Novel Triterpene Sulfates from *Fusarium compactum* Using a Rhinovirus 3C Protease Inhibitor Screen

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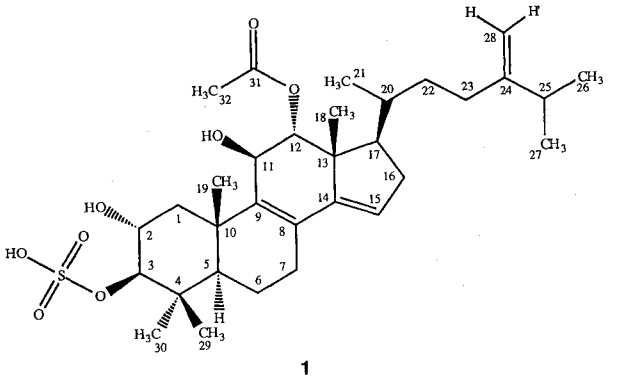
Two novel triterpene sulfates have been isolated from *Fusarium compactum* by bioactivity-directed fractionation using an assay which measures the inhibition of proteolytic activity of rhinovirus 3C protease on a fluorogenic peptide substrate. The compounds were purified by countercurrent and reverse phase chromatographies. NMR, MS, UV and IR studies revealed two triterpene sulfates, uncommon metabolites of terrestrial fungi.

In the course of screening microorganisms for the production of bioactive metabolites, a *Fusarium compactum* sp., isolated from an ant hill soil sample collected near the village of Rantan, Nigeria, was found to produce rhinovirus 3C protease inhibition activity. The 3C protease, one of two virally encoded proteases, is responsible for cleaving the initially translated rhinoviral polyprotein at eight locations to form the mature structural proteins and viral replicative enzymes.[1] There are more than 100 known serotypes of human rhinovirus,[2] and they are thought to be responsible for 40~60% of the common colds.[3] In principle, compounds which inhibit the proteolytic action of the 3C protease would disrupt replication of the rhinovirus, and could lead to an effective drug treatment for the common cold.

Using an assay which measures the inhibition of 3C protease activity on a synthetic fluorogenic substrate, bioactivity-directed fractionation of a stationary fermentation extract of *Fusarium compactum* (AB 2194I-103) provided two triterpene sulfates. Triterpene sulfates had not been reported as bioactive metabolites from terrestrial fungi until Vesonder and Burmeister isolated them from several *Fusarium* species in a search for potentially phytotoxic secondary metabolites.[4,5] It was found that these metabolites are produced in relative abundance when terrestrial *Fusarium* species are grown on grain substrates.[5] In our study, Shredded Wheat was used as a solid support for the growth of the fungus.

The major bioactive compound isolated in this study, A-108835 [4,4,24-trimethylcholesta-Δ8,9; 14,15; 24,28-trien-2α,3β,11β,12α-tetrol-12-acetate, 3-sulfate] (**1**), is diastereomeric with 4,4,24-trimethylcholesta-8,14,24(28)-trien-2α,3β,11α,12β-tetrol-12-acetate, 3-sulfate, a compound isolated as the sodium salt from *Fusarium graminearum* grown on white corn grits.[4] They differ only by the stereochemistry at the C-11 and C-12 positions. The structure of A-108835 was confirmed by NMR, MS, UV and IR studies. The minor compound, A-108836 [4,4,24-trimethylcholesta-Δ7,8; 9,11; 24,28-trien-2α,3β,12α-triol-12-*O*-methyl, 3-sulfate] (**2**) differs



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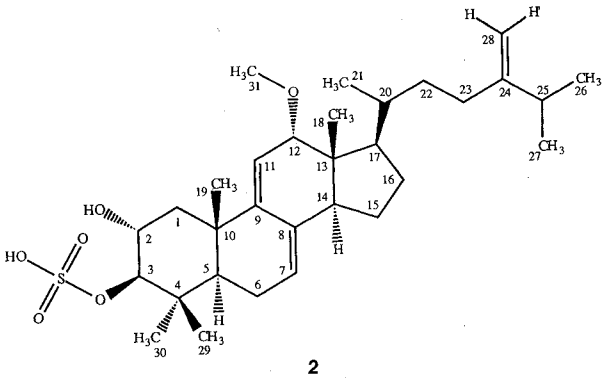

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from A-108835 in the arrangement of the heteroannular diene; encompassing the 7,8; 9,11 positions of rings B and C instead of the 8,9; 14,15 positions of the C and D rings. In addition, A-108836 is missing the hydroxyl at C-11 and has a methoxy group at C-12 instead of an *O*-acetyl. In this manuscript, the characterization and fermentation of the producing strain and the isolation, structural determination and some biological data are outlined for these triterpene sulfates.

Characterization of the Producing Strain

Strain AB 2194I-103 grew rapidly, and colonies attained a diameter of 75~80 mm after seven days on potato dextrose agar (PDA, Difco). The culture produced abundant cottony aerial mycelia, and the colonies were white (N 9.5) to pale orange yellow (10Y/R 8/4). The reverse was colored moderate orange (7.5Y/R 6/10) in the center to pale yellow (2.5Y 8/4) at the edge. On corn meal agar (Difco), colonies grew to 65~68 mm in diameter. The mycelia was very sparse, colorless and translucent. No soluble pigment was produced on either of these media.

Strain AB 2194I-103 did not sporulate on either medium at seven days, but it did form spores as it aged. After a month on PDA, a mount of the hyphae in lactophenol showed macroconidia, microconidia and chlamydospores. The macroconidia measured 3~4×70~100 μm and had 8~11 septa. The apical cells were long, whip-like and typically curved. The basal cells were foot shaped. Microconidia were 1.2~2×8~14 μm, usually with a single septum, but occasionally with none. Chlamydospores were produced terminally, intercalary and in clumps. They were spherical to elliptical and typically measured 6~10×8~10 μm.

Fermentation

RVI-136 was produced by solid state fermentation in a modification of the procedure described previously.[6] *Fusarium compactum* AB 2194I-103 was maintained as frozen mycelium at −70°C and used at 1% to inoculate 500-ml Erlenmeyer seed flasks containing 100 ml of a tomato paste-oat flour medium described by GOETZ *et al.*[7] The seed flasks were incubated on a rotary shaker at 225 rpm at 28°C for 72 hours. Four 20-liter glass carboys were used as the fermentation vessels. Spoon Size Shredded Wheat, which had been defatted with ethyl acetate, was dispensed at 300 g per carboy, and the carboys were sterilized for 45 minutes at 121°C. The fermentation medium consisting of glycerol 1.2%, molasses 1.2%, peptone (Difco) 0.48%, yeast extract (Difco) 0.12%, NaCl 2.4%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.4%, $FeSO_4 \cdot 7H_2O$ 2.4 ppm, and $CuSO_4 \cdot 5H_2O$ 0.8 ppm was prepared in distilled water. The medium was dispensed at 360 ml in 1-liter Erlenmeyer flasks and sterilized for 45 minutes at 121°C. For inoculation of each carboy, 60 ml of the 72-hour seed growth was mixed with 360 ml of fermentation medium. The inoculated medium was transferred to the Shredded Wheat in the carboy. Following mixing to distribute the inoculum, the carboys were incubated at 20°C for twenty-one days.

Extraction and Isolation

At harvest, 300 ml of acetone was poured onto the fungal growth in each carboy. After six hours, 375 ml of ethyl acetate-toluene (1:1) was added to each vessel. The carboys were held at 4°C overnight. Solvent was drawn off and the marc in each carboy was soaked and extracted three times with an additional 1 liter of acetone. All extracts were combined and concentrated on a circulating flash evaporator until 1 liter of an essentially aqueous residue remained. This was then extracted with four 1/2 volumes of ethyl acetate. Based on bioassay, the first three ethyl acetate extracts were combined and concentrated under vacuum and the oily residue was subjected to preparative droplet countercurrent chromatography in the solvent system H_2O-MeOH-$CHCl_3$-CCl_2=CHCl (2:5:3:2), with the lower phase stationary. The active fractions; 11~50 (10 ml fractions), were combined, concentrated to a residue (2.36 g) and subjected to C_{18} flash chromatography (Bondesil 40 μm, Analytichem International, 100 g of packing). The active components were eluted in the 80~100% methanol/water fractions, which were concentrated under vacuum to yield 1 g of material. One half of this material was chromatographed on a 2.5×31 cm Lichroprep RP-8 Lobar column (E. Merck) using a gradient from H_2O-MeOH (53:47) to H_2O-MeOH (40:60) over 2500 ml (10 ml fractions). Two active bands, fractions 195~198 (63.4 mg) and 209~211 (26.0 mg), were each subjected to HPLC on a C_{18} Partisil 10 ODS-3 column (M-9, Whatman) in the solvent systems H_2O-CH_3CN (60:40) and H_2O-CH_3CN (56:44) respectively. The more polar compound, A-108835 (32 mg), was identified as 4,4,24-trimethylcholesta-Δ8,9; 14,15; 24,28-trien-2α,3β,11β,12α-tetrol 12 acetate, 3-sulfate] (**1**). The minor bioactive compound, A-108836 (7 mg), was identified as [4,4,24-trimethylcholesta-Δ7,8; 9,11; 24,28-trien-2α,3β,12α-triol 12-*O*-methyl, 3-sulfate] (**2**).

Characterization and Determination of Structure

A-108835 and A-108836 are soluble in common organic solvents such as chloroform, ethyl acetate, acetone and methanol. High resolution negative ion FAB mass spectrometry gave an exact measured mass of 593.3140 (calc. 593.3148, $C_{32}H_{49}O_8S$) for A-108835 (**1**) and 549.3230 (calc. 549.3250, $C_{31}H_{49}O_6S$) for A-108836 (**2**), indicating molecular formulae of $C_{32}H_{50}O_8S$ and $C_{31}H_{50}O_6S$ respectively. Both compounds showed UV spectra suggestive of heteroannular dienes (Table 1). In the FTIR spectrum, a very strong $\nu_{max} = 1240\,cm^{-1}$ band, characteristic of a sulfate group, was seen for both compounds (Figs. 1 and 2).

All NMR spectra were recorded in CD_3OD which gave good dispersal of signals with no coincident chemical shifts in either the proton or the carbon spectra. The ^{13}C NMR of A-108835 identified thirty-two carbons, characterized in conjunction with a DEPT experiment, as an ester carbonyl, six olefinic carbons, four oxygen substituted methines, six aliphatic methylenes, seven methyl groups and one vinylic methylene (Table 2). The degree of hydrogen deficiency, when considered with the carbon hybridizations implied from CMR data, and the

Table 1. The UV spectral data for A-108835 and A-108836.

A-108835 λ_{max} (ε)	A-108836 λ_{max} (ε)
242 (13,300, sh)	240 (9,700, sh)
248 (14,300)	245 (10,500)
256 (10,200, sh)	253 (7,900, sh)

Fig. 1. The FTIR spectrum of A-108835.

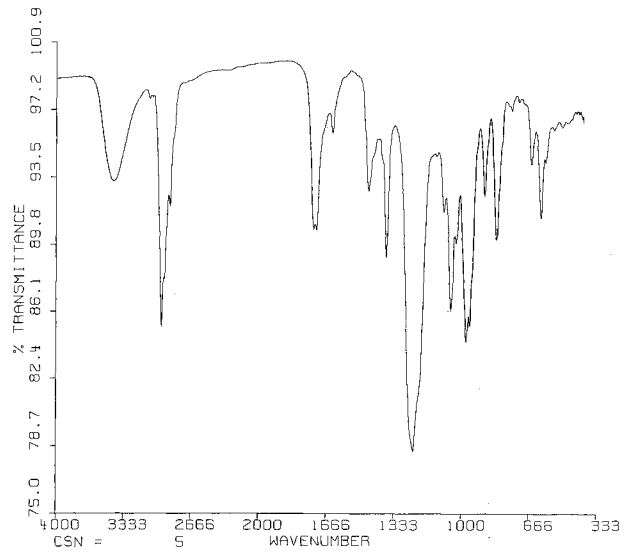


Fig. 2. The FTIR spectrum of A-108836.

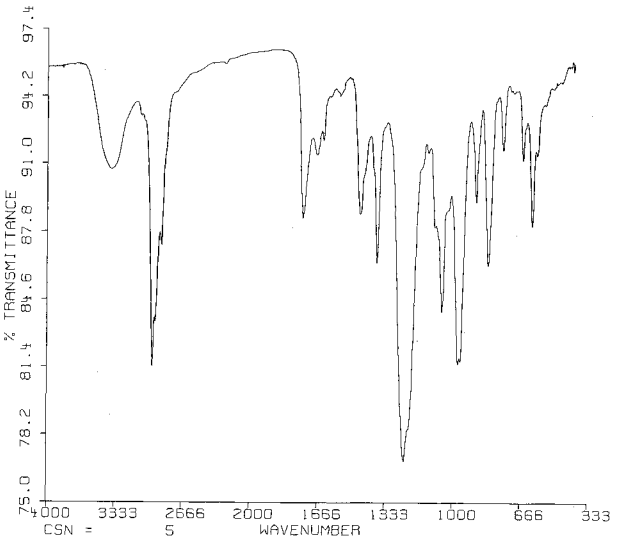


Table 2. The ^{13}C NMR chemical shift data for A-108835 and A-108836.

Number	A-108835[a]	A-108836[a]	Diastereomer of A-108835[b]
1	44.4 CH_2	45.3 CH_2	43.3
2	69.0 CH	68.9 CH	68.0
3	91.9 CH	91.5 CH	79.1
4	40.9 Q	40.6 Q	40.2
5	51.9 CH	50.3 CH	50.8
6	19.3 CH_2	24.4 CH_2	18.6
7	27.9 CH_2	125.2 CH	27.1
8	126.7 Q	136.4 Q	125.3
9	139.5 Q	151.5 Q	139.4
10	38.9 Q	39.3 Q	38.2
11	69.4 CH	117.4 CH	68.6
12	79.9 CH	81.4 CH	79.1
13	48.1 Q	46.8 Q	47.3
14	148.5 Q	46.2 CH	147.8
15	122.0 CH	23.8 CH_2	120.8
16	36.3 CH_2	29.1 CH_2	35.7
17	50.4 CH	47.6 CH	49.5
18	17.1 CH_3	12.2 CH_3	16.9
19	23.5 CH_3	23.6 CH_3	22.0
20	34.5 CH	37.0 CH	34.1
21	18.7 CH_3	18.1 CH_3	18.3
22	35.7 CH_2	35.9 CH_2	35.0
23	32.0 CH_2	32.1 CH_2	31.3
24	157.5 Q	157.8 Q	156.7
25	34.9 CH	34.9 CH	33.8
26	22.5 CH_3	22.5 CH_3	22.1
27	22.3 CH_3	22.3 CH_3	22.0
28	107.0 CH_2	106.9 CH_2	106.6
29	17.9 CH_3	18.0 CH_3	16.9
30	29.3 CH_3	29.3 CH_3	28.9
31	172.1 Q	55.9 CH_3	171.9
32	21.0 CH_3	—	21.1

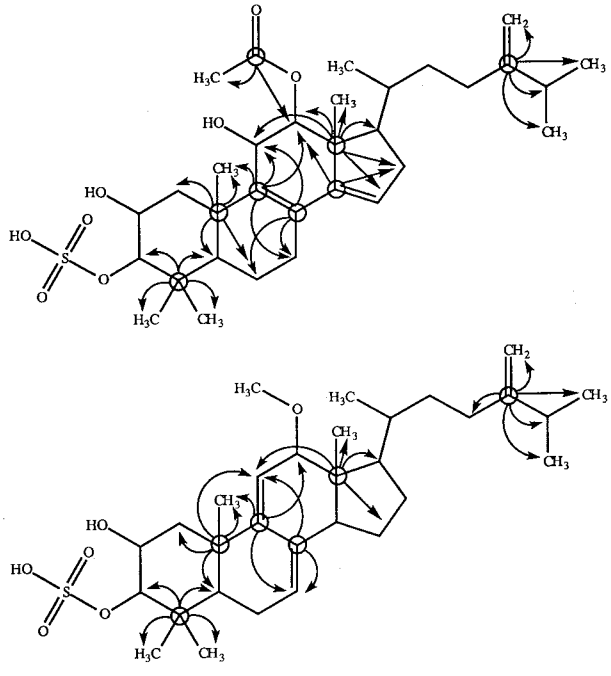
[a] 125 MHz, including distortionless enhancement by polarization transfer (DEPT) summary in CD_3OD.
[b] Taken from Ref. 4.

Table 3. The ^{1}H NMR chemical shift data for A-108835 and A-108836.

Number	A-108835[a]	Number	A-108836[a]
1 (α, β)[b]	1.25, 2.39	1 (α, β)	1.44, 2.37
2	3.99	2	3.98
3	3.88	3	3.87
5	1.27	5	1.32
6 (α, β)	1.80, 1.71	6 (α, β)	2.20, 2.14
7 (α, β)	2.42, 2.31	7	5.61
11	4.22	11	5.82
12	5.06	12	3.59
		14	2.54
15	5.59	15 (α, β)	1.80, 1.40
16 (α, β)	2.43, 2.06	16 (α, β)	1.97, 1.34
17	1.95	17	2.03
18	1.08	18	0.50
19	1.31	19	1.07
20	1.66	20	1.43
21	0.92	21	0.98
22	1.59, 1.16	22	1.58, 1.18
23	2.12, 1.93	23	2.13, 1.92
25	2.24	25	2.23
26	1.03	26	1.04
27	1.01	27	1.02
28 (H, H')	4.67, 4.73	28 (H, H')	4.65, 4.72
29	0.91	29	0.96
30	1.11	30	1.09
		31	3.37
32	1.99		

[a] 500 MHz in CD_3OD.
[b] Relative stereochemistry based on ROESY experiments.

Fig. 3. Summary of HMBC data for quaternary carbons of A-108835 and A-108836.



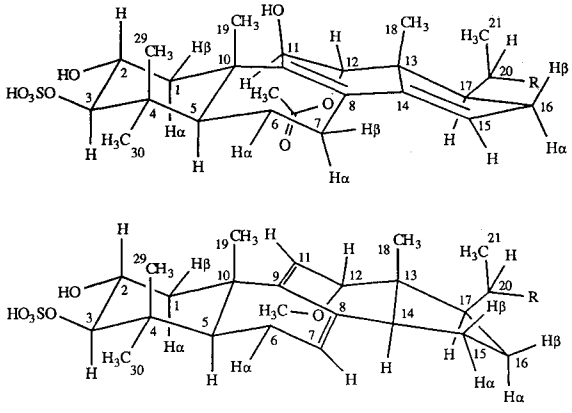
presence of a sulfate group, require a tetracyclic structure. Three carbon-carbon double bonds are indicated by the six olefinic sp^2 carbons in the ^{13}C NMR, and two of these must be part of a heteroannular diene. Seven aliphatic methyl groups, including four methyl singlets, are seen in the ^{1}H NMR (Table 3). From this information one must conclude that A-108835 is a triterpene or steriod type molecule.

The ^{13}C NMR HMQC/HMBC data for A-108835 show that the terminal vinyl group (Δ24, 28) is on the side chain next to the isopropyl methine (C-25, 34.9 ppm) that ends the chain (Fig. 3). ^{1}H NMR COSY/TOCSY experiments show that the only proton on the heteroannual diene system (15-H, δ 5.59) is coupled through a methylene (C-16, 36.3 ppm) to a methine (C-17, 50.4 ppm) that bears the side chain. HMBC couplings from the quaternary carbons C-13 and C-14 (48.1, 148.5 ppm) to this system of coupled protons, as well as to the 17.1 ppm C-18 methyl singlet (18-CH_3, δ 1.08), resolve the D ring and side chain of the molecule. An acetate methyl (32-CH_3, δ 1.99) is coupled to a 172.1 ppm carbonyl that shows a coupling to the δ 5.06 12-H proton. The C-9 (139.5 ppm) and C-14 quaternary carbons of the diene show 3-bond couplings to this same proton. The C-8 (126.7 ppm) and C-9 quaternary carbons of the diene show couplings to the δ 1.31 19-CH_3 and a pair of coupled methylenes (C-6, C-7). The C-10 (38.9 ppm) quaternary carbon that bears the 19-CH_3 is tied into both the H-5/CH_2-7 and the CH_2-1/H-3 proton coupling systems. The same is true for the lanosterol-like C-4 (40.9 ppm) dimethyl quaternary carbon. This information defines a classical tetracyclic triterpene skeleton. A ^{13}C NMR MeOH/MeOD solvent shift experiment showed that only two hydroxyl groups were present on A-10885; at the C-2 and C-11 positions. The presence of a sulfate group on C-3 is indicated by the 91.9 ppm C-3 chemical shift, molecular formula consideration and the IR data.

A-108836 also contains a C-2 hydroxy and C-3 sulfate group on the A ring, but has significant changes with regard to the heteroannular diene system arrangement in the B, C and D rings. The ^{13}C NMR HMQC/HMBC and ^{1}H COSY/TOCSY data for A-108836 show that the diene has changed to the 7, 8; 9, 11 positions of rings B and C instead of the 8, 9; 14, 15 positions of the C and D rings (Fig. 3). A-108836 is missing the hydroxyl at C-11 and has a methoxy group at C-12 instead of an *O*-acetyl.

A-108835, [4,4,24-trimethylcholesta-Δ8,9; 14,15; 24,28-trien-2α,3β,11β,12α-tetrol-12-acetate, 3-sulfate] (**1**), is diastereomeric with the reported structure of [4,4,24-trimethylcholesta-8,14,24(28)-trien-2α,3β,11α,12β-tetrol-

Fig. 4. Relative stereochemistry of the A, B, C and D rings of A-108835 and A-108836.



12-acetate, 3-sulfate] (sodium salt).[1] They differ only by the stereochemistry at the C-11 and C-12 positions. In a ROESY experiment, both A-108835 and A-108836 show a 1-3-5 triaxial set of NOE cross peaks between the 2-H, 19-CH_3 and 29-CH_3 protons. The 3-H proton shows 1-3 diaxial cross peaks to 1α-H and 5-H, and 3-H and 5-H show cross peaks to the 30-CH_3. These data indicate that in A-108835 and A-108836 the 2-OH is in the α position, and the 3-OSO_3H is in the β position (Fig. 4). For both compounds, a cross peak is seen between the 12-H and 18-CH_3 protons, and the 12-H and 21-CH_3 protons. In A-108836, a 1-3 diaxial cross peak is seen between 14-H and 17-H protons. These data indicate a placement of the 12-substituent ($OCOCH_3$ and OCH_3) in the α position for both A-108835 and A-108836. In A-108835, the 11-H proton shows strong NOE cross peaks to both the 1α-H and 1β-H protons, as well as to the 32-CH_3, indicating placement of the 11-OH in the β position. The 11-H is an olefinic proton in A-108836. The CMR data for A-108835, A-108836 and the reported data for the diastereomer of A-108835 are presented in Table 2 and are supportive of the structural assignments.

Bioactivity

[4,4,24-Trimethylcholesta-8,14,24(28)-trien-2α,3β,11α,12β-tetrol 12 acetate, 3-sulfate] has been shown to be a phytotoxic compound which will inhibit seed germination of wheat and tomato, and to have some activity against fungi.[4] Our studies tested A-108835 against several fungal pathogens such as *Candid albicans*, *Torulopsis glabrata*, *Cryptococcus albidus* and *Aspergillus niger*, and found no activity at 100 μg/ml. The compound had no Gram-negative antibacterial activity at 100 μg/ml and only scattered weak Gram-positive activity against some *Staphylococcus aureus* and *Streptococcus* strains in the range of 6~50 μg/ml. The compound exhibited an IC_{50} value of 48 μg/ml against rhinovirus 3C protease from strain 1B.

Materials and Methods

The Producing Strain

The producing fungus, strain AB 2194I-103, was isolated from an ant hill soil sample collected near the village of Rantan, Nigeria. Strain AB 2194I-103 was identified as *Fusarium compactum* Wollenw. & Reinking *sensu* Gordon by KERRY O'DONNELL at the National Center for Agricultural Utilization Research. The culture was grown for characterization at 20°C under continuous fluorescent light for seven days. It was deposited at the National Center for Agricultural Utilization Research, United States Department of Agriculture, 1815 North University Street, Peoria, Illinois, 61604 U.S.A. The accession code at this depository is NRRL 25020. The codes in parentheses following color names in the strain characterization section are Munsell color notations[9] and the color names were taken from the book "Color: Universal Language and Dictionary of Names".[10]

Rhinoviral 3C Protease Fluorogenic Substrate

The fluorogenic substrate Asp-Glu(EDANS)-Met-Ser-Ala-Ile-Phe-Gln-Gly-Pro-Ile-Ser-Lys(DABCYL)-Asp was synthesized using a solid phase synthetic procedure[11] and an automated peptide synthesizer (Model 430A, Applied Biosystems, Inc.). The substrate was purified by reverse-phase HPLC with a C_{18} column and a gradient of acetonitrile in water with 0.1% TFA.

Assay

The rhinovirus 3C protease inhibition assay was conducted with f-Met 3C protease from human rhinovirus strain 1B which had been cloned and expressed in *E. coli* and purified. The assay was performed in a 96-well format using white Micro-fluor "U" shaped plates (Dynatech, Chantilly, VA) and a Fluoroskan II fluorescence plate reader (ICN Biomedicals) equipped with excitation and emission wavelength filters of 355 and 485 nm, respectively. Rhinoviral 3C protease (0.12 μM) was preincubated with test solutions for 30 minutes at room temperature in buffer containing 50 mM potassium phosphate pH 7.5, 200 mM NaCl, 2 mM EDTA, and 1 mg/ml BSA. Reactions were started by the addition of rhinoviral 3C protease fluorogenic substrate (10 μM final concentration) and the fluorescence increases were monitored for 30 minutes using DeltaSoft II version 4.1 software (BioMetallics) and a Macintosh computer. The resulting enzymatic reaction velocities were compared with uninhibited controls in order to measure inhibitory activity.

Instrumentation

The droplet countercurrent device used in the isolation of A-108835 and A-108836 consists of 100 spiral Teflon loops with an approximate total volume of 800 ml. Approximately 90% of this volume is retained as stationary phase during use, when a mobile phase flow rate of 1 to 1.5 ml/minute is maintained.

NMR spectra were acquired employing a Varian Unity 300 or 500 spectrometer. Mass spectra were recorded on a Finnigan-MAT-TSQ700 or VG70-SEQ spectrometer. UV spectra were recorded on a Hitachi U-2000 spectrophotometer, and IR spectra on a Nicolet 5SXC FT-IR instrument.

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