Serratiochelin, a New Catecholate Siderophore from *Serratia marcescens**

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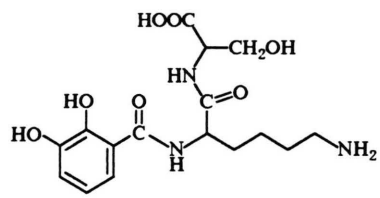
The catecholate siderophore serratiochelin isolated from an iron deficient culture medium of *Serratia marcescens* TW was characterized by mass spectrometry and NMR and by GC/MS analysis of the hydrolysis products as 1-(2,3-dihydroxybenzamido)-3-[4 *S*,5 *R*-2-(2,3-dihydroxyphenyl)-5-methyl-2-oxazoline-4-carboxamido]propane. The structure of serratiochelin was confirmed by synthesis. The bacterial strain also produces chrysobactin.

Introduction

In any natural environment of microbial activity siderophores are present produced by microorganisms to supply the cell with iron. Siderophores using 2,3-dihydroxybenzoyl (DHB) units for Fe^{3+} complexation are combined in the catecholate group. Since the first description of a catecholate siderophore (itoic acid (Ito and Neilands, 1958)) many others have been isolated and characterized. They contain up to 3 DHB units and are, therefore, subdivided into tricatecholates (*e.g.* enterochelin (O'Brien and Gibson, 1970; Pollack and Neilands, 1970), protochelin (Taraz *et al.*, 1990)), dicatecholates (*e.g.* azotochelin (Corbin and Bulen, 1969)), and monocatecholates (*e.g.* chrysobactin (Persmark *et al.*, 1989)).

The present paper deals with the isolation and characterization of catecholate siderophores of *Serratia marcescens* TW. This strain produces two catecholates under iron deficiency: chrysobactin (**1**) isolated previously from a culture medium of *Erwinia chrysanthemi* (Persmark *et al.*, 1989) and serratiochelin (**2a**) a hitherto unknown dicatecholate.



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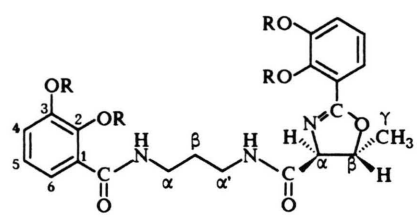

2a: R=H
2b: R=CH_3

Results and Discussion

The strain *Serratia marcescens* TW produces catecholate siderophores as shown by the UV spectrum of a cell-free culture medium and proved by the addition of aqueous $FeCl_3$ solution to the culture, which causes a color change to violet. The catecholates were isolated by adsorption on XAD-2 resin and desorbed with CH_3OH/H_2O and subsequently with CH_3OH. The first fraction contains chrysobactin, which was identified by NMR, amino acid analysis and FAB-MS, the CH_3OH fraction **2a.** Both catecholates were purified by chromatography on polyamide.

The UV spectrum of a solution of **2a** in CH_3OH shows absorption maxima at 316 ($\log \varepsilon = 3.8$) and 254 nm ($\log \varepsilon = 4.3$) characteristic for catecholates.

* Part LIX of the series "Bacterial Constituents". For part LVIII see Jacques, P., Gwose, I., Seinsche, D., Taraz, K., Budzikiewicz, H., Schröder, H., Ongena, M., and Thonart, P. (1993). Nat. Prod. Letters *3*, 213–218.

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Catecholate siderophores are known to be sensitive to oxidation (Hider, 1984) and polymerization (Leong and Neilands, 1982). Therefore **2a** was permethylated (**2b**) before hydrolysis and subsequently TAB (N/O-trifluoroacetyl-*n*-butyl ester)-derivatized to determine its composition by GC/MS. The derivatives of threonine (Thr), 2,3-dihydroxybenzoic acid (DHBA) and 1,3-diaminopropane (DAP) were identified by their mass spectra.

Thr is L-configurated as determined by co-injection of TAP (N/O-trifluoroacetyl-2-propyl ester)-derivatized **2b** hydrolysate with TAP-D,L-Thr as reference.

Analysis of the EI mass spectra of **2a** ($M^{+\cdot}$, *m/z* 429) and **2b** ($M^{+\cdot}$, *m/z* 485) leads to the following conclusions:

a) The molecular mass of **2b** is 56 u higher than that of **2a.** This difference corresponds to 4 additional methyl groups introduced by methylation of the hydroxyl groups of **2a.** Thus **2a** contains two DHB units (no salicylo unit as in parabactin (Peterson and Neilands, 1979) was detected by GC/MS in the hydrolysate of **2b**). This result agrees with the ^{1}H NMR spectrum of **2a** (Fig. 1), which shows two AMX spin systems between 6.6 and 7.2 ppm.

(b) The molecular mass of **2a** is 18 u lower than calculated for a non-cyclic molecule composed of 1 Thr, 2 DHBA, and 1 DAP which suggests a structure formed by the loss of one molecule of H_2O (*e.g.* an additional ester or amide bond). Since **2a** contains Thr and DHBA the presence of an oxazoline ring as in agrobactin (Ong *et al.,* 1979), parabactin (Peterson and Neilands, 1979), vibriobactin (Griffiths *et al.,* 1984), and fluvibactin (Yamamoto *et al.,* 1993) seemed to be a possibility. This assumption is supported by the ion *m/z* 192 in the mass spectrum of **2a** (Fig. 2) (*m/z* 220 for **2b**). Other characteristic fragments are *m/z* 137 (2,3-dihydroxybenzoyl cat-

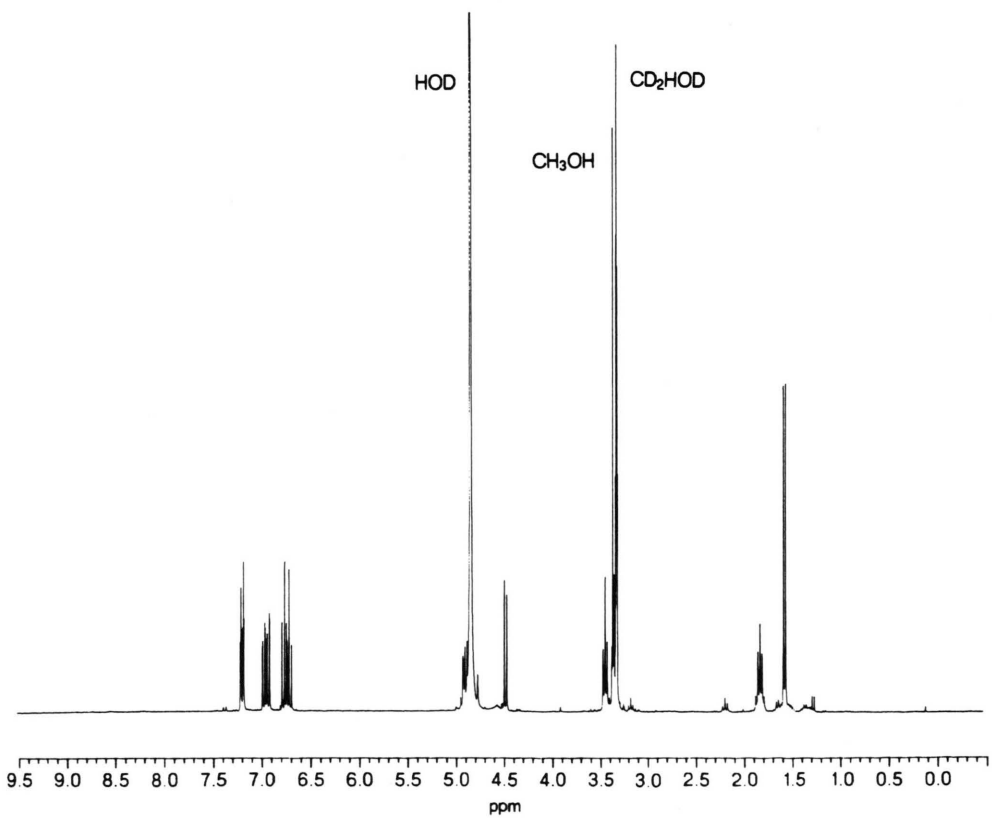

Fig. 1. 300 MHz ^{1}H NMR spectrum of **2a** in CD_3OD.

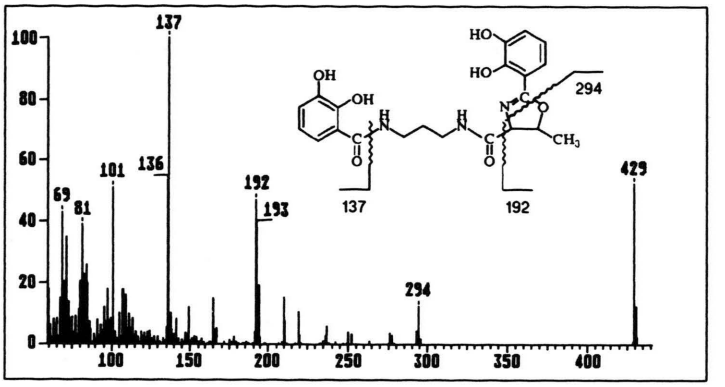

Fig. 2. EI mass spectrum (70 eV) of **2a**.

ion) accompanied by *m/z* 136 (H-loss, typical for *o*-hydroxybenzoic acid derivatives (Budzikiewicz *et al.*, 1967)). Loss of 2,3-dihydroxybenzoyl nitrile leads to *m/z* 294.

The ^{1}H and ^{13}C NMR data of **2a** are summarized in Tables I and II. The ^{1}H NMR spectrum (Fig. 1) shows two overlapping AMX spin systems between 6.6 and 7.2 ppm. Correlation with the 2,3-dihydroxybenzoyl (DHB) and the 2,3-dihydroxyphenyl oxazoline (DHPO) systems was possible by $^3J_{C,H}$ cross peaks (*e.g.*, between H-5 at 6.7 ppm and C-1 at 116.6 ppm). Comparison of the chemical shifts of Thr protons for **2a** with these of agrobactin and agrobactin A (Peterson *et al.*, 1980) allows a differentiation between an open N-benzoyl and a cyclic (oxazoline) structure in favor of the latter.

Table I. ^{1}H NMR data of **2a**.

Proton	δ [ppm]	Mult.[a]	Coupling constant |*J*| [Hz]
DHB[b]			
H-4	6.91	dd	$^4J = 1.4$, $^3J = 7.9$
H-5	6.70	t	$^3J = 8.0$, $^3J = 8.0$
H-6	7.18	dd	$^4J = 1.6$, $^3J = 8.1$
DHPO[b]			
H-4	6.96	dd	$^4J = 1.6$, $^3J = 7.9$
H-5	6.74	t	$^3J = 7.9$, $^3J = 7.9$
H-6	7.18	dd	$^4J = 1.7$, $^3J = 8.0$
Thr[c]			
α-CH	4.45	d	$^3J = 7.5$
β-CH	4.85	m	
γ-CH_3	1.55	d	$^3J = 6.3$
DAP			
α-CH_2[d]	3.42	t	$^3J = 6.6$
α′-CH_2[d]	3.33	t	$^3J = 6.6$
β-CH_2	1.80	qi	$^3J = 6.6$

[a] *Abbreviations:* d, doublet; t, triplet; q, quartet; qi, quintet; m, multiplet; dd, double doublet.
[b] Assignments by homonuclear resonance experiments and long range couplings.
[c] Comparison values for agrobactin (4.6, 5.4, and 1.4 ppm (Peterson *et al.*, 1980)), agrobactin A (N-(2,3-dihydroxybenzoyl)-Thr unit) (5.0, 4.2, and 1.2 ppm (Peterson *et al.*, 1980)), and fluvibactin (4.85, 5.29, and 1.43 ppm (Yamamoto *et al.*, 1993)).
[d] Correlation by long range couplings with the corresponding CO groups.

Table II. ^{13}C NMR data of **2a**.

Carbon	δ [ppm]
DHB	
C-1[a,b]	116.6
C-2[a]	150.4
C-3[a]	147.4
C-4[c]	119.7
C-5[c]	119.6
C-6[c]	118.5
CO[b]	171.8
DHPO	
C-1[a,b]	111.8
C-2[a]	149.6
C-3[a]	146.8
C-4[c]	120.4
C-5[c]	120.0
C-6[c]	119.9
O<u>C</u>N[b]	168.4
Thr	
α-CH	75.8
β-CH	80.7
γ-CH_3	21.4
CO[b]	173.2
DAP	
α-CH_2[a,c]	37.5
α′-CH_2[a,c]	37.6
β-CH_2	30.3

[a] Assignment by ^{1}H,^{13}C long range couplings.
[b] See text.
[c] Assignment by ^{1}H,^{13}C COSY.

The fact that the CH protons of the oxazoline ring are *trans* (as shown by a differential NOE experiment) confirms that **2a** comprises Thr (and not *allo*-Thr) in agreement with the GC/MS data.

The signals of the amide and imino ether ^{13}C-atoms (Table II) can be assigned by comparison with reference compounds: The signal at 173.2 ppm belongs to the amide bond of Thr (*cf.* 172.7 ppm for peptidically bound Thr (Wüthrich, 1976)) as confirmed by a $^3J_{C,H}$ correlation with the α′-CH_2 protons of DAP (3.33 ppm), and a $^2J_{C,H}$ correlation with the α-CH proton of Thr. The signal at 171.8 ppm belongs to the DHB unit (*cf.* 170.8 and 171.5 ppm for protochelin (Taraz *et al.*, 1990), 171.6 ppm for **8c**). The signal at 168.4 ppm has to be attributed to the oxazoline ring (confirmed by a $^3J_{C,H}$ correlation with the α-CH proton of Thr (*cf.* 166.8 ppm for fluvibactin (Yamamoto *et al.*, 1993)). The signal at 116.6 ppm (quarternary by DEPT) can be attributed to C-1 of DHB by comparison with analogous signals in the spectrum of protochelin (Taraz *et al.*, 1990) (between 116.6 and 117.3 ppm), **8c** (116.5 ppm), and fluvibactin (Yamamoto *et al.*, 1993) (115.7 ppm). Therefore the signal belonging to a quarternary C (DEPT) at 111.8 ppm has to be attributed to C-1 of DHPO (*cf.* 110.9 ppm for fluvibactin (Yamamoto *et al.*, 1993)).

All these data agree with the structure **2a** proposed for serratiochelin. The final proof was obtained by its synthesis (Scheme 1). Identity of synthetic **2a** with the natural product was shown by MS, UV, and NMR. The route chosen for the synthesis of **2a** was similar to the one described for agrobactin (Bergeron *et al.*, 1985).

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Experimental

UV/VIS spectroscopy

Perkin-Elmer (Überlingen, Germany) Lambda 7 or Perkin-Elmer Hitachi 200 spectrophotometer.

NMR spectroscopy

Bruker (Rheinstetten, Germany) AM 300 or AC 300 NMR spectrometer (300 MHz ^1H NMR, 75.5 MHz $^{13}C\{^1H\}$ NMR), samples were dissolved in CD_3OD, signals were assigned by homonuclear decoupling experiments, reverse $^1H,^{13}C$ correlation (Aue *et al.*, 1976) (spectral widths: 3650 Hz (12 ppm) in F_2 and 11,013 Hz (146 ppm) in F_1; after 2 dummy scans each t_1 experiment was made of 104 scans with additional GARP decoupling during acquisition), and long range couplings (determined in a not decoupled HMBC (heteronuclear multiple-bond correlation) (Bax and Summers, 1986); spectral widths: 3650 Hz (12 ppm) in F_2 and 12,788 Hz (169 ppm) in F_1; 4 starting dummy scans, 64 scans for each t_1, and a 80 ms delay ($J_{C,H}$ = 6.3 Hz) for evolution of long range couplings).

Mass spectrometry

FAB-MS: Varian (Bremen, Germany) MAT 731 equipped with a Ion Tech. Ltd. (Teddington, U.K.) FAB gun, Xe was used as collision gas, glycerol as matrix; EI-MS: Varian MAT 212; GC/MS: Kratos (Manchester, U.K.) MS 25 RFA with a Carlo Erba (Milano, Italy) Mega-GC HRGC 5150, FS-SE-54-CB-0.1 capillary column (25 m × 0.32 mm ID) (CS, Langerwehe, Germany), temp. program: 70 °C (3 min) – 270 °C (7 min), 10 °/min.

HPLC

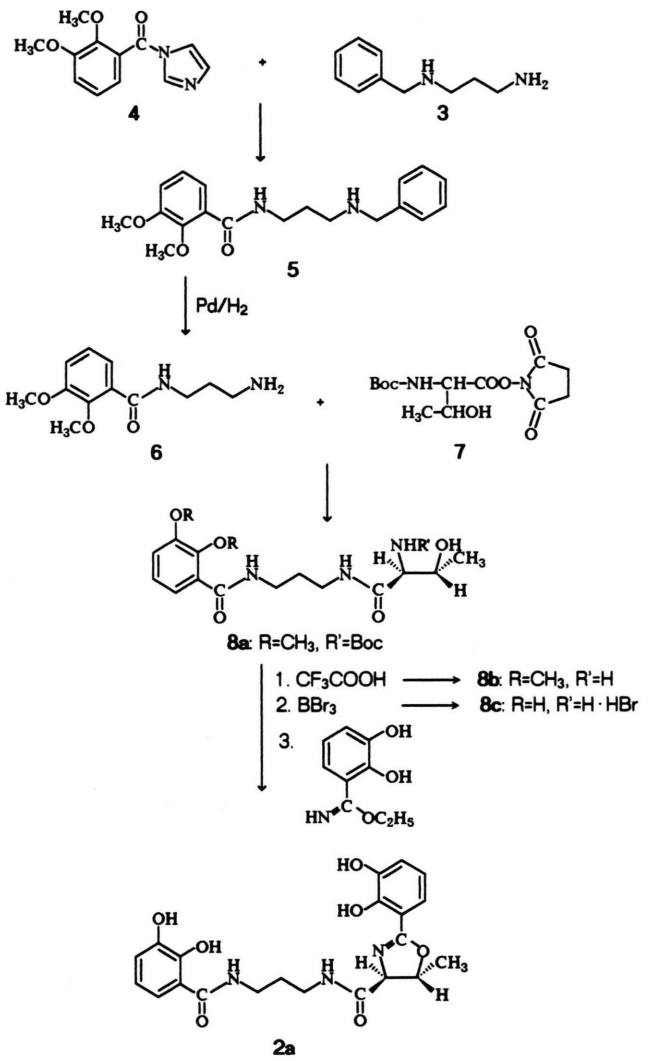
Chromatographic pump 8800 (DuPont, Bad Nauheim, Germany) and a UV spectrophotometer (DuPont).

GC

Carlo Erba HRGC 4160 with a FID detector and equipped with a Shimadzu (Kyoto, Japan) Chromatopac C-R 3 A integrator, FS-WCOT-chirasil-L-Val-0.12 capillary column (25 m × 0.25 mm ID) (Chrompack, Middelburg, Netherlands), temp. program: 70 °C (5 min) – 190 °C (10 min), 5 °/min, carrier gas was He at a pressure of 7×10^4 Pa, the temperature of the injector was 250 °C and of the detector (FID) 300 °C.

Chromatography

ISCO-UA-5 absorbance monitor (ISCO, Lincoln, U.S.A.).



Scheme 1. Synthesis of serratiochelin.

Growth conditions

Serratia marcescens TW was grown in a medium of low iron content composed per liter of 13 g Na-gluconate; 4 g KH_2PO_4, brought to pH 7.2 by adding 40% aqueous KOH solution; 5 g $(NH_4)_2SO_4$; 0.5 g $MgSO_4 \cdot 7\,H_2O$. Cultures were incubated in 3000 ml fermenters containing 1000 ml of the medium which were mounted on a rotary shaker at room temp. for 45 to 50 h (maximum of catecholate production as determined by extinction measurement at 330 nm of a centrifuged culture sample), after which the cultures were acidified to pH 5.4 with 6 N hydrochloric acid and the cells were removed by tangential filtration.

Isolation and purification of **2a**

The supernatant was passed slowly through a XAD column (type 2, particle size 0.3–1 mm; Serva, Heidelberg, Germany). After washing with H_2O (approximative 5 l) adsorbed chrysobactin was eluted with CH_3OH/H_2O (1/1; v/v) and subsequently **2a** with CH_3OH. Fractions were evaporated to dryness under reduced pressure at 25 °C. **2a** was dissolved in a minimal volume of CH_3OH and chromatographed on a polyamide 6 S column (Riedel DeHaën, Seelze, Germany) with CH_3OH/H_2O (4/1; v/v) (detection: absorption at 254 and 310 nm). The catecholate containing fraction was evaporated *i.v.* to dryness and re-chromatographed on a fresh

polyamide 6 S column with CH_3OH/H_2O (1/1; v/v). The main fraction was evaporated *i.v.* to dryness and **2a** was stored at −25 °C.

Methylation of **2a** was performed as described elsewhere (Taraz *et al.*, 1990). The reaction mixture was evaporated to dryness, the residue dissolved in CH_3OH and **2b** was purified by HPLC on a polygosil 60−10 column (Macherey & Nagel, Düren, Germany) with hexane/2-propanol (7/2; v/v; 10.5 ml/min).

Hydrolysis of **2b**

2b was hydrolyzed with 6 N hydrochloric acid (24 h, 110 °C) and TAB-derivatized as described elsewhere (Taraz *et al.*, 1990). The reaction mixture was analyzed by GC/MS and the components were identified by their EI-mass spectra and retention times. As a reference compound DAP was trifluoroacetylated and characterized by GC/MS.

Configuration of Thr

The hydrolysate of **2b** was TAP-derivatized (Taraz *et al.*, 1990) and analyzed gas chromatographically by co-injection with D,L-Thr derivatized in the same way.

Synthesis

Reactions were performed under N_2 where necessary. Solvents were distilled and dried according to usual methods.

1-Amino-3-(2,3-dimethoxybenzoyl)aminopropane (**6**). To a solution of 2,3-dimethoxybenzoylimidazole (**4**) prepared by stirring 1.82 g 2,3-dimethoxybenzoic acid and 1.62 g N,N′-carbonyldiimidazole in 30 ml dry CH_2Cl_2 for 1 h (ceasing CO_2 evolution) at room temp. 1.66 g N-(3-aminopropyl)benzylamine (**3**) (Ueda and Ishizaki, 1967) were added. The reaction mixture was stirred for 24 h, diluted with 100 ml CH_2Cl_2 and washed with 100 ml 1.25 N aqueous NaOH solution and 100 ml H_2O. After drying with Na_2SO_4 the solvent was distilled off. The oily 1-benzylamino-3-(2,3-dimethoxybenzoyl)aminopropane (**5**) ($M^{+\cdot}$, *m/z* 328) (1.96 g) without further purification was dissolved in 140 ml ethanol and hydrogenated with 10% Pd on C (prehydrogenated for 2 h) for 3 days. The catalyst was filtered off and the solvent removed by distillation. Yield 0.87 g (62%) of oily **6** ($M^{+\cdot}$, *m/z* 238).

1-(L-N-t-Butoxycarbonyl-threonyl)-3-(2,3-dimethoxybenzoyl)aminopropane (**8a**). At 0 °C to a solution of 0.71 g **6** in 100 ml dry dimethylformamide (DMF) 1 g N-(L-N-*t*-butoxycarbonyl-threonyloxy)succinimide (**7**) (Bergeron and Kline, 1984) in 30 ml dry DMF were quickly added under stirring. After warming to room temp. and stirring for 48 h the solvent was distilled off *i.v.* The residue was dissolved in 70 ml CH_2Cl_2, washed three times with 20 ml 3% aqueous hydrochloric acid and six times with 20 ml H_2O, dried with Na_2SO_4, freed from the solvent by distillation and purified by HPLC on polygosil 60-2540 C_{18}, (Macherey & Nagel) with CH_3OH/H_2O (3/2, v/v). Yield 623 mg (47%) of a viscous oil (**8a**) ($M^{+\cdot}$, *m/z* 439).

1-(2,3-Dimethoxybenzoyl)amino-3-L-threonylaminopropane (**8b**). After stirring 220 mg **8a** for 1 h at room temp. with 100 ml trifluoroacetic acid the reagent was distilled off. The residue was dissolved in CH_2Cl_2 and the solvent distilled off, this procedure was repeated several times. Finally the residue was dissolved in 100 ml CH_2Cl_2, washed three times with 50 ml of an ice-cold 3 M aqueous Na_2CO_3 solution, dried with Na_2SO_4 and freed from the solvent by distillation. Yield 139 mg (82%) of a viscous oil (**8b**).

1-(2,3-Dihydroxybenzoyl)amino-3-L-threonylaminopropane hydrobromide (**8c**). At 0 °C 136 mg **8a** in 30 ml dry CH_2Cl_2, were dropped under stirring to 15 ml of a 1 M solution of BBr_3 in CH_2Cl_2. After warming slowly to room temp. the mixture was stirred for 16 h, again cooled to 0 °C and 15 ml ice-cold H_2O were added dropwise. After stirring for 1 h at room temp. the aqueous layer was separated and brought to dryness *i.v.* The residue was dissolved several times in CH_3OH, brought to dryness and finally chromatographed on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) with CH_3OH/H_2O (7/3; v/v). Yield 109 mg (70%) of a glassy solid (**8c**) ($[M+H]^+$ by FAB-MS, *m/z* 312).

1-(2,3-Dihydroxybenzamido)-3-[4S,5R-2-(2,3-dihydroxyphenyl)-5-methyl-2-oxazoline-4-carboxamido]propane (**2a**). 78 mg **8c** and 181 mg 2,3-dihydroxybenzimido acid ethyl ester (Bergeron *et al.*, 1985) in 35 ml dry CH_3OH were refluxed for 40 h. The reaction mixture was brought to dryness *i.v.* and the residue chromatographed on Sephadex LH-20 with CH_3OH/H_2O (7/3; v/v). Yield 53 mg (62%) of **2a** which proved identical with natural **2a** (MS, ^1H and ^{13}C NMR, chromatographic behavior).

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