMR to compounds isolated from ristocetin.[6,7]

Amino acid **7**: ^1H NMR (D_2O) δ 6.54 (m, 2H), 6.67 (m, 1H), 7.04–7.27 (m, 3H), the α protons were obscured by the H_2O peak at δ 4.82; ^{13}C NMR (D_2O) δ 58.8 (α, α'–C), 106.7 (4'–C), 109.5 (6'–C), 111.4 (2'–C), 119.6 (5–C), 122.8 (2–C), 127.5 (6–C), 127.6 (1–C), 137.7 (1'–C), 144.4 (4–C), 150.2 (3–C), 159.2 (3'/5'–C), 160.5 (5'/3'–C), 173.6 (both C=O's).

Hydrolysis of the aglycone in HCl. Aglyco-A35512B (0.20 g) was hydrolyzed with constant boiling HCl (20 ml) at 104° for 23 hr. Workup and ion-exchange chromatography were carried out as for the HI hydrolysis. Acid **6**, (26.9 mg) and **2d** (38.5 mg) were obtained with **2d** being eluted just after acid **6**.

Amino acid **2d**: ^1H NMR (D_2O) δ 4.69 (s, 1H), 5.12 (s, 1H), 6.57 (d, J = 2.7 Hz, 1H), 6.69 (d, J = 2.7 Hz, 1H), 7.03–7.25 (m, 3H); ^{13}C NMR (D_2O) δ 57.0 (α'–C), 59.3 (α–C), 107.9 (4'–C), 111.0 (2'–C), 116.6 (6'–C), 119.7 (5–C), 122.6 (2–C), 127.8 (6–C), 128.6 (1–C), 135.5 (1'–C), 144.3 (4–C), 150.0 (3–C), 155.1 (5'–C), 158.4 (3'–C), 173.3 (C=O'), 174.5 (C=O).

Degradation of amino acids **7** and **2d**. Acids **2d** (16.9 mg) and **7** (12.6 mg) were converted to their N-acetyl O-methyl derivatives by treatment with Ac_2O (0.12 ml) in MeOH (0.5 ml) at room temp. overnight followed by evaporation and treatment of the residue with excess diazomethane. The derivatives were filtered through short columns of silica gel (TLC grade) (eluant: CH_2Cl_2–MeOH, 96:4) and converted to the O-methylated free amino acids by refluxing 18 hr in 1 N HCl. These were degraded by treatment with "Clorox" (NaOCl) to the corresponding nitriles which were hydrolyzed and esterified to give bis(esters) **3d** and **8**.[4a] The esters were purified by TLC (pentane–EtOAc, 98:2) or column chromatography (pentane–EtOAc, 9:1). Bis(ester) **3d** (2.2 mg, 13%): TLC, ^1H NMR, and low resolution MS were identical to synthetic material (see below). HRMS: Calc for $C_{18}H_{17}O_7Cl$: 380.0663. Found: 380.0639. Bis(ester) **8** (2.2 mg, 18%): TLC, ^1H NMR and low resolution MS were identical to synthetic material (see below). HRMS: (Found: 346.1049. Calc for $C_{18}H_{18}O_5$: 346.1052.)

Synthesis of 2-chloro-3,5-dihydroxybenzoic acid. The title compound was prepared by a slight modification of the method of Lock and Nottes.[15] 3,5-Dihydroxybenzoic acid (3.2 g, 21 mmol), SO_2Cl_2 (2.8 g, 21 mmol), S_2Cl_2 (0.2 ml) and $AlCl_3$ (20 mg) were refluxed in anhyd ether (160 ml) for 4 hr. Evaporation of the solvent and recrystallization of the residue (H_2O) gave 1.6 g (41%) of the acid, m.p. 255–257° (Lit.[15] m.p. 249°).

Synthesis of esters **10** *and* **11a**. 2-Chloro-3,5-dihydroxybenzoic acid (4.8 g, 25 mmol) was treated with excess CH_2N_2 until TLC (pentane–EtOAc, 7:3) showed 2 main spots (no starting material), the slower of which gave a positive test with diazotized benzidine. The mixture was separated by flash chromatography (pentane–EtOAc, 8:2) to give 2.4 g (44%) of **10**, m.p. 128–131°, and 1.75 g (30%) of **11a**, a thick oil.

Ester **10** (*methyl 2-chloro-3-methoxy-5-hydroxybenzoate*): *m.p.* 133–135° after recrystallization from EtOAc–pentane; ^1H NMR (d_6-acetone) δ 3.86 (s, 3H), 3.88 (s, 3H), 6.73 (d, J = 2.7 Hz, 1H), 6.80 (d, J = 2.7 Hz, 1H), 8.89 (OH); ^{13}C NMR (d_6-acetone) δ 52.5, 56.8, 103.8, 109.2, 112.1, 133.8, 157.5, 157.6, 166.9. MS *m/e* 216 (82), 185 (100). (Found: C, 50.18; H, 4.24. Calc for $C_9H_9O_4Cl$: C, 49.90; H, 4.19%.)

Ester **11a** (*methyl 2-chloro-3,5-dimethoxybenzoate*):[16] ^1H NMR (d_6-acetone): 3.85 (s, 3H), 3.89 (s, 3H), 3.92 (s, 3H), 6.80 (d, J = 2.9 Hz, 1H), 6.85 (d, J = 2.9 Hz, 1H); ^1H NMR ($CDCl_3$) δ 3.79 (s, 3H), 3.86 (s, 3H), 3.91 (s, 3H), 6.61 (d, J = 3 Hz, 1 H), 6.82 (d, J = 3 Hz, 1 H); ^{13}C NMR (d_6-acetone) δ 52.6, 56.2, 56.9, 103.0, 107.0, 113.4, 133.9, 157.4, 159.9, 166.7. MS *m/e* 230 (100), 199 (83).

Synthesis of ester **9**. Ester **11a** (1.0 g, 4.3 mmol) was added to a soln of sodium ethanethiolate [prepared by adding ethanethiol (1.5 ml, 20 mmol) to a suspension of NaH (1.0 g, 21 mmol) in 40 ml of DMF].[17] The mixture was heated under N_2 at 75° for 28 hr, poured into cold dilute HCl, and extracted 3 times with EtOAc. The extracts were combined, dried, evaporated and esterified with methanolic HCl (reflux 6 hr). After removal of the solvent, the residue was purified by flash chromatography (CH_2Cl_2 eluant) to give 0.335 g (36%) of **9**, m.p. 68–69°.

Ester **9** (*methyl 2-chloro-3-hydroxy-5-methoxybenzoate*): m.p. 76–78° after recrystallization from EtOAc–pentane; ^1H NMR (d_6-acetone) δ 3.80 (s, 3H), 3.87 (s, 3H), 6.75 (d, J = 3 Hz, 1H), 6.83 (d, J = 3 Hz, 1H), 9.14 (OH); ^{13}C NMR (d_6-acetone)δ 52.6, 56.1, 105.9, 108.1, 111.8, 133.5, 155.4, 159.8, 166.8; MS *m/e* 216 (73), 185 (100). (Found: C, 50.02; H, 4.28. Calc for $C_9H_9O_4Cl$: C, 49.90; H, 4.19%.)

Synthesis of bis(ester) **3d**. The Na salt of **10** was prepared by addition of the ester (0.432 g, 2 mmol) to a soln of NaOMe [prepared from Na (45 mg, 1.95 mmol)]. After removal of the MeOH, the salt was dissolved in dry pyridine (10 ml); methyl 3-bromoanisate[18] (0.732 g, 3 mmol) and Cu_2Cl_2 (0.125 g) were added.[9] The mixture was heated under reflux for 18 hr, cooled and poured into cold 3 N HCl (10 ml). The acidic soln was extracted 3 times with EtOAc–MeOH (9:1). The combined extracts were washed once with brine, dried and evaporated. Starting materials were removed by sublimation. The dark residue was dissolved in CH_2Cl_2 and passed through a column of silica gel. The eluant was evaporated and purified by TLC (pentane:EtOAc, 8:2) to give **3d**, (9.5 mg, 1.2%): ^1H NMR (d_6-acetone) δ 3.82 (s, 3H), 3.85 (s, 3H), 3.90 (s, 3H), 3.91 (s, 3H), 6.68 (d, J = 2.8 Hz, 1H), 6.92 (d, J = 2.8 Hz, 1H), 7.30 (d, J = 8.6 Hz, 1H), 7.69 (d, J = 2.0 Hz, 1H), 7.93 (dd, J = 8.6 + 2.0 Hz, 1H); ^{13}C NMR (d_6-DMSO) δ 51.5, 52.1, 55.9, 56.6, 104.6, 108.4, 113.2, 113.8, 121.9, 122.6, 127.6, 132.5, 142.8, 155.0, 156.2, 156.6, 165.1(× 2); MS *m/e* 380 (100), 349 (45).

Synthesis of bis(ester) **3c**. The synthesis was carried out as for **3d** with the following quantities: **9** (0.216 g, 1 mmol), Na (21 mg, 0.91 mmol), methyl 3-bromoanisate (0.366 g, 1.5 mmol), Cu_2Cl_2 (66 mg), pyridine (4.5 ml). After workup and purification by TLC (CH_2Cl_2–pentane, 7:3), 0.85 mg (0.2%) of **3c** was obtained: ^1H NMR (d_6-acetone) δ 3.76 (s, 3H), 3.84 (s, 3H), 3.92 (s, 6H), 6.50 (d, J = 2.9 Hz, 1H), 7.06 (d, J = 2.9 Hz, 1H), 7.30 (d, J = 8.5 Hz, 1H) 7.58 (d, J = 2 Hz, 1H), 7.92 (dd, J = 8.5 + 2.0 Hz, 1H); MS *m/e* 380 (100), 349 (51). HRMS: (Found: 380.0650. Calc for $C_{18}H_{17}O_7Cl$: 380.0663.)

Synthesis of bis(ester) **8**. The synthesis was carried out as above with the Na salt of methyl 3-hydroxy-5-methoxybenzoate[19] (0.099 g, 0.49 mmol), methyl 3-bromoanisate (0.159 g, 0.65 mmol), Cu_2Cl_2 (25 mg) in pyridine (1.5 ml). Workup as above and purification by column chromatography on silica gel (pentane–$CHCl_3$, 7:3) gave **8** (23.6 mg, 14%) as a clear oil: ^1H NMR (d_6-acetone) δ 3.83 (s, 3H), 3.84 (s, 6H), 3.89 (s, 3H), 6.74 (t, J = 2.4 Hz, 1H), 7.02 (J = 2.4 + 1.5 Hz, 1H), 7.24 (dd, J = 2.4 + 1.5 Hz, 1H), 7.29 (d, J = 8.5 Hz, 1H), 7.67 (J = 2.2 Hz, 1H), 7.92 (dd, J = 8.5 + 2.2 Hz, 1H); ^{13}C NMR (d_6-acetone) δ 52.2, 52.5, 56.1, 56.6, 108.2, 109.6, 110.0, 113.8, 123.7, 124.2, 128.7, 133.4, 144.5, 156.8, 160.2, 162.0, 166.4, 166.6; MS *m/e* 346 (100), 315 (20).

Reduction of amino acid **2d**. Acid **2d** (20.2 mg), prepared by hydrolysis of 200 mg aglycone in 2 N HCl (24 hr, 107°),[13] was reduced with PtO_2 (20 mg) as the catalyst. After removal of the catalyst by filtration, the filtrate was lyophilyzed and separated by ion exchange chromatography to give **12** (5.6 mg) as the only isolable amino acid: $[\alpha]_D^{24}$ 0° (*c* 0.20, 0.2 N HCl).

Preparation and reduction of N-isopropyl amino acid **13**. A35512B (0.500 g) was stirred overnight with acetone (1.0 ml) and $NaCNBH_3$ (0.174 g) in H_2O (2.0 ml, pH 7).[12] The reaction was acidified and lyophilyzed. The aglycone was prepared as above to give N-isopropylated aglyco-A35512B. The aglycone was hydrolyzed in 2 N HCl (24 hr, 106°);[13] the hydrolysate was separated by ion-exchange chromatography to give **13** (25.4 mg): ^1H NMR (D_2O + DCl) δ 1.23 (d, J = 6.3 Hz, 6H), 3.24 (m, J = 6.3 Hz, 1H), 5.04 (s, 1H), 5.43 (s, 1H), 6.64 (m, 2H), 7.04–7.31 (m, 3H); $[\alpha]_D^{22}$ -24.7° (c 1.01, 0.2 N HCl).

Reduction of **13** (17.8 mg) with PtO_2 as above followed by ion-exchange chromatography gave **14** (3.2 mg): ^1H (D_2O)δ 1.36 (d, J = 7 Hz), 1.40 (d, J = 7 Hz), 1.84 (m), 3.47 (m, J = 7 Hz), 3.57 (d, J = 5 Hz, α-CH); $[\alpha]_D^{22}$ -13.9° (c 0.64, 0.2 N HCl) [synthetic *R*(−)-N-isopropylcyclohexylglycine prepared from *R*(−)-phenylglycine had an identical ^1H NMR spectrum and $[\alpha]_D^{22}$ -18° (*c* 0.74, 0.2 N HCl)] followed by cyclohexylglycine (**13**, 3.2 mg): ^1H NMR (D_2O) δ 1.12–1.29 (m), 1.67 (m), 3.85 (d, J = 5 Hz, α-CH); $[\alpha]_D^{24}$ + 12.5° (*c* 0.32, 0.2 N HCl) (Lit.[20] $[\alpha]_D^{24}$ -35° (*c* 1.0, 5 M HCl) for *R* isomer).

Note added in proof. Dr. N. Jones (Eli Lilly) has informed us that the structure of diester **3d** has been confirmed by X-ray crystallography.

Acknowledgements—We wish to thank Dr. M. Debono, D. E. Dorman, N. Jones and A. H. Hunt at Eli Lilly for the supply of A35512B, access to unpublished information, and many helpful discussions. High resolution mass spectra were provided by Dr. Brian Sweetman. This research was supported by the U.S. Public Health Service (GM-25793). The VG Micromass 7070 mass spectrometer was purchased with funds from the U.S. Public Health Service (GM-27557).

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